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FUNCTIONAL AND COMPARATIVE STUDIES OF HAEMOPROTEINS FROM POLAR FISHES

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ABBREVIATIONS

Å: Ångstrøm

Abs: Absorbance

ACC: Antarctic Circumpolar Current

ATP: Adenosine triphosphate

Cygb: Cytoglobin

BPG: 2,3-Biphosphoglycerate

DEAE: diethylaminoethyl

DLS: dynamic light scattering

DTT: Dithiothreitol

EDTA: Ethylene-diamino-tetra-acetic acid

FPLC: Fast Protein Liquid Chromatography

GTP: Guanosine triphosphate

Hb: Haemoglobin

HbA: Human haemoglobin

HbS: Sickle cell haemoglobin

HEPES: 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid

hxHb: hexacoordinate haemoglobin

Fe^{2+}: Iron atom (ferrous)

MALDI-TOF: Matrix-Assisted Laser Desorption Ionization-Time Of Flight

Mb: Myoglobin

MES: 2-(N-morpholine)-ethane sulfonic acid

MS: Mass spectrometry

mya: Million years ago

Ngb: neuroglobin

nHill: Hill coefficient

PITC: Phenyl-isothiocyanate

p_{50}: O_2 partial pressure required to achieve half-saturation

RBC: red blood cell

ROS: radical oxygen species

RP-HPLC: Reverse-phase high-performance liquid chromatography

RT-PCR: Reverse-transcriptase polymerase chain reaction

SCA: Sickle Cell Anemia

TB: terrific broth

TFA: Trifluoracetic acid

TRIS: Tris-hydroxymethyl-methylamine

Summary

Proteins, such as the members of the globin superfamily, are sensitive to temperature and their properties are the result of a long process of adaptation to the conditions encountered during the species evolution. The globin superfamily comprises globular proteins that reversibly bind gaseous ligands such as O_2, CO and NO to a haem prosthetic group, Fe-protoporphyrin IX. The globins of this family are the components of classical haemoglobin (Hb) and myoglobin (Mb), but also of neuroglobin, cytoglobin (Cygb), globin X, globin Y and eye-globin.

In this study, particular attention has been given to biochemical and physico-chemical characterisation of two proteins. On one hand, the O_2 transport systems from two Arctic fish species (*Lycodes reticulatus* and the cod *Gadus morhua*) have been investigated. On the other, Cygb has been studied from two Antarctic fish species, one belonging to the family Channichthyidae (icefish) lacking Hb and Mb (*Chaenocephalus aceratus*) and one red-blooded species belonging to the family Nototheniidae (*Dissosticus mawsoni*).

The Arctic and Antarctic regions have the low temperature in common but differ in geographic position and history. The Antarctic is a continent isolated by the Polar Front, a circular oceanic system, and the temperatures are constantly close to -1.87°C. In contrast, the Arctic is essentially an ocean that lies between North America, Greenland, Europe and Asia. There are strong currents with high temperature variations. The Arctic and Antarctic icthyofaunas are very different. In the Antarctic, a single group of teleost fishes is dominant, the suborder Notothenioidei, that includes eight families. The modern family Channichtyidae is particularly interesting because its species have coulorless blood, lacking Hb and in some cases Mb. In contrast, in the Arctic there are six marin groups, nobody being dominant. Given a shorter evolutionary time at polar temperatures, than the Antarctic ichthyofauna, Arctic fish may provide valuable information on the effects of environmental temperature on specific physiological and biochemical traits. It is note that fish Hbs offer the possibility to investigate functional differentiation and molecular adaptations in species living in a large variety of environmental conditions.

In this study, the structural and functional characterisation of the hemolysate of *L. reticulatus* (family Zoarcidae), living on the sea floor near the coasts of northern Europe and North America is reported. The hemolysate shows only a single α chain, whereas

polymorphism of two β chains, which differ by only four residues corresponding to two Hbs. For such a high identity, complete purification of the two Hbs was not achieved and the functional studies were carried out on the hemolysate. The latter showed a low Bohr effect and no Root effect. The Hbs tend to form high-molecular mass polymers at physiological pH and low temperature (4°C), as shown by gel-filtration chromatography and dynamic light scattering. The elucidation of the primary structure has allowed to establish correlation between functional behaviour (no Root effect) and structural properties (polymerisation). In fact, it was demonstrated that Cys residues are present in high number and tend to form intermolecular disulphide bridges as shown by mass spectrometry.

Recently, an unusual process of Hb polymerisation (sickling), which occurs *in vivo* in red blood cells of several Arctic species of the family Gadidae, was discovered and reported in the literature. The *G. morhua* Hb polymerisation showed pH- and concentration-dependence in the deoxygenate state *in vitro*, suggesting that polymerisation may be an adaptive response to extreme and stressful environmental conditions. Therefore, Arctic fish Hbs appear to be very useful models for studying sickling disorders and Hb-polymerisation processes.

The second topic of the thesis were two Cygbs from Antarctic fish. Cygb is a cytoplasmatic protein found in almost all tissues and characterized by endogenous hexacoordination of the haem. The function is not clear. Involvement in protection from oxidative stress, in NO metabolism, in collagen synthesis and in defence mechanisms of cancer cells was hypothesised. Cygb was found in both: in red-blood *D. mawsoni* and in the icefish *C. aceratus*.

The Cygbs were cloned, expressed and purified and a preliminary characterisation was carried out. It was demonstrated that they are hexacoordinated independently of pH- and temperature, similar to human Cygb. Understanding the role of the Cygb genes in species lacking Hb and Mb is a very important task necessary to elucidate of the function of this protein.

CHAPTER 1

Introduction

1.1 Polar regions

The polar oceans are often considered extreme environments because temperatures are close to the freezing point and the life is possible only for few selected species. Temperature and its fluctuations affect other physico-chemical parameters, such as pH, salinity, gas solubility, pressure, viscosity and redox potential, that entail extreme environmental conditions in the polar regions and constitute an important driving force for the species survival.

The Arctic and Antarctic regions are more dissimilar than similar. They have in common the cold temperature but differ for geographic and historical characteristics.

In late Paleozoic, about 250 million years ago (mya), land masses were assembled within a single large continent called Pangea that split, about 200 mya, into Laurasia in the northern hemisphere and Gondwana in the southern one. Fragmentation of Gondwana into the modern southern continents initiated 135 mya, and the Antarctic continent reached its current geographic location approximately 65 mya. The Drake Passage completed the isolation (Kennett, 1977) and produced the Antarctic Circumpolar Current (ACC) and the Polar Front, a circular oceanic system that produce permanent turbulence (Fig. 1.1a). Just north of the Front, the water temperature has an abrupt rise of about 3°C, a critical factor for ecosystem isolation and adaptation. The Antarctic water has tested slow temperature transition from 15°C, in the early Tertiary, to -1.87°C, today (Eastman, 1993, 2005).

The Arctic is most covered by the sea and lies between North America, Greenland, Europe and Asia. The Arctic Ocean is almost completely surrounded by land and contains two basins (Fig 1.1b).

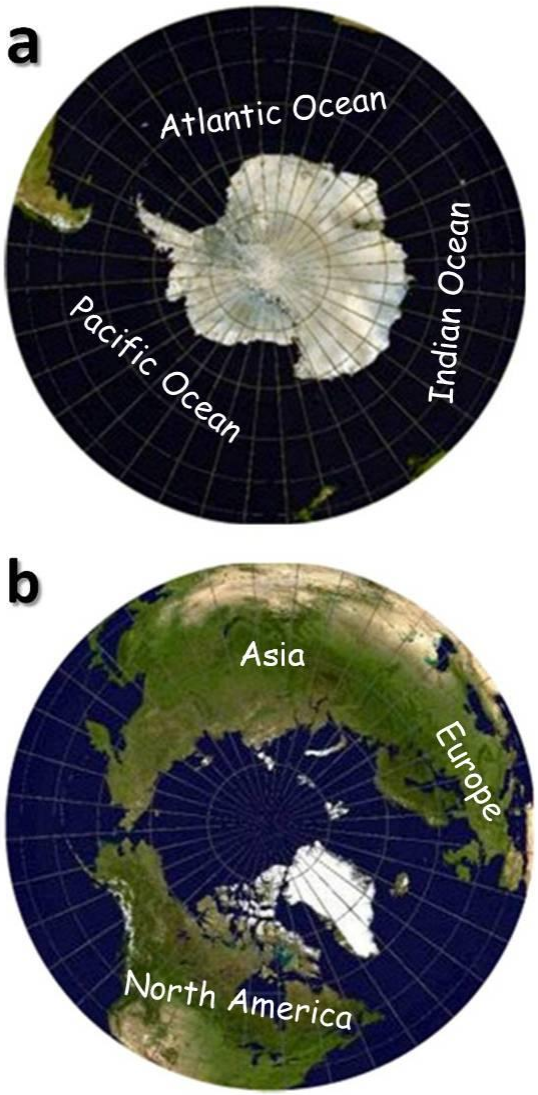

Fig. 1.1: a) Antarctic region; **b)** Arctic region

The Europe separated from Greenland in the late Cretaceous but the exchange of water between the Arctic and the Atlantic Ocean was not possible until 27 mya. The history of the Arctic Ocean during the Cenozoic (0-65 mya) is unknown and researchers have long debated the timing, extent and nature of the onset of Northern Hemisphere Glaciation. Recent evidence, based on a Cenozoic palaeo-oceanographic record, revises the timing of the earliest Arctic cooling events, strongly supporting a "bipolar symmetry" in climate cooling (Moran et al., 2006). According to this revision, the earliest Arctic cooling events are dated approximately 45 mya. During the Miocene, about 10-15 mya, Arctic land masses reached their present positions and it is commonly accepted that, only at this time, temperatures dropped below freezing as suggested by the unipolar ice-sheet model (Perlmutter and Plotnick, 2003). However, there are conflicting views about when cooling

led to the formation of Arctic sea-ice. Ice cores from both Antarctica and Greenland show that during the past 400,000 years interglacial temperatures were between 2-5ºC higher and sea levels 4-6 m higher than they are today (Severinghaus et al., 1998; Rohling et al., 2008).

Repeated glaciation of the whole Arctic until about 11,000 years ago, when the last ice age ended, enforced repeated exchange of the Arctic fauna with temperate species.
This is in contrast to what happened in the Southern Ocean, where most of the species were effectively isolated after the establishment of the ACC. Therefore, the Antarctic and Arctic fish faunas are very different and allow examination of convergent evolutionary trends to similar environmental conditions at levels of biological organisation. The modern ichthyofaunas differ in age, endemism, taxonomy, biodiversity and range of physiological tolerance to environmental parameters (Eastman, 1997)

In Antarctic are present five groups account for about 74% of the Antarctic fauna (notothenioids, mycthophids, liparids, zoarcids and gadiforms) with an unique dominant group of teleost fishes, the notothenioids (Eastman, 1997). During the cooling of the Southern Ocean, this suborder experienced extensive radiation about 24 mya (Near, 2004) and exploited the diverse frozen habitats. Probably, the Antarctic has the oldest and most isolated marine species in the world (Dayton, 1994). In ten million years the Antarctic notothenioids have lost the ability to cope with higher temperatures and now they live at temperatures between 2°C and -1.8°C. The suborder Notothenioidei reflects the evolutionary adaptive changes in the molecular and cellular machinery, e.g. an efficient microtubule assembly (Detrich, 1989, 2000) and loss of heat-shock response (Hofmann, 2000). The suborder Notothenioidei includes eight families: Bovichtidae, Pseudaphritidae, Eleginopidae, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae and Channichthyidae (Balushkin, 1992; Pisano, 1998; Lecointre, 2004). Bovichtidae (except one species), Pseudaphritidae, Eleginopidae and some species of Nototheniidae inhabit north of the Antarctic Polar Front and probably, this divergence took place relatively recently between 10-15 mya and 2.5 mya, when a portion of notothenioid stock became isolated in the Southern Ocean south of the Antarctic Polar Front (Bargelloni, 1994; Ritchie, 1997). Moreover, every family has red-blooded species with the exception of the family Channichthyidae with all 16 species without Hb (Ruud, 1954) and 6 species also without Mb (Grove, 2004). This family, for the loss of Hb and the characteristic colourless blood, is called “icefish” (Ruud, 1954). Several modifications of the cardiovascular system

of icefish compensate for the lack of Hb in the blood. In fact, loss of Hb and Mb, associated with NO-oxygenase activity and subsequent
elevation of NO levels, may explain the unique cardiovascular and physiological traits of icefish (Sidell and O'Brien, 2006).

In Arctic, there is not a predominant group like the Antarctic notothenioids, but coexist six different groups that are equally dominant and comprise 58% of the Arctic fauna (zoarcoids, gadiforms, cottids, salmonids, pleuronectiforms and chondrichthyans). The Arctic fauna includes 416 species in 96 families, about 52% larger than Antarctic fauna. Despite the different histories and age of the polar ecosystems, gadiforms and zoarcids are the only groups that are present in both poles with 27 families, 35 genera and 10 species common for both (Eastman, 1997). The Arctic fish fauna consists of eurythermal (they can resist wider temperature variations) and euryhaline (they can tolerate salinity variations) boreal marine and freshwater fish.

1.2 Globin superfamily

Proteins, such as the members of the globin superfamily, are sensitive to temperature and their properties are the result of a long adaptation to the conditions encountered during the species evolution.

The globin superfamily comprises globular proteins that reversibly bind gaseous ligands like O_2, CO and NO with a haem prosthetic group, the Fe-protoporphyrin IX. Globins are present in all kingdoms: archea, bacteria, fungi, plants, protists and animals (Hardison 1996; 1998).

Until a few years ago only two globins were known to be present in vertebrates: haemoglobin (Hb) and myoglobin (Mb). Recently, other globins were discovered in vertebrates: neuroglobin (Ngb) and cytoglobin (Cygb), that are widespread between all vertebrates (Burmester et al., 2004), globin X, only in fish and amphibians (Fuchs et al., 2006; Roesner et al., 2005), globin Y, in *Xenopus* tissues, (Fuchs et al., 2006) and eye-globin, in chicken (Kugelstadt et al., 2004; Blank et al., 2011).

Hb is a hetero-tetrameric protein composed by two α and two β chains. This protein is present in erythrocytes and transports O_2 and other gaseous ligands in the circulatory system (Perutz 1990; Brunori 1999; Imai 1999; McMahon et al., 2002).

Mb is a monomeric protein that is present in cardiac and striated muscle. It acts as an O_2 buffer, facilitates O_2 diffusion and is involved in the removal of NO (Wittenberg and Wittenberg, 1989, 2003; Brunori, 2001; Flögel et al., 2001).

In 2000 Ngb was localised in neuronal tissues from mouse and human brain (Burmester et al., 2000). It is a monomeric protein of about 16 kDa with high affinity for O_2 (Fago et al., 2004), widely express in the brain (Mammen et al., 2002; Reuss et al., 2002; Geuens et al., 2003; Hundahl et al., 2005, 2008a) and retina (Schmidt et al., 2003; Hundahl et al., 2005, 2008b).

More recently, Cygb was found in almost all kind of tissues (Burmester et al., 2002). Cygb has a monomeric unit of about 21 kDa and, similarly to Ngb, has a high affinity for O_2. It is a cytoplasmatic protein, however it has also been found in the nuclei of neurons (Schmidt et al., 2003).

Particular attention was addressed to Hbs and Cygbs.

1.2.1 Haemoglobin

Hb is a tetrameric protein composed by two α and two β subunits. It is the main O_2 carrier in the vertebrates and each subunit binds only one of this ligand. Each subunit has similar three-dimensional structure. α and β subunits of adult human Hb (HbA) have 141 and 146 amino-acid residues, respectively (Fig. 1.2). In the β subunits there are eight α-helices called with the letters from A to H, while in the α subunits the D α-helix misses.

Each subunit binds an O_2 molecule by a prosthetic group, the haem, responsible for the red colour of blood. The haem is a complex Fe-protoporphyrin IX, which consists of a tetrapyrrole ring bound to four methyl groups, two vinyl groups and two propionate side chains. Haem is harboured within the globin fold, organised into a two layer structure, called "three-over-three" α-helical sandwich. The haem is surrounded by E, F, G and H helices. In deoxygenated Hb the Fe^{2+} is pentacoordinated and is bound to four N-atoms of the pyrrole ring and to proximal His in F8 position (HisF8). Fe^{2+} lies approximately 0.4 Å outside the porphyrin plane because Fe^{2+} is slightly too large to fit into the well-defined hole within the porphyrin ring.

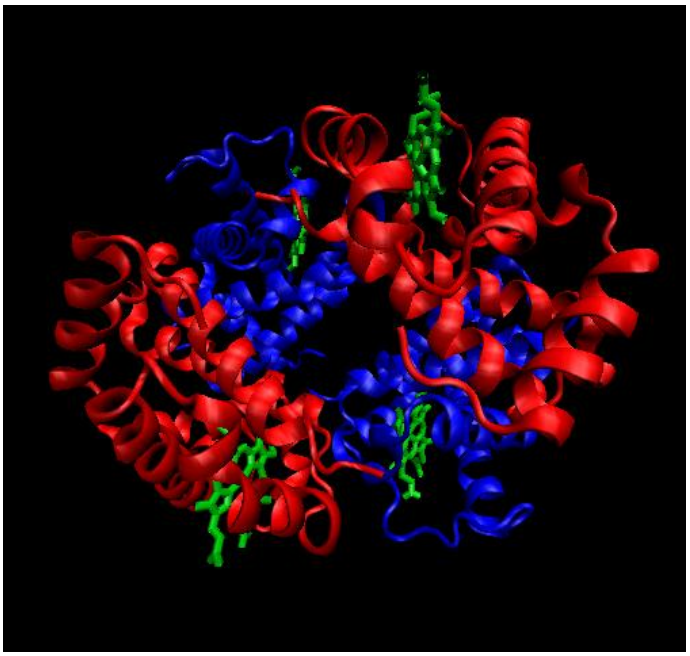

Fig. 1.2: Quaternary structure of HbA (IBP code 2HHB; Fermi et al., 1984)

When O_2 binds the Fe^{2+} in sixth coordination position there is an electronic rearrangement, so that the Fe^{2+} become smaller and can enter into the porphyrin plane. The binding of O_2 is stabilised by a hydrogen bond by distal His in position E7 (HisE7). The haem environment is shown in Fig. 1.3

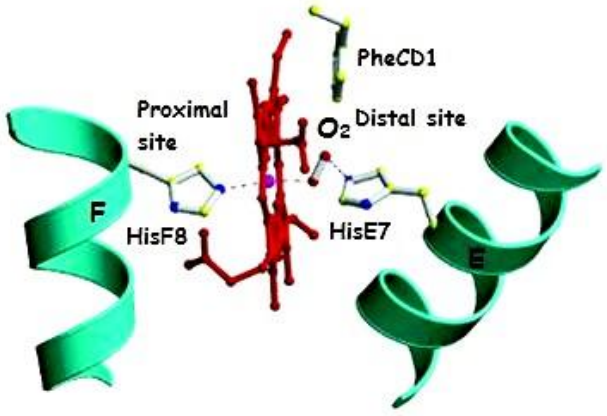

Fig. 1.3: Haem environment (Taken after Pesce et al., 2002)

The three-dimensional structure of Hb can be seen like two identical αβ dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$) that associate to form the tetramer (Perutz, 1965). Because of the binding between a ligand and the haem, the protein is subjected to allosteric conformational transition (Monod, 1965; Perutz, 1987). The two-state allosteric model of Monod, Wyman

and Changeux (MWC) assumes that the Hb is only in two states, corresponding to a low-affinity structure named T (tense) and a high-affinity structure named R (relax) (Monod, 1965). With the T→R transition, the two dimers rotate about 15 degree with respect to one another but their structure is relatively unchanged. The only conformational shifts are localised into the interface between the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers. The rearrangement of the dimer interface provides a pathway for communication between subunits, enabling the cooperative binding of O_2.

The O_2 affinity of Hbs is lowered by protons, chloride, carbon dioxide, and organic phosphate, i.e 2,3–biphosphoglycerate (BPG) in mammals and adenosine triphosphate (ATP) or guanosine triphosphate (GTP) in teleost fish, all of which are present in the red cell. They are known collectively as allosteric effectors. (Perutz, 1998).

In vertebrates, O_2 affinity of Hbs is strongly pH dependent and this phenomenon is called *alkaline Bohr effect* (Riggs, 1988). During the cell metabolism, CO_2 and lactic acid are released thus lowering the tissue pH. As the proton concentration increases, more O_2 will be provided to ensure adequate O_2 supply. It is possible to understand the physiological relevance of this effect when one considers that the tissues highly active produce acidic substances which enhance O_2 unloaded from Hb. During the oxygenation, the T state is converted to R and the *cooperativity*, expressed by the Hill coefficient *n* (nHill), is used as a measure of this conversion. In many teleost fishes Hbs, displaying the *Root effect*, at low pH the nHill changes from 3 (at alkaline pH) to 1. In this case, the O_2 affinity decreases to such an extent that the Hbs cannot be fully saturated at very high O_2 pressure and the cooperativity is completely lost, so the O_2 capacity of blood reduces by almost 50% compared to an alkaline pH. For this reason the Root effect can be considered an exaggerated Bohr effect (Brittain, 2005). Probably, the Root effect in fishes is connected with the presence of at least one of two anatomical structures with high O_2 pressure: the rete mirabile and the choroid rete. The first structure supplies the gland that inflates the swimbladder with O_2, while the second is a vascular structure which supplies O_2 to the retina (Wittenberg and Wittemberg, 1974).

During the Root effect the low-affinity T state is stabilised by high proton concentration (Perutz, 1987) and the transition T→R is inhibited causing a drastic reduction in the nHill. Large conformational changes occur at the dimer $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces. Some polar residues seem to be involved in Root effect, because of formation of salt bridges. In particular, the residues are: Lys β(EF6), Ser β(F9), Glu β(FG1), Arg β(H21) and His β(HC3) (Perutz and Brunori, 1982). Different theories about the residues involved

in Root effect are supposed. On one hand, it is hypothesised that the pH-dependent R→T transition is due to placed positive-charge clusters at the allosteric β_1/β_2 interface (Mylvaganam, 1996), on the other hand, another possible theory is the overstabilisation of the T state by inter-Asp hydrogen bond at the α_1/β_2 interface (Mazzarella, 2006a) and modulated by salt bridges between histidyl residues (Mazzarella, 2006b). The aspartyl triad (Asp95α, Asp101β and Asp99β) is present in the primary structure of all fish Hbs, but not in mammalian Hbs, where Asp95α is replaced by Glu. The Asp-Asp interaction has been found in the deoxygenated structure of Antarctic *Trematomus bernacchii* and tuna Root–effect Hb, but not in the deoxygenated form of the non-Root-effect HbI from trout, where the interaction between the two aspartyl residues is mediated by a water molecule (Yokoyama et al., 2004).

Currently, despite more than three decades of studies, it is yet virtually impossible to ascribe the real explanation of the Root effect to substitutions of a few amino-acid residues. Indeed, the situation is highly complex, and is probably linked to the combination and interplay of a number of factors in the architecture of the globin tetramer.

1.2.1.1 Sickle Cell Hb

A single point mutation in HbA can be the cause of particular disease due to changes in interactions between molecule and substrate or in interaction with the environment. An example is the Sickle Cell Anemia (SCA) in the man that is associated with the expression of the abnormal mutant sickle cell Hb (HbS). HbS was one of the first human disease proteins extensively studied. The genetic basis of SCA is the substitution of a single DNA nucleotide in the sixth codon (GAG→GTG) (Nagel and Steinberg, 2001). The single point mutation in the β chain, where polar Glu in position 6 is replaced by non-polar Val (Ingram, 1957), induces the formation of a twisted 14-member polymer fiber that reduces the solubility of the protein in the deoxygenated state causing cell sickling. The formation of these fibers requires protein concentration greater than 170 mg/ml, thus fiber formation occurs at physiological conditions (Ferrone et al., 2004).

Structural analysis of HbS fibers by single crystal X-ray diffraction, fiber X-ray diffraction and electron microscopy provide important information concerning the basic fiber architecture (Eaton and Hofrichter, 1990). Electron microscopy has revealed that the

HbS fiber is composed of 14 filament-strands that associate as half-staggered pairs (Dykes et al., 1979). Molecules within each strand align one another via axial contacts, and the two strands are stabilised by lateral contacts involving β Val6 (Wishner et al., 1976). The lateral contacts involve the mutant Val in the A helix and β Phe85 and β Leu88 in the EF corner region in two different HbS molecules (Harrington et al., 1997) (Fig. 1.4).

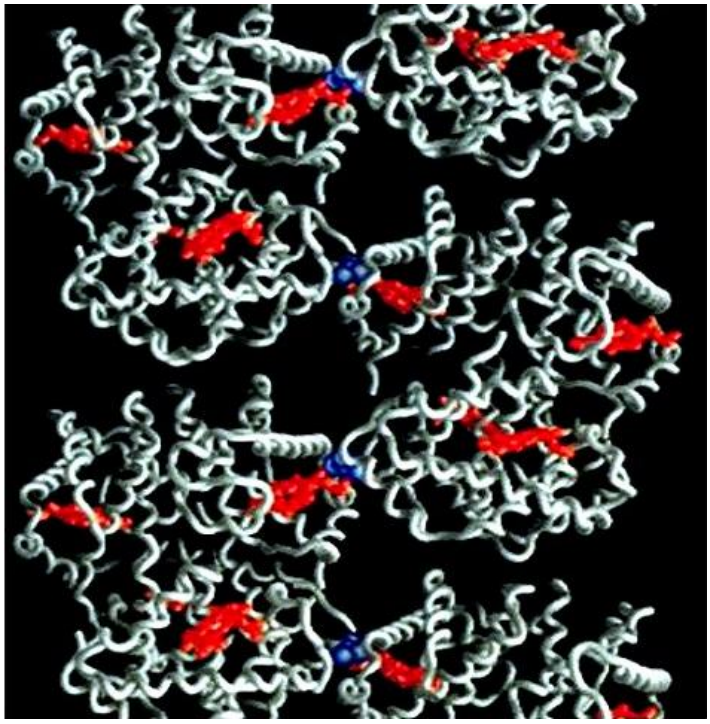

Fig. 1.4: Double strand of HbS molecules with haem group in red and Val residues in blue (Taken after Harrington et al., 1997)

HbS polymerisation has been found to occur by a two-pathway mechanism, divided into two steps: homogeneous and heterogeneous nucleation. The homogeneous pathway requires formation of an unstable aggregate, called homogeneous nucleus, which rate-limits the reaction. Once a polymer has been nucleated, a second pathway becomes available and new nuclei may also form on the surface of a polymer (Fig. 1.5), which they do more easily than in solution (Samuel et al., 1990).

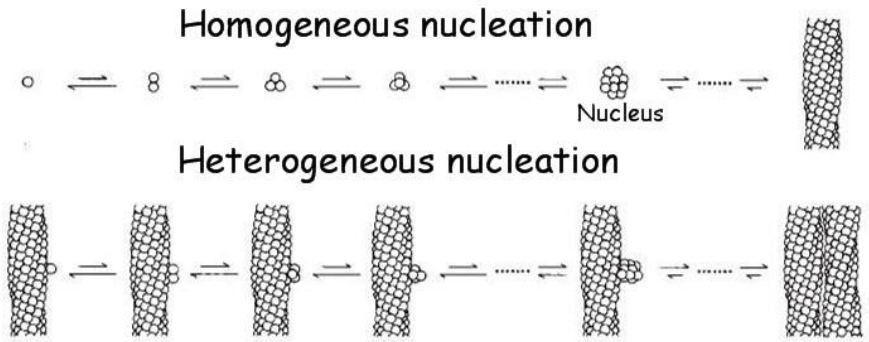

Fig. 1.5: Double nucleation mechanism for HbS polymer formation (Taken after Ferrone et al. 2004)

HbS polymers form an extremely viscous gel, responsible for the peculiar deformation of the red blood cells (RBCs) (Galkin, 2004). In fact, the increased stiffness of HbS fibers is the reason for the wide variety of shapes that deoxygenated RBCs acquire (Christoph et al., 2005; Ferrone, 2004; Statius van Eps, 1999). Moreover, because of increased stiffness, the circulation of sickle cells through the body's narrow blood vessels is often obstructed resulting in infarctions and organ damage (Aprelev et al., 2005; Embury, 2004; Hoffbrand et al., 2006). Moreover, over stroke due to occlusion of large cerebral arteries is one of the main complications of sickle-cell disease (Hillery and Panepinto, 2004; Routhieaux et al., 2005; Zennadi et al., 2008; Zermann et al., 1997).

1.2.2 Cytoglobin

Cygb shares 30% amino acid sequence identity with Mb, suggesting a common evolutionary ancestry (Burmester et al., 2002). Human Cygb is a globin of 190 amino acids with the classical vertebrate folding "three-over-three" α-helical sandwich and the antiparallel sets of helices A/E/F and B/G/H/ that are involved in this particular arrangement (Fig.1.6).

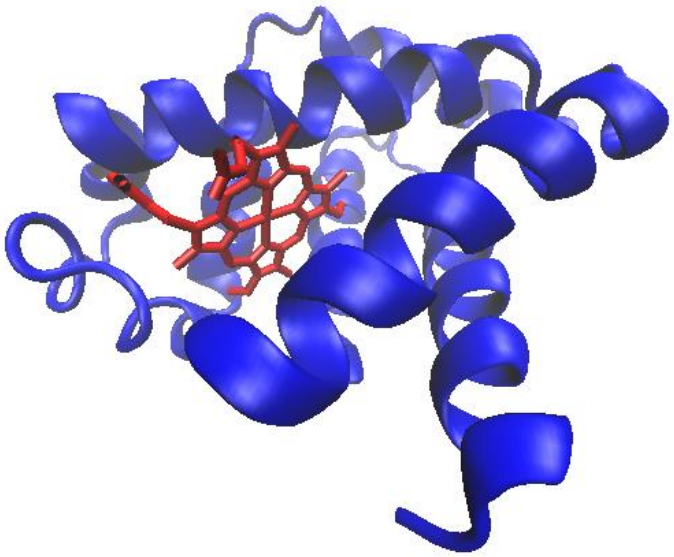

Fig. 1.6: Monomeric unit of Cygb (Sugimoto et al., 2004)

The N- and C-terminal regions of about 20 residues each one have an high conformational flexibility (de Sanctis et al., 2004). Conflicting results were published on human Cygb structure. Crystal structures show an asymmetric unit including two Cygb described as dimer (de Sanctis et al., 2004; Sugimoto et al., 2004). In contrast, in a recent study by mass spectrometry and size exclusion chromatography with multi-angle laser light scattering, the Cygb was found as a monomer with an intramolecular disulfide bridge between two Cys residues (CysB2 and CysE9) (Lechauve et al., 2010).

Cygb, similar to other hemoproteins, binds O_2 and other ligands with different affinity dependent on the redox-states (Burmester et al., 2002; Fago et al., 2004). In fact, the measured P_{50} value is about 1 torr at pH 7.0 and 20°C with a disulfide bridge between two Cys residues (Fago et al., 2004) and decreases by a factor of about 2 when the Cys residues are reduced (Hamdane et al., 2003). In other words, the oxidation of thiol groups increases ligand affinity.

Another important characteristic of the Cygb is the hexacoordination of the haem that binds the distal His at 6-coordination position, similarly to the Ngb (Fig.1.7).

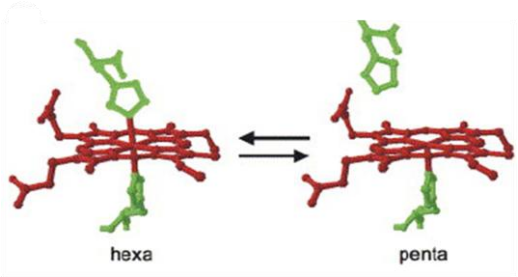

Fig. 1.7: Hexacoordination of the haem with reversible binding of the distal His

In this case, the distal His is capable of reversible dissociation to allow the stable binding of exogenous ligands like O_2 and CO. The hexacoordinate haemoglobins (hxHbs) are in plants, animals and cyanobacteria (Duff et al., 1997; Burmester et al., 2000; Scott and Lecomte, 2000) but our knowledge on their functional role is based mainly on *in vitro* reactions with recombinant proteins. However, there is growing evidence linking hxHbs with NO scavenging and a protective role during hypoxia (Sun et al., 2001; Hargrove, 2000). Moreover, the hexacoordination entails an enhanced thermal stability with a melting temperature (T_m) of 95°C for the ferric form, about 15°C more than Mb.

Cygb is a cytoplasmic hemoprotein in almost all cell types and it is present in the nuclei of neurons (Schmidt et al., 2003). Currently, the functions are not very clear. The high affinity of Cygb for O_2 and the low concentration *in vivo* (µM) suggest a function restrict to O_2-requiring cellular reactions unrelated to mitochondrial respiration (Fago et al., 2004). Moreover, Cygb was shown to be overexpressed in oxidative stress and hypoxic conditions *in vitro* and *in vivo* (Fordel et al., 2004 and 2006; Burmester et al., 2004; Guo et al., 2007; Li et al 2007) that proposes an involvement in protection from oxidative stress (Fordel et al., 2006). Other plausible functions are the involvement in the NO metabolism, like NO dioxygenase when coupled to suitable electron donors (Gardner et al., 2010), in the collagen synthesis in fibroblasts and related cells (Schmidt et al., 2003) and in the defence mechanisms that allow cancer cells to survive in hypoxic microenvironments (Emara et al., 2010).

1.3 Hbs and Cygbs in fish

1.3.1 Hb in fish

The comparison of the biochemical and physiological adaptations of cold-adapted Antarctic and Arctic fishes with sub-Antarctic and temperate fishes has been a powerful tool to understand whether an extreme environment has required specific adaptations (Verde et al., 2006; di Prisco et al., 2007). Moreover, fishes of the two polar regions have undergone different regional histories driving the physiological diversities.

Because of cold temperature, the O_2 solubility in the Antarctic water is higher than in temperate seas, therefore its uptake and transport are not limiting steps for Antarctic fish. Notothenioids developed an important hematological difference from temperate and tropical species, in having fewer erythrocytes, reduced Hb concentration and multiplicity and quite low O_2 affinity of Hbs.

The Hb content of erythrocytes is variable and in some species seems positively correlated with life style (Eastman, 1993). In general, the vast majority of notothenioids species have a single Hb with minor Hbs that are vestigial remnants (about 5% of the total). In comparison with temperate species, Antarctic notothenioids have lost globin diversity because of thermostable environment, where the need for more Hbs may be reduced (Verde et al., 2006). An extreme example of adaptation is the Channichthyidae family which has species with blood without Hb (Eastman, 1993). The loss of Mb and Hb in icefish becomes explicable by the exploitation of high O_2 solubility and low metabolic rates in the cold, where an enhanced fraction of O_2 supply occurs through diffusive O_2 flux. Icefish developed compensatory adaptations that reduce tissue O_2 demand and enhance O_2 transport. O_2 delivery to tissues occurs by transport of the gas physically dissolved in the plasma.

Unlike Antarctica, Arctic fishes, being exposed to seasonal temperature variations, exhibit higher physiological plasticity, high biodiversity and many species display Hb multiplicity. An example is the blood of the spotted wolfish (*Anarhichas minor*) of the family Anarhichadidae (suborder Zoarcoidei) which contains three major Hbs (Hb1, Hb2 and Hb3). The three Hbs display differences in pH and organophosphate regulation and O_2 binding dependent on temperature (Verde et al., 2002). Similar situation is present in the

gadids *Gadus morhua* (Atlantic cod), *Arctogadus glacialis* (Arctic cod) and *Boreogadus saida* (polar cod) (Verde et al., 2006). Nine globin genes were discovered in *G. morhua* and expressed simultaneously in adult fish. This finding suggests that the *G. morhua*, similarly to temperate species, could respond to environmental challenges, by altering the level of expression of the genes (Borza et al., 2009; Wetten et al., 2010).

Another important study conducted on the Atlantic cod Hbs showed their ability to polymerise in particular stress conditions. Recently, Koldkjær and Berenbrink (2007) have demonstrated extensive *in vivo* sickling of RBCs of whiting *Merlangius merlangus* after capture stress without any apparent hemolysis and showed its subsequent recovery by high cooperative proton binding *in vitro* and reduction of extracellular pH *in vivo*. The Hb polymerisation causes the sickling process similarly to sickle cell disease in human (Hárosi et al., 1998).

1.3.2 Cygb in fish

Fish and mammals Cygbs differ for number, length and sequence. Unlike mammals that have only one Cygb, in different teleost fishes (*Danio rerio*, *Oryzias latipes*, *Tetraodon nigroviridis* and *Takifugu rubripes*), two distinct paralogous Cygb genes (Cygb-1 and Cygb-2) have been found (Fuchs et al., 2005). The two Cygb genes diverged in teleost evolution, suggesting a large-scale duplication event. Cygb-1 has from 174 to 179 amino acids, while Cygb-2 has from 179 to 196 residues. The sequence identity among Cygbs-2 and mammalian Cygbs shows that Cygb-2 is more closely related to mammalian Cygb than fish Cygb-1 (Fuchs et al., 2005).

Interestingly, the position of Cys is not conserved in teleosts and, for this reason, it is possible that the O_2 affinity does not display dependence on the redox state as in mammalian Cygbs.

qRT-PCR analyses in *Danio rerio* have shown that both Cygb mRNAs have a broad expression profile in many tissues and Cygb genes exposed to mild or severe hypoxia have little change in their expression (Fuchs et al., 2005; Roesner et al., 2006). Cygb-2 detected at highest levels in neural tissues like brain and eye and was stronger expressed in almost all tissues (Fuchs et al., 2005). Currently, a biochemical

characterisation of these proteins is not reported in literature and is as well as their possible structures and functions.

1.4 Objectives and research strategy

This thesis addressed two major topics: the mechanism of Hb aggregation in Arctic fishes and the functional role of Cygb in red-blood Antarctic fishes and icefishes.

Particularly, the main objectives of my PhD thesis were:

- The description of the O_2 system transport of *Lycodes reticulatus*, family Zoarcidae, and characterisation of its Hb polymerisation. The polymerisation was studied by biochemical techniques, Dynamic Light Scattering (DLS) and mass spectrometry. The aggregation was compared to that of the cod *G. morhua*, family Gadidae.

- The cloning, expression, purification and preliminary characterisation of one of two different Cygbs of *Chaenocephalus. aceratus* (family Channichthyidae) and *Dissosticus mawsoni* (family Nototheniidae). Understanding the role of the Cygb genes in species without Hb and Mb is very important and useful to the comprehension of the function of this protein, not yet clear.

In order, to accomplish the research activity, according to the above reported strategy, collaboration with several Institutions has been activated:

- Mass spectrometry experiments in collaboration with Prof. P. Pucci, University of Naples "Federico II", Italy.
- DLS experiments in collaboration with Prof. L. Paduano, University of Naples "Federico II", Italy.
- Cygb genes cloning in collaboration with Prof. C. Cheng, University of Illinois at Urbana-Champaign, USA.
- Cygbs expression and purification in collaboration with Prof. Sylvia Dewilde, University of Antwerp, Belgium.

CHAPTER 2

Materials and methods

2.1 Materials

CO was purchased from SON, Società Ossigeno Napoli spa; sodium dithionite, dithiothreitol (DTT), 4-vinylpyridine and Tris-hydroxymethyl-methylamine (Tris) were from Sigma Aldrich (Steinheim, Germany). Trypsin (EC 3.4.21.4) treated with L-1-tosylamide-2-phenylethylchloromethylketone from Cooper Biomedical; acetonitrile from Delchimica; oligonucleotides from the other chemicals were from Merck AG (Darmstadt, Germany), were analytical or reagent grade and without further purification.

2.2 Methods

2.2.1 Arctic fish Hbs

Specimens, hemolysates: Adult *G. morhua* and *L. reticulatus* were collected by bottom and midwater trawling from the R/V *Jan Mayen* (*L. reticulatus*: Greenland, 72°00'N, 21°01'W; *G. morhua*: Svalbard, 78°13'N). Blood was taken by heparinised syringes from the caudal vein. Saline-washed RBCs were kept frozen at -80°C until use.

Hemolysates were prepared from the erythrocytes, separated from the blood plasma by centrifugation (1067xg, 5 minutes) and washed twice with cold isotonic solution (10 mM TRIS-HCl pH 7.6, 1.7% NaCl). Lysis of erythrocytes was carried out by incubation in ipotonic solution (10 mM TRIS-HCl pH 7.6), followed by centrifugation for 20 minutes at 17065xg to discard membranes, cellular components and nucleic acids from the

supernatant and stripped of organophosphates with a Sephadex-G25 column (GE-Healthcare Bio-Sciences). All steps were carried out at 0−4°C (Tamburrini et al., 1994).

Globin separation: Separation of *L. reticulatus* globins was carried out by reverse-phase high-performance liquid chromatography (RP-HPLC) of stripped hemolysate on micro-Bondapak-C_{18} (0.39 cm×30 cm; Waters) columns, equilibrated with 45% acetonitrile, 0.3% Trifluoracetic acid (TFA) (Solvent A) and 90% acetonitrile, 0,1% TFA (Solvent B); absorbance at 546 nm and 280 nm was monitored (Verde et al., 2006). Addition of 100 mM DTT avoided polymerisation.

Amino-acid sequencing of α globin: Alkylation of sulfhydryl groups with 4-vinylpyridine, deacetylation of the α-chain N terminus and tryptic digestion were carried out as described (D'Avino and di Prisco, 1989; Tamburrini et al., 1996). Sulfydryl groups were treated with phenyl-isothiocyanate (PITC), Edman's reagent. Globins were solubilised in 500 mM TRIS-HCl pH 7.8, 2 mM EDTA, 6 M guanidine-HCl; cystine disulfide bridges were split by DTT in stoichiometric excess (10:1). After 1-hour incubation at 37°C, 4-vinylpyridine was added in stoichiometric excess (30:1) over DTT and the sample was incubated at room temperature for 45 minutes. The reaction was stopped by adding DTT in stoichiometric excess (2.5:1) over 4-vinylpyridine. Alkylated globins were purified by reverse-phase HPLC, on micro-Bondapak-C18 column equilibrated with 45% acetonitrile, 0.3% TFA (Solvent A) and 90% acetonitrile, 0,1% TFA (Solvent B). Tryptic digestion was carried out at 37°C, in 50 mM TRIS-HCl pH 8.0 and adding the trypsin (1 mg/ml in 1 mM HCl) three times every two hours, starting with a ratio 1:100 (enzyme:substrate) and reaching 1:33. The reaction was stopped by heating the solution at 100°C for a few minutes. Tryptic peptides were purified by RP-HPLC with a μBondapak C_{18} column (0.39 × 30 cm; Waters Associates), equilibrated with 0.1% TFA in water (Solvent A) and 0.08% TFA in 99.92% acetonitrile (Solvent B). Sequencing was performed with an Applied Biosystems Procise 492 automatic sequencer, equipped with on-line detection of phenylthiohydantoin amino acids.

Cloning and sequence analysis of β^2 globin cDNA. Total RNA was isolated from the spleen of *L. reticulatus* using TRI Reagent (Sigma) (Chomczynski et al., 1987). The cDNA of the β^2 globin was amplified by reverse transcriptase- polymerase chain reaction (RT-PCR) using oligonucleotides designed on the N-terminal regions as direct primers and

at the adaptor primer as the reverse primer (primer forward, AARTGGACNGAYAAAGA, and primer reverse pk72, CGGAGATCTCCAATGTGATGGGAAATTC). Amplifications of cDNA were performed with 2.5 units Taq DNA polymerase, 5 pmol each of the 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. Standard molecular biology techniques (Sambrook et al., 1989) were used in the isolation, restriction, and sequence analysis of plasmid DNA.

Purification of L. reticulatus *Hb*: Different purification attempts were tried to obtain the Hbs purified to homogeneity.

a. The hemolysate was loaded on MONO Q column using
   1. **(A)** 10 mM Tris-HCl pH 7.6 **(B)** 10 mM Tris-HCl pH 7.6 and 1 M NaCl
   2. **(A)** 10 mM Tris-HCl pH 7.6 **(B)** 10 mM Tris-HCl pH 7.6 and 400 mM NaCl
   3. **(A)** 10 mM Tris-HCl pH 7.6 and 1 mM DTT **(B)** 10 mM Tris-HCl pH 7.6, 1 mM DTT and 1 M NaCl
   4. **(A)** 10 mM Tris-HCl pH 7.6 and 1 mM DTT **(B)** 10 mM Tris-HCl pH 7.6, 1 mM DTT and 400 mM NaCl
   5. **(A)** 10 mM Tris-HCl pH 7.6 and 1 mM DTT **(B)** 10 mM Tris-HCl pH 7.6, 1 mM DTT and 250 mM NaCl

b. The same hemolysate was loaded on DEAE 52 fast flow column with
   1. **(A)** 10 mM Tris-HCl pH 7.6 **(B)** 10 mM Tris-HCl pH 7.6 and 300 mM NaCl

Mass spectrometry . Mass mapping of the α and β^1 chains of *L. reticulatus* was carried out by overnight trypsin digestion of the native protein in 50 mM ammonium bicarbonate buffer pH 8.0 at 37°C.

For disulfide bridges assignments, Hb aggregates purified by gel filtration were concentrated and digested overnight with trypsin in the same buffer used for chromatography (10 mM ammonium acetate pH 7.3) at 37°C.

In both cases, the peptide mixtures were directly analysed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and TOF-TOF mass spectrometry (MS) on an AB Sciex 4800 MALDI TOF-TOF mass spectrometer. Tandem MS analyses (MS/MS) were carried out on selected signals to confirm amino-acid sequences.

Gel filtration. DTT was added to CO-hemolysates of *L. reticulatus* and *G. morhua* in 10 mM Tris-HCl pH 7.6, at final concentration of 100 mM. The Hb concentration in the hemolysate was 0.06 mM on a haem basis. Gel filtration was carried out by Fast Protein Liquid Chromatography (AKTA-FPLC) with a Superose 6 column (GE-Healthcare). Elution was performed at 4°C, in 10 mM Tris-HCl pH 7.6, 200 mM NaCl.

UV-Visible spectroscopy. To evaluate the oxidation state of Hbs and calculate their concentration, UV-Visible electronic absorption spectra were acquired from 700 to 250 nm in a Cary 300 UV-Visible spectrophotometer (Varian). A typical absorption spectrum of HbA is characterised by strong maximum at 415-419 nm (Soret) and two maxima at 540 (ε = 13.4) and 569 nm (ε = 13.4) for HbCO, 541 (ε = 13.5) and 576 nm (ε = 14.6) for HbO_2.

Dynamic Light Scattering. Dynamic light scattering (DLS) experiments were performed with 0.06 mM (on a haem basis) *L. reticulatus* and *G. morhua* hemolysates, filtered through 0.22-μm Millipore filters, in 100 mM Tris-HCl/MES in the pH range 6.6 - 9.0 at 4°C. CO-hemolysates (800 μL) were flushed with CO and sodium dithionite was added at a final concentration of 1 mM. Deoxy hemolysates were prepared by photolysis of the CO-hemolysates. Samples were then placed in a dry box filled with nitrogen following addition of few crystals of sodium dithionite. The cuvettes were sealed with rubber caps.

DLS was performed with a setup of a Photocor compact goniometer, a SMD 6000 Laser Quantum 50 mW light source operating at 5325 Å, and a PMT and correlator from Correlator.com. All measurements were performed at 4.00±0.2°C in a thermostatted bath.
In DLS, the intensity autocorrelation function $g^{(2)}(t)$ is measured and related to the electric field autocorrelation $g^{(1)}(t)$ by the Siegert relation (Berne and Pecora, 2000):

$$g^{(2)}(t) = 1 + \beta \left| g^{(1)}(t) \right|^2 \qquad \text{(Eq. 1)}$$

where β (≤ 1) is the coherence factor, which accounts for the deviation from ideal correlation and depends on the experimental geometry. The parameter $g^{(1)}(t)$ can be written as the Laplace transform of the distribution of the relaxation rate Γ used to calculate the translational diffusion coefficient *D*

$$g^{(1)}(t) = \int_{-\infty}^{+\infty} \tau A(\tau) \exp\left(-\frac{t}{\tau}\right) \mathrm{d}\ln\tau \qquad \text{(Eq. 2)}$$

where $\tau = 1/\Gamma$. Laplace transforms were performed using a variation of CONTIN algorithm incorporated in Precision Deconvolve software. From the relaxation rates, the *z*-average of the diffusion coefficient *D* may be obtained as (Berne and Pecora, 2000)

$$D = \frac{\Gamma}{q^2} \qquad \text{(Eq. 3)}$$

where $q = 4\pi n_0/\lambda \sin(\theta/2)$ is the modulus of the scattering vector, n_0 is the refractive index of the solution, λ is the incident wavelength and θ represents the scattering angle. Provided that the solutions are quite dilute, the Stokes–Einstein equation, which rigorously holds at infinite dilution for spherical species diffusing in a continuum medium, may be used to evaluate the hydrodynamic radius R_H of the aggregates.

$$R_H = \frac{kT}{6\pi\eta D} \qquad \text{(Eq. 4)}$$

where *k* is the Boltzmann constant, *T* is the absolute temperature and η is the medium viscosity. We note that R_H in Eq. 4 for not spherical particles represents the radius of equivalent spherical aggregates with the same diffusion coefficient (Tyrrell and Harris, 1984). The number of tetramers in each aggregate was obtained by dividing the volume of aggregates by the tetramer volume.

O_2 binding. Hemolysate stripping was carried out by passage through a column of Sephadex G-25 (PD-10 Amersham), equilibrated with 10 mM HEPES pH 7.6. After this procedure, salts and organic phosphates have been removed. O_2 equilibria were measured in 100 mM MES/HEPES in the pH range 6.3–8.7, at 5 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 haem basis. An average standard deviation of ± 3% for values of O_2 affinity was calculated; experiments were performed in duplicate. To obtain stepwise O_2 saturation, a modified gas-diffusion chamber was used, coupled to cascaded Wösthoff pumps for mixing pure nitrogen with air (Weber et al., 1987). The pumps are connected to a

spectrophotometer Eppendorf, 1101 M model. Values of pH 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 effect of ATP was measured at a final ligand concentration of 3 mM, in excess with respect to tetrameric Hb. O_2 affinity (measured as p_{50}) and cooperativity (nHill) were calculated by linear fitting of the Hill plot.

For each experiment, one aliquot of CO-hemolysate was thawed, converted to the oxy form by exposure to light and O_2, and immediately used; For this purpose, the CO-hemolysate solution was placed in an ice bath and the gas phase was 100% O_2. Under gentle stirring, hemolysate was exposed to a light source (Sylvania Model SG-50 with a DWY lamp). No oxidation was detectable spectrophotometrically, indicating that final Met-Hb formation was negligible (<2%).

2.2.2 Antarctic fish Cygb

Cloning and sequencing of Cygb cDNA: Total RNA was isolated from *C. aceratus* brain and *D. mawsoni* retina and the Cygb cDNA was cloned in SmaI site of pBSII KS (-). The subcloning of Cygb cDNAs was tested in two different expression vectors: pET3a and pBAD. A PCR was performed on the plasmids using the 5' primer (GGGAATTCCATATGGAGAGGATGCAGGGAGAGG for pET3a and CCGCTCGAGATGGAGAGGATGCAGGGAGAGG for pBAD), with a NdeI and XhoI restriction sites for pET3a and pBAD respectively, and the 3' primer (CGCGGATCCTCACCCACTTGAGCTTGAG for pET3a and CCGGAATTCTCACCCACTTGAGCTTGAG for pBAD) containing a BamHI and EcoRI restriction sites for pET3a and pBAD, respectively. The PCR products were cleaned and cut with the restriction enzymes, then ligated into the expression vectors. The sequences were checked and the constructs were verified in correct position.

Expression of Cygb: Recombinant expression plasmids were transformed in the *Escherichia coli* BL21(DE3)pLysS (Invitrogen). The cells were grown overnight at 37°C in 6 ml L-broth (10 g/L tryptone, 5 g/L yest extract and 0.5 g/L NaCl) with 200 mg/L ampicillin and 30 mg/L chloramphenicol. The grown cultures were poured into a flask containing 250 ml terrific broth (TB) medium (1.2% bactotryptone, 2.4% yeast extract,

0.4% glycerol, 17 mM KH_2PO_4 and 72 mM K_2HPO_4 $3H_2O$), 200 mg/L ampicillin and 30 mg/L chloramphenicol. The cultures were shaken at 160 rpm at 25°C. The induction was at A_{600} > 1.0 O.D by the addition of isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.4 mM. The expressions were continued overnight. The grown cells were harvested (20 min at 3220 x g) and resuspended in 12 ml lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM ethylenediaminetetracetic acid (EDTA), 0.5 mM DTT).

Purification of Cygb: The resuspended cells were exposed to three freeze-thaw steps and sonicated (1 min at 60 Hz and 3 sec pulses) in ice until completely lysed. The extracts were clarified by low and high speed centrifugation. Different purification attempts were tried before to find the best conditions.

1. The samples were purified before by 60% ammonium sulfate precipitaton. The pellets were dissolved in 5 mM Tris-HCl pH 7.5 and dialysed overnight against the same buffer.
2. The dialysed material was mixed in bulk with an excess diethylaminoethyl (DEAE) Sepharose matrix in a funnel. The unbound material was eluted with 5 mM Tris-HCl pH 7.6 and then the Cgbs were eluted with 300 mM NaCl and 5 mM Tris-HCl pH 7.5. Afterwards, the Cygbs were concentrated.
3. The concentrated material was loaded on a Sephacryl S-200 high resolution column. The column was equilibrated at 4°C in 50 mM Tris-HCl pH 7.6, 150 mM NaCl and 5 mM EDTA. The fractions with Cygb were joined and concentrated.
4. The material was loaded on HiTrap DEAE FF column, trying two different buffers:
   (A) 20 mM Tris HCl pH 7.6 **(B)** 20 mM TrisHCl pH 7.6 and 300 mM NaCl
   (A) 20 mM Sodium Phosphate pH 6.8 **(B)** 20 mM Sodium Phosphate pH 6.8 and 300 mM NaCl
5. The same material was loaded on Superdex TM-75 column. The buffer was the same used for the Sephacryl S-200 high resolution column
6. The same sample was loaded on Q Sepharose FF and Mono Q columns, using two different types of buffer:
   (A) 20 mM Tris HCl pH 7.6 **(B)** 20 mM Tris HCl pH 7.6 and 1.0 M NaCl
   (A) 20 mM Tris HCl pH 7.0 **(B)** 20 mM Tris HCl pH 7.0 and 1.0 M NaCl
7. The sample was collected, joined and concentrated, after loaded on Mono S column using:

(A) 50 mM Sodium Phosphate pH 6.8 **(B)** 50 mM Sodium Phosphate pH 6.8 and 1.0 M NaCl

8. All sample was loaded on Superdex TM-200 column with 50 mM Tris-HCl pH 7.6, 150 mM NaCl and 5 mM EDTA

UV-Visible spectroscopy: To experience the coordination state of Cygb and check the possible pH- and temperature- dependence, UV-Visible electronic absorption spectra were acquired from 700 to 350 nm in a Cary 300 UV-Visible spectrophotometer (Varian). The pH range tested was 6.0 – 9.0 and the temperature range was 4°C – 80°C. The buffers used were 20 mM Tris-HCl/MES. The spectra were acquired in the absence and presence of exogenous ligands such as CO. Samples were about 5 μM on a heme basis in 100 mM buffer at different pH. The ferric form was slowly reduced by ten-fold excess sodium dithionite after bubbling nitrogen for 15 min in 1-cm optical-pathway cuvettes. The CO form was achieved by equilibration of reduced samples under 1 atm of CO for 15 min.

CHAPTER 3

Results and discussion

3.1 Arctic fish Hbs

3.1.1 Globins and primary structure

Only a single Hb was detected in the fresh hemolysate of *L. reticulatus* by cellulose acetate electrophoresis, although the Blue Native PAGE of frozen and thawed CO-hemolysate revealed multiple bands (Fig. 3.1), suggesting the formation of polymers during freezing.

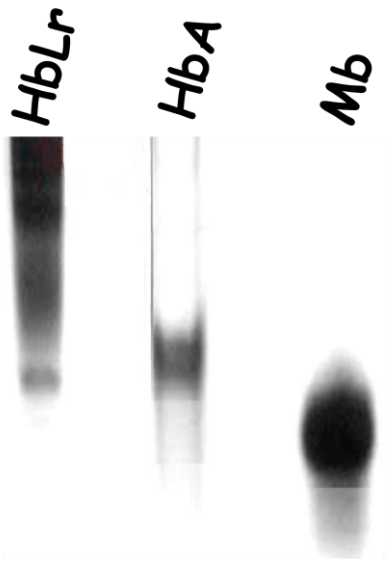

Fig. 3.1: Blue Native PAGE of *L. reticulatus* (HbLr, on the left) with HbA (in the middle) and Mb (on the right) as markers.

The RP-HPLC profile of the CO-hemolysate in the polymerised form (Fig. 3.2a) showed the presence of the α chain and of an unresolved peak corresponding to the β chains, as established by MS and N-terminal amino-acid sequencing. In the presence of

100 mM DTT, RP-HPLC (Fig 3.2b) displayed three different globins, namely the α chain and two different β chains (β^1 and β^2), exhibiting slight heterogeneity.

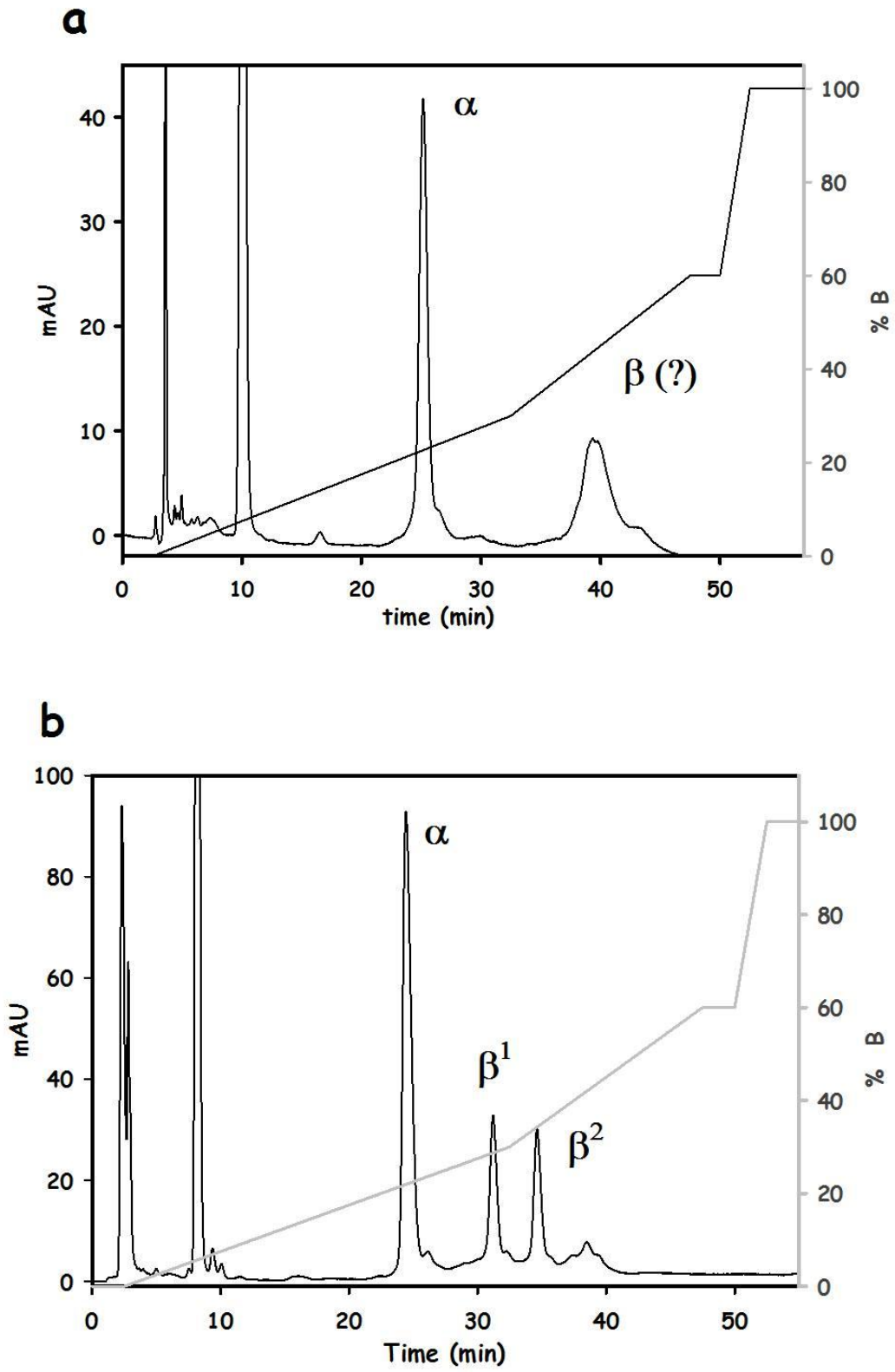

Fig. 3.2: The RP-HPLC profiles of a) the CO-hemolysate in the polymerized form b) the CO-hemolysate in presence of DTT

The amino-acid sequences of the α and β chains of *L. reticulatus*, in comparison with other sequences, are reported in Fig. 3.3a and 3.3b. The primary structure of the α chain was established by alignment of tryptic peptides, homology with fish globins and confirmed by their mass mapping. DNA sequencing, obtained from RNA isolated from *L. reticulatus* spleen, was utilized only for the β^2 chain. The β^1 chain was digested with trypsin and its peptide mixture was directly analyzed by MALDI-TOF and TOF-TOF MS, to obtain whole amino-acid sequencing. The accurate mass values of the tryptic peptides were mapped onto the anticipated amino-acid sequence of the β^2 chain used as template. MS/MS analyses were carried out on the selected signals displaying mass differences from the β^2 peptides, leading to the definition of their sequences. The molecular masses were 15,663.3 ± 0.3 Da for the α and 16,121.5 ± 0.3 and 16,067.4 ± 0.6 Da for the β^1 and β^2 chains, respectively, in perfect agreement with the theoretical values calculated as the basis of the primary structures. The N terminus of the α chain was not available to Edman degradation because of the presence of a blocking acetyl group. The two β chains differ in only four positions, that are Ala44 → Thr, Ser50 → Thr, Ala51 → Pro and Leu58 → Pro. For the sake of simplicity, I refer to only one Hb, and not to two Hbs, because I assume that this heterogeneity defines a genetic variant and not a functionally distinct Hb. In fact, because of a few mutations all attempts to purify non-polymerized Hb to homogeneity were unsuccessful. The globins have several substitutions, important for Bohr and Root effects, with respect to other from vertebrate. Among the functionally important residues suggested to be involved in the molecular mechanism of the Bohr and Root effects in fish Hbs (Camardella et al., 1992), Ser β93 F9, Glu β94 FG1, and Gln β144 HC1 are conserved in the β chains, whereas His β146 HC3 is replaced by Cys. Of the Asp α48 CD6/His α55 E3 and His β69 E13/Asp β72 E16 pairs, supposed to contribute to the Root effect in fish Hbs (Mazzarella et al., 2006a; Yokoyama et al., 2004), only the latter is conserved. In the $\alpha_1\beta_2$ ''dovetailed'' switch region formed in HbA by Pro α44 CD2, Thr α38 C3, Thr α41 C6, and His β97 FG4, Pro α44 CD2 is replaced by Ser and Thr α38 C3 by Gln. Val β60 E4, considered to be invariant in vertebrates, including most teleosts, is replaced by Ile. Val β67 E11, usually present at the distal side of the haem, is replaced by Ile. This substitution may produce functional subunit heterogeneity, as reported in Hb of temperate *Chelidonichthys kumu* (Fago et al., 1993) and in cathodic Hb of Antarctic *Trematomus newnesi* (Mazzarella et al., 2006b). In HbA mutants, the bulky side chain of Ile β67 E11 blocks the access of O_2 to the β chain, significantly lowering the association (and equilibrium) constant in both the T (Nagai et al., 1987) and R states (Mathews et al., 1989).

In deoxy HbA, Val β67 E11 overlaps the ligand binding site and is considered to play a key role in controlling the O_2 affinity. The α and β chains of *L. reticulatus* contain several Cys residues often absent in other teleosts, in positions α105 G11, α131 H13, β31 B13, β109 G11, β121 GH4, and β146 CH3. The previously published (Verde et al., 2006) amino-acid sequences of the two α and the two β chains constituting the three Hbs of *G. morhua* are also reported in Fig. 3.3a and 3.3b. Similar to *L. reticulatus*, they are unusually rich in Cys. Despite the general trend toward reduction in His content in teleost Hbs (Berenbrink et al., 2005), the β^2 chain of *G. morhua* contains two extra His residues, His β10 A7 and His β77 EF1 (Verde et al., 2006). These residues are absent in most fish Hbs with the exception of *L. reticulatus* β globins, which have His β77 EF1. Recently, high number of globin genes (four α and five β) has been found in *G. morhua* species, suggesting a response to environmental challenges and altering their level expression (Borza et al., 2009). Moreover, Andersen showed that the *G. morhua* β^1 globin polymorphism (Met55Val and Lys62Ala) leads two distinct behaviors: a) to low O_2 affinity at high temperatures for those fish populations that inhabit the cold Arctic waters (with Val55-Ala62) and b) to no temperature-dependence for the non-Arctic populations (Met55-Lys62) (Andersen et al., 2009). At high temperatures, in Arctic fish as *G. morhua* the biosynthesis of the Val55-Ala62 globin is increased by a molecular compensatory mechanism to maintain the total O_2-carryng capacity (Gamperl et al., 2009). This is an example of co-evolution of structural and regulatory adaptation with a relationship between temperature and functional molecular variation (Star et al., 2011).

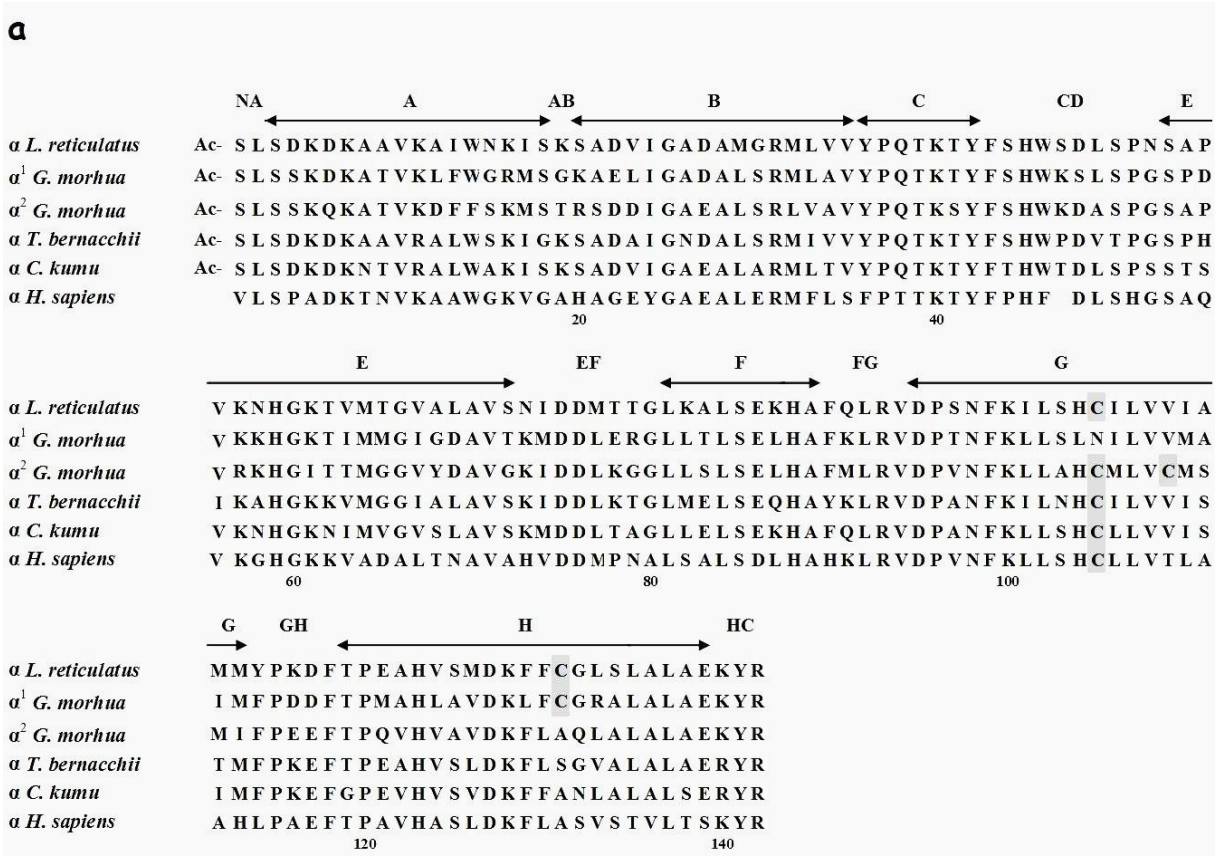

Fig. 3.3a: Amino-acid sequence of the α chain of *L. reticulatus* and *G. morhua* (Verde et al. 2006), Antarctic *T. bernacchii* (Camardella et al., 1992) and temperate *C. kumu* (Fago et al., 1993). Cys residues are in light grey boxes.

b

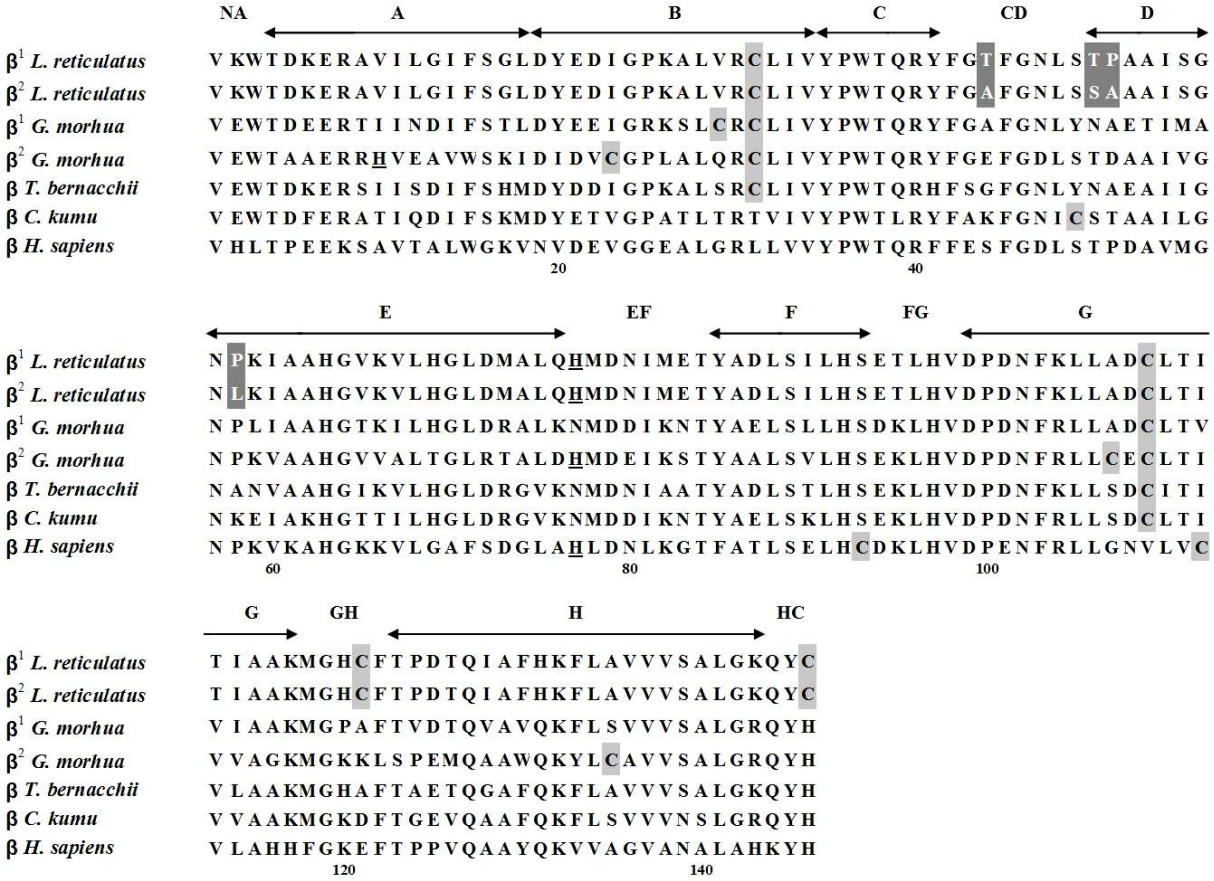

Fig. 3.3b: Amino-acid sequence of the β chains of *L. reticulatus* and *G. morhua* (Verde et al. 2006), Antarctic *T. bernacchii* (Camardella et al., 1992) and temperate *C. kumu* (Fago et al., 1993). Cys residues are in light grey boxes. The differences between the two β chains of *L. reticulatus* are in white, in dark grey boxes.

3.1.2 Purification attempts

The manipulation of the *L. reticulatus* hemolysate was tough. The primary structure analysis revealed a polymorphism that made the purification not feasible, despite the numerous attempts. Thus, the hemolysate was used to characterise the aggregation behaviour.

During the first year several purification attempts were extensively conducted, trying several strategies by different chromatography columns and conditions.

The results of the purification procedures were unsuccessful. In fact, HPLC chromatography of different fractions collected during the purification attempts showed the coexistence of all the globins. Therefore, the primary strategy to analyse the isolated component Hbs was replaced by an alternative strategy, in which the hemolysate was considered. Despite this strategy does not factorize the contribution of each component to the overall functional behavior, it provides anyway a picture of the physiologically relevant properties (related to the coexistence of all the components in the blood).

The difficulty in purifying the *L. reticulatus* hemolysate also in presence of DTT indicates that the unsuccessful purification can be due to the high sequence identity of the two β chains (different for only 4 residues) and not in the formation of aggregates.

3.1.3 The polymerisation process

Gel filtration of the CO-hemolysate of *L. reticulatus*, in 10 mM Tris-HCl pH 7.6, revealed multiple large peaks, suggesting formation of polymers (Fig 3.4a). The first three fractions contained higher-molecular-mass components, whose spectral features excluded re-oxidation of the iron. The last small fraction contained non- polymerised Hb that had identical elution volume to that of HbA. The chromatogram suggested formation of polymers of different molecular size. Polymerisation essentially appeared to depend upon formation of inter-molecular disulfide bonds because the first three fractions disappeared upon addition of DTT (Fig. 3.4b) and were replaced by the tetramer. The RP-HPLC of the tetramer obtained from gel filtration showed three well separated peaks, an α chain and the two β chains in equal amounts, similarly to the globin pattern of the hemolysate in the presence of DTT. An important note is that special attention was paid when DTT was

added to *L. reticulatus* Hb solutions, because of Hb degradation (greenish colour) followed by precipitation after some hours later the addition.

In contrast, gel filtration of the CO-hemolysate of *G. morhua* at pH 7.6 in the absence of DTT revealed a much lower amount of polymers (Fig. 3.5a) and the high-molecular-mass components did not decrease upon addition of 120 mM DTT (Fig. 3.5b). Presumably, the absence of DTT effect is not due to involvement of the Cys residues, despite their high number in both chains, but to different type of interactions, not really clear at the moment. In the first extensive study of the O_2-transport system of three Arctic species of the family Gadidae, namely the Arctic cod *Arctogadus glacialis*, the polar cod *Boreogadus saida*, and the Atlantic cod *G. morhua* (Verde et al., 2006), these fish have identical multiplicity of Hbs. The ion-exchange chromatography of the three hemolysates yielded similar elution patterns, showing one broad band, indicative of unresolved Hbs. Many procedures were attempted to purify the different components to homogeneity, but they were unsuccessful, with the exception of the third component. Hence, concentration-dependent equilibria between dimers or pH-dependent aggregation between tetramers were hypothesised (Verde et al., 2006).

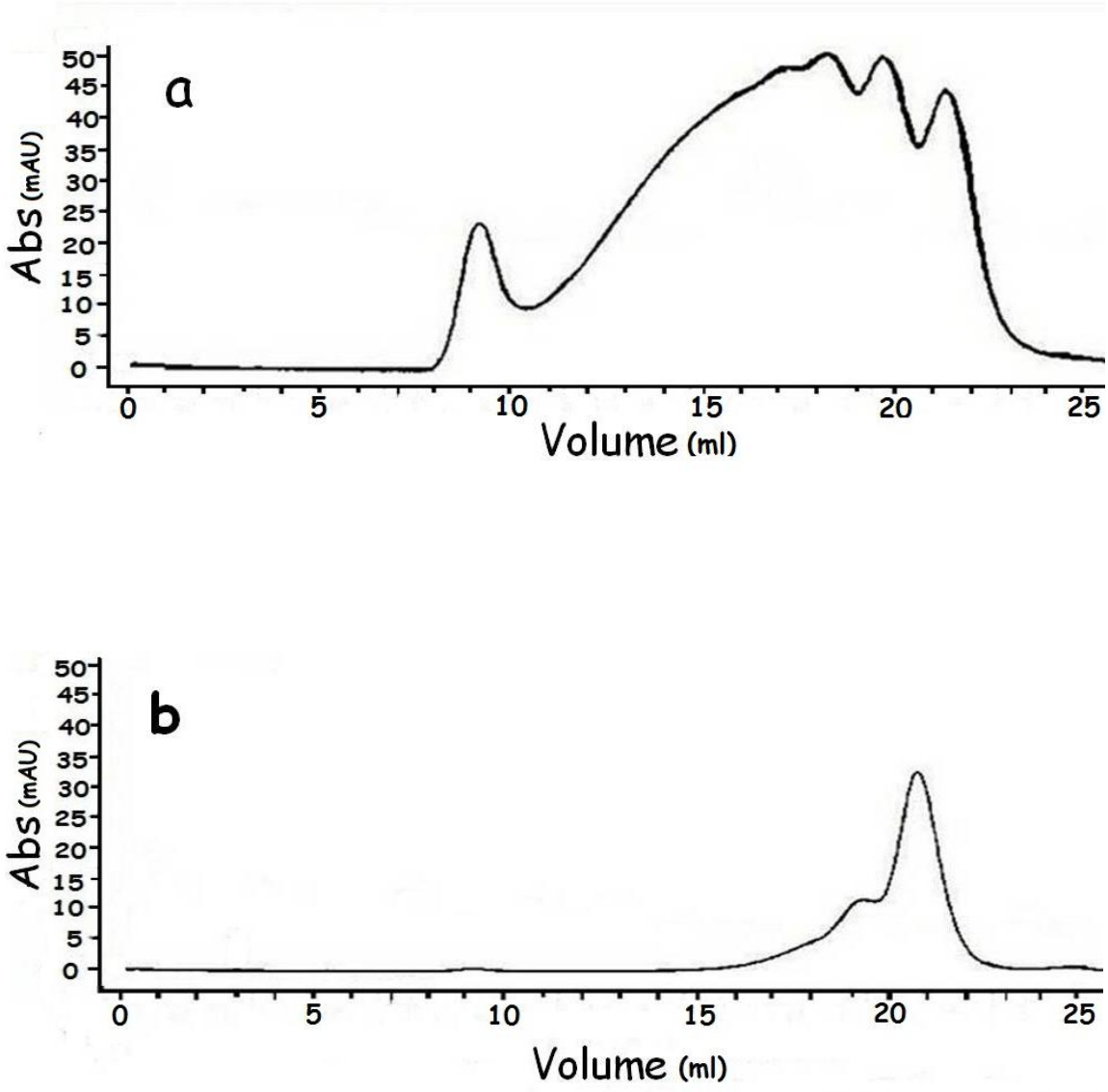

Fig. 3.4: Gel filtration of the CO-hemolysate of *L. reticulatus* a) without DTT and b) upon addition of DTT

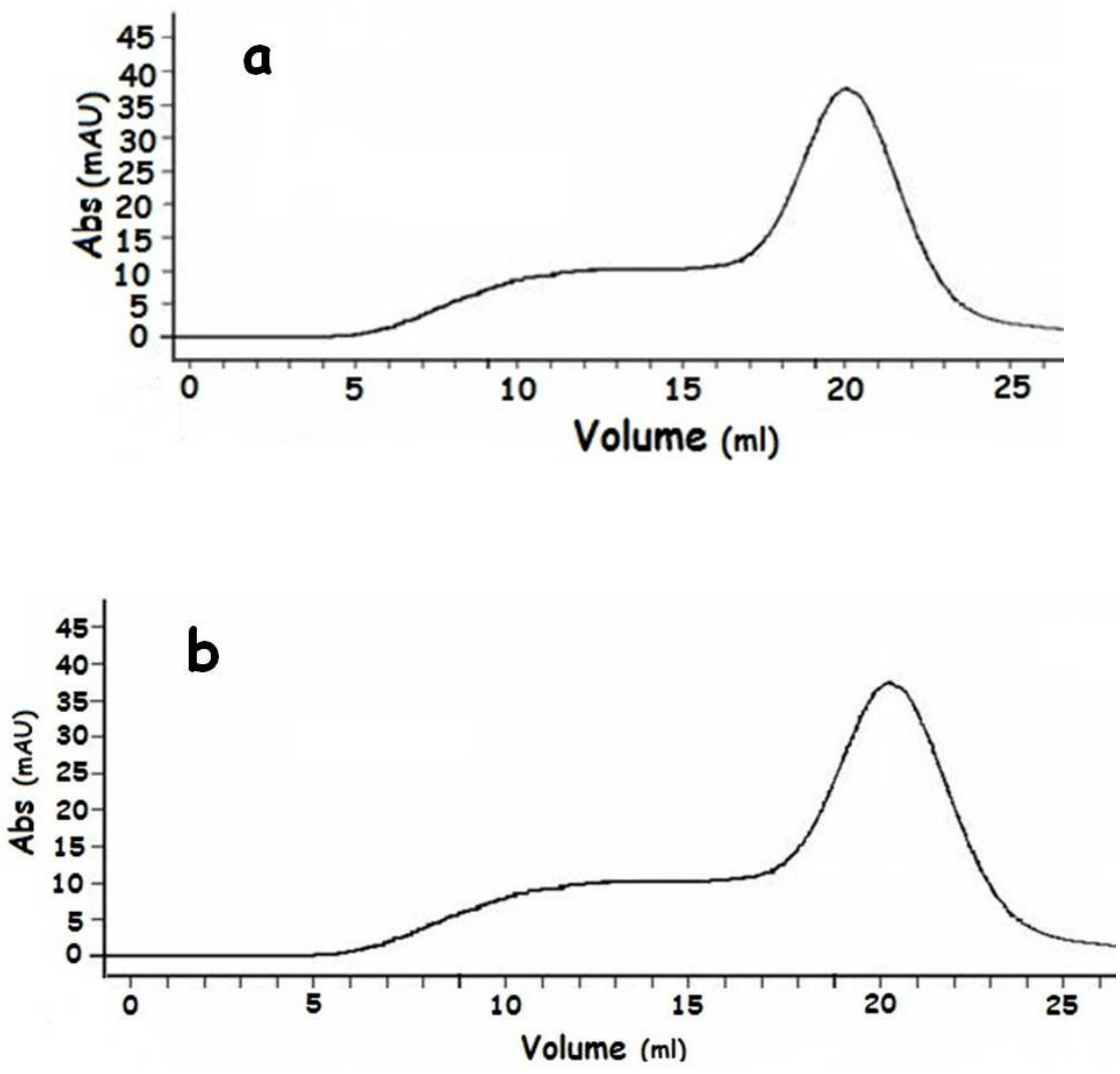

Fig 3.5: Gel filtration of the CO-hemolysate of *G. morhua* a) without DTT and b) upon addition of DTT

3.1.4 Mass spectrometry of *L. reticulatus* globins

MS experiments were conducted in collaboration with the Prof. P. Pucci of the University of Naples, "Federico II". The chemical nature of the oligomers of *L. reticulatus* Hb was investigated by mass mapping of their tryptic peptides. The high-molecular-mass, DTT-reduced aggregates were isolated by gel filtration and directly digested with trypsin. The peptide mixture was analysed by MALDI-TOF MS (Fig. 3.6), revealing the occurrence of peptides belonging to both α and β globin chains. A number of mass signals in the spectra could not be associated to any linear peptide within the amino-acid sequence of the globins and were tentatively interpreted as disulfide-containing fragments. On the basis of their unique mass values, these signals were identified as S-S bridged peptides and their assignments are listed in Table 1, together with the Cys pairs involved in the cross-links. Selected signals were submitted to MS/MS analyses in order to confirm the assignments.

Mass spectral analyses confirmed the hypothesis that the Hb oligomeric species of *L. reticulatus* were essentially formed by intermolecular S-S bridges. Further support to this hypothesis is also provided by homology modelling, indicating that the distances between pairs of Cys residues are incompatible to form intramolecular S-S bridges (L. Boechi, personal communication). As expected, the vast majority of the Cys residues involved in disulfide-bridge formation belong to the β globins, suggesting higher reactivity of these residues than those of the α chain, a well known behaviour similar to human globins. A single Cys of the α chain, Cys α105 G11 was indeed found involved in an S-S bridge with Cys β146 CH3. The almost identical sequences of the two β chains impaired to ascertain which chain was involved in each bridge, with the exception of the peptide pair associated with the mass value at m/z 4490.2 (see Table 1). This signal corresponds to the β^2 peptide 31-59 joined to the β^1 (or β^2) fragment 105-117, as the two β globins showed different sequences in the 44-58 region. Many S-S bridges were formed by C-terminal Cys β146 CH3 of the β chain. This behaviour is similar to that found in the human variant Hb Rainier, where βC-terminal Tyr is substituted by Cys, leading to an intramolecular disulfide with Cys β93 F9 (Carbone et al., 1999).

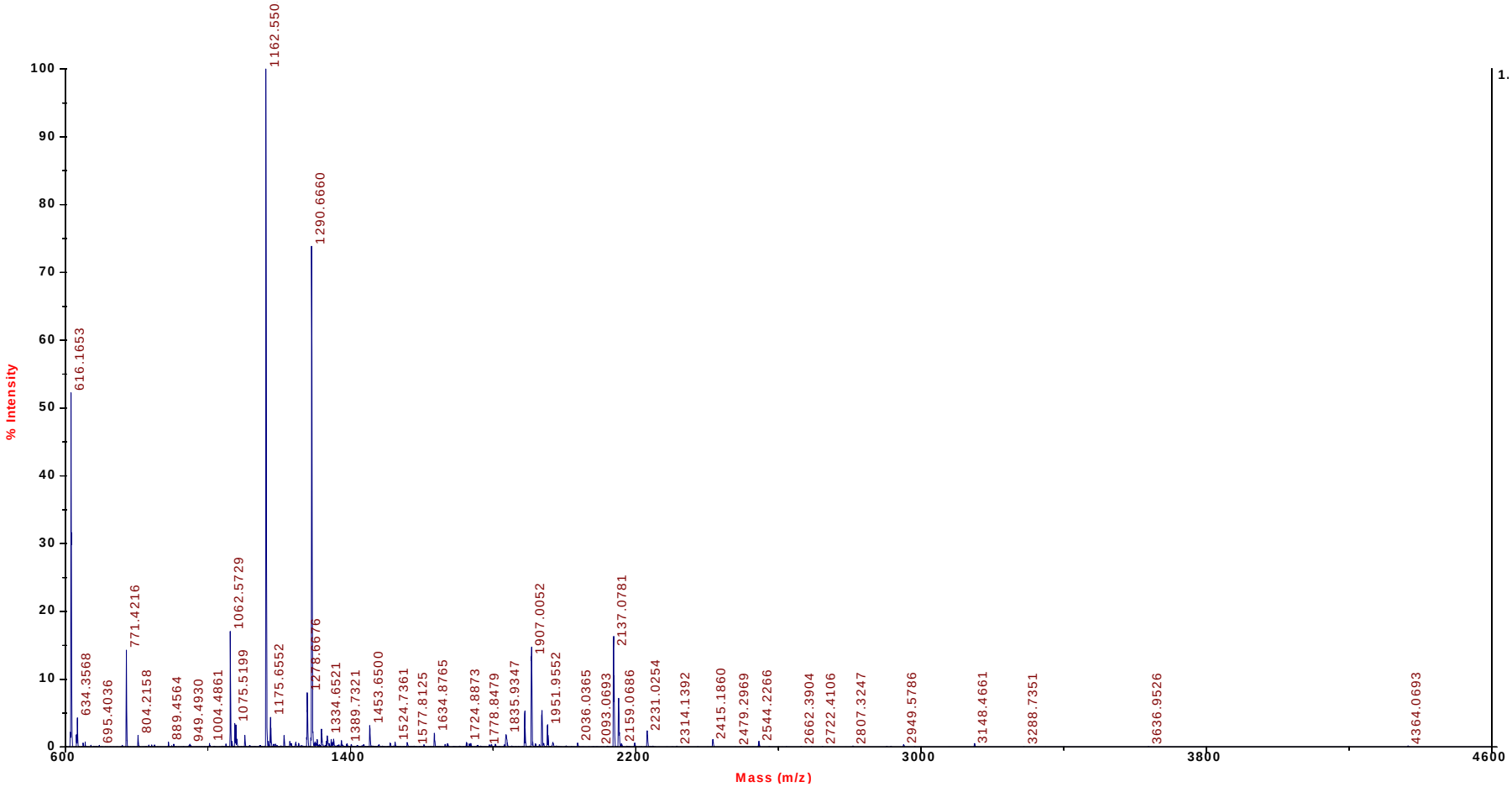

Fig. 3.6: Peptide mixture of oligomers of *L. reticulatus* Hb analysed by MALDI-TOF MS

Table 1

Mass signals of S-S bridged peptides and Cys residues involved in tryptic hydrolysis of high-molecular-weight aggregates of *L. reticulatus* Hb

MH^+	Peptide pair	Cys residues involved
4490.2	β^2 (31-59) + β (105-117)	β^2 Cys 31-β Cys109
2621.4	β (31-40) + β (105-117)	β Cys31-β Cys109
1688.8	β (31-40) + β (144-146)	β Cys31-β Cys146
1755.9	β (105-117) + β (144-146)	β Cys109-β Cys146
3075.5	β (105-117) + β (118-132)	β Cys109-β Cys121
3598.7	α (101-128) + β (144-146)	α Cys105-β Cys146
3462.5	β (118-132) + β (118-132)	β Cys121-β Cys121

3.1.5 Dynamic Light Scattering

The DLS experiments were carried out in collaboration with the Prof. L. Paduano of the University of Naples "Federico II". Globin association in the hemolysates of *L. reticulatus* and *G. morhua* as a function of coordination state (CO and deoxy), pH (6.6-9.0) and addition of 120.0 mM DTT (final concentration) at 4°C, was also investigated by DLS. The hemolysates showed multimodal distributions of three-four aggregates, named **I**, **II**, **III** and **IV** according to increasing size. HbA was used as control of non-aggregating globin, with a hydrodynamic radius (R_H) 3.5 ± 0.2 nm.

At pH 7.6 and in the absence of DTT, the *L. reticulatus* CO-hemolysate showed three aggregates of different size (**II** at 8.5 ± 0.8, **III** at 34 ± 5, and **IV** at 85 ± 12 nm) (Fig. 3.7A). Addition of DTT (Fig. 3.7B) fastly led to formation of an additional species (**I**) and a significant variation of the aggregation size (**I** at 3.3 ± 0.5, **II** at 14 ± 1, **III** at 33 ± 5, and **IV** 174 ± 90 nm). Upon DTT removal, almost instantaneous disappearance of **I** and slow return to the initial aggregation distribution occurred. The additional diffusing particle **I,** as a consequence of DTT-induced reduction of disulfide bridges, can be associated to the single Hb tetramer of *L. reticulatus* (3.3 nm) (Pan et al., 2007). Due to technical limitations (at higher concentrations multiple scattering occurs making analysis of the results unreliable) it was not possible to investigate the effect of concentration on aggregation.

Indeed, DLS experiments confirmed the significant role of intermolecular disulfide bridges in the aggregation behaviour of the hemolysate of *L. reticulatus* and defined the multimodal aggregate distribution (Table 2), showing the ability to produce polymers of the large number of Cys residues in the α and β chains of the *L. reticulatus* hemolysate. R_H in both deoxy and CO hemolysates were quite invariant upon pH variation (from 6.6 to 9.0), suggesting no crucial involvement of protonable groups in the aggregation mechanism.

In the absence of DTT, the CO-hemolysate of *G. morhua* (Fig. 3.7C) showed three aggregates (**I** at 3.4 ± 0.3, **II** 88.0 ± 5.0, **III** 421 ± 12 nm). The number of aggregates and their R_H were insensitive to DTT (Table 2), suggesting that involvement of Cys in the aggregation mechanism is not crucial, despite their high content, which is comparable in number (but not in position) to that of the *L. reticulatus* sequence. Moreover, the dependence on the concentration was demonstrated. In fact, as expected, at higher Hb concentration, the relative population **II**/**I** and **III**/**I** increased.

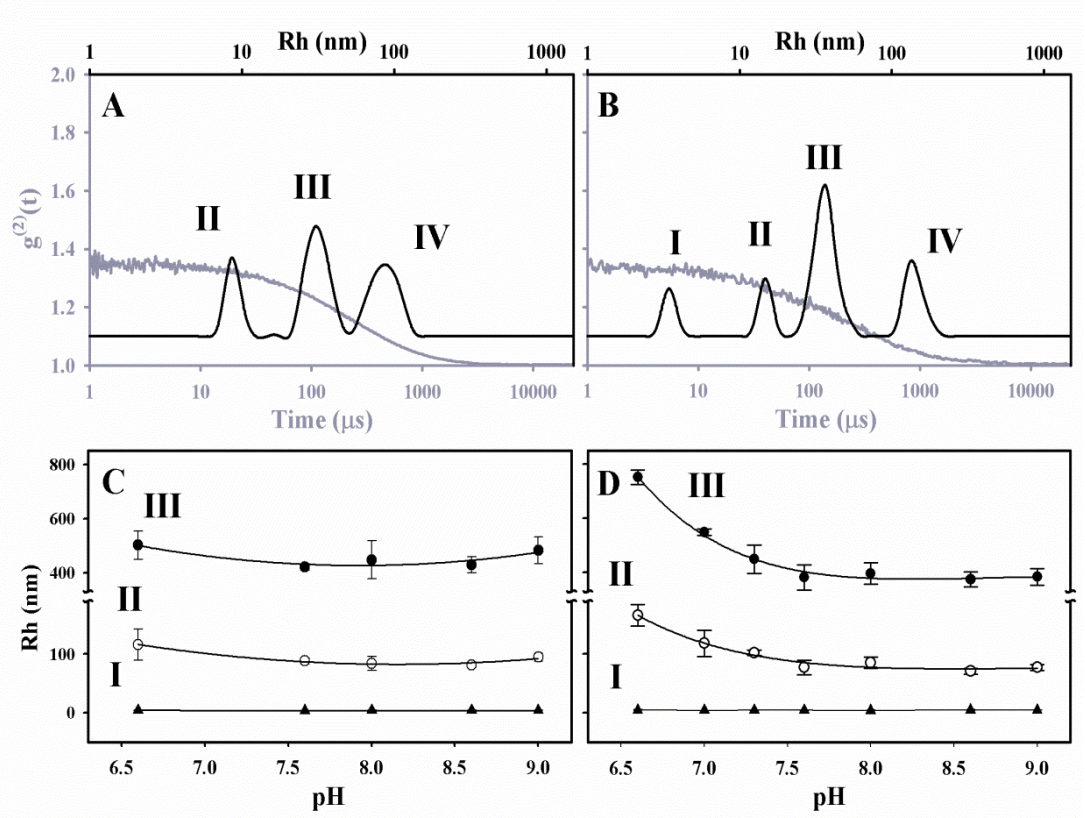

Fig. 3.7: DLS characterisation of the hemolysates of *L. reticulatus* (A, B) and *G. morhua* (C, D). (A) CO and (B) deoxy state of *L. reticulatus* Hb with the distribution of aggregates as a function of hydrodynamic radius, R_H, (in black), and examples of the correlation function, $g^{(2)}(t)$, as a function of time for both states (in gray). (C) CO and (D) deoxy state of *G. morhua* Hb with R_H as a function of pH for each multimodal distribution of aggregates (**I**, **II** and **III**).

In contrast to *L. reticulatus*, the hemolysate of *G. morhua* exhibited modulation of the aggregation behaviour. In fact, the *G. morhua* hemolysate showed dependence of aggregation behaviour on pH, particularly in the deoxy state (Fig. 3.7D). As pH decreased, the *G. morhua* population **I** distribution decreased in favour of the larger aggregates **II** and **III**, whose R_H increased (Table 2). The dependence on pH suggests a significant role of protonable groups on the surface of the Hb in the deoxy state in the aggregation mechanism. Indeed, despite the general trend of reduction in His content in teleost Hbs, suggested to be an important step in the evolution of the O_2-transport system (Berenbrink et al., 2005; Pan et al., 2007), the analysis of the amino-acid sequences of *G. morhua* globins (Verde et al., 2006) indicates that one of the two β globins contains two extra His residues (His β7 A7 and His β77 EF1) located on the surface of the protein (Koldkjaer and Berenbrink, 2007; Berenbrink, 2006).

Table 2

R_H of each *L. reticulatus* and *G. morhua* aggregate in CO and deoxy state at different pH. In bold, R_H after DTT addition. In brackets, number of Hb tetramer in the aggregate.

	L. reticulatus								***G. morhua***					
	CO state				**Deoxy state**				**CO state**			**Deoxy state**		
pH	**R_H (nm) I**	**R_H (nm) II**	**R_H(nm) III**	**R_H (nm) IV**	**R_H (nm) I**	**R_H (nm) II**	**R_H (nm) III**	**R_H (nm) IV**	**R_H (nm) I**	**R_H (nm) II**	**R_H (nm) III**	**R_H (nm) I**	**R_H (nm) II**	**R_H (nm) III**
6.6	–	7.8 ± 0.5 (11)	32 ± 5 (7.6E2)	93 ± 3 (1.8E4)	4.5 ± 0.4 (2)	17 ± 3 (1.1E2)	78 ± 7 (1.1E4)	385 ± 93 (1.3E6)	3.9 ± 0.4 (1)	116 ± 26 (3.6E4)	501 ± 51 (2.9E6)	4.3 ± 0.6 (1)	166 ± 18 (1.1E5)	752 ± 27 (9.9E6)
7.0	–	–	–	–	–	–	–	–	–	–	–	3.5 ± 0.1	118 ± 22	548 ± 12
												3.5 ± 0.6 (1)	**121 ± 60** (3.8E4)	**507 ± 150** (3.8E6)
7.3	–	–	–	–	–	–	–	–	–	–	–	3.9 ± 0.3 (1)	102 ± 4 (2.5E4)	449 ± 53 (2.1E6)
7.6	–	8.5 ± 0.8	34 ± 5	85 ± 12	4.9 ± 0.6	20 ± 3	71 ± 7	354 ± 66	3.4 ± 0.3	88 ± 5	421 ± 12	3.8 ± 0.3	77 ± 13	382 ± 47
	3.3 ± 0.1	**14 ± 1** (14)	**33 ± 5** (9.2E2)	**174 ± 90** (1.4E4)	**3.3 ± 0.6** (3)	**13 ± 4** (1.8E2)	**49 ± 4** (8.3E3)	**139 ± 4** (1E6)	**3.5 ± 0.1** (1)	**89 ± 6** (1.6E4)	**453 ± 29** (1.7E7)	**3.4 ± 0.6** (1)	**86 ± 9** (1.1E4)	**400 ± 20** (1.3E6)
8.0	–	–	–	–	–	–	–	–	3.5 ± 0.3 (1)	84 ± 12 (1.3E4)	447 ± 70 (2E7)	3.4 ± 0.3	85 ± 10	397 ± 40
												3.5 ± 0.3 (1)	**72 ± 4** (1.4E4)	**360 ± 6** (1.4E6)
8.6	–	6.9 ± 0.3	23 ± 2	103 ± 12	4.9 ± 0.3	20 ± 3	80 ± 11	289 ± 78	3.6 ± 0.4	81 ± 4	429 ± 30	4.2 ± 0.6	71 ± 5	375 ± 28
	3.6 ± 0.9	**19 ± 5** (8)	**102 ± 10** (2.8E2)	**269 ± 68** (2.5E4)	**3.3 ± 0.2** (3)	**12.7 ± 2** (1.8E2)	**60 ± 10** (1.2E4)	**240 ± 90** (5.6E5)	**3.2 ± 0.3** (1)	**86 ± 6** (1.2E4)	**424 ± 50** (1.7E7)	(1)	(8.3E3)	(1.2E6)
9.0	–	–	–	–	–	–	–	–	3.6 ± 0.3 (1)	95 ± 7 (2E4)	482 ± 50 (2.6E6)	4.0 ± 0.2 (1)	77 ± 5 (1.0E4)	384 ±30 (1.3E6)

3.1.6 O_2 binding

The single non-polymerised Hb without DTT is impossible to obtain because of the high reactivity of Cys residues and the difficulty in blocking them. Moreover, the DTT is oxidised by air and interferes with the O_2 binding measurements, for this reason the functional studies were only performed on the polymerised form of *L. reticulatus* Hb. The O_2 binding experiments were performed at 5°C and 10°C, in the absence and presence of allosteric physiological effectors, chloride and organophosphates (ATP) (Fig. 3.8 and 3.9). The Bohr effect was low, and the effectors did not significantly enhance it. In the presence and in absence of the effectors for every investigated pH, the nHill was close to 1.5, reflecting low levels or lack of subunit cooperativity. Therefore, the key role of polymerisation in the lack of Bohr effect in *L. reticulatus* Hb cannot be uniquivocally deduced, because Cys in place of His at the C terminus of this Hb may also substantially decrease such effect. In fact, in human HbA, the main Bohr groups are N-terminal Val α1 NA1 and C-terminal His β146 HC3, which account for about 30% and 50–65% of Bohr effect, respectively (Perutz and Brunori, 1982), but fish Hbs have acetyl-Ser in position α NA1, therefore, the decreased Bohr effect observed in *L. reticulatus* with respect to other fish Hbs may be due to the His → Cys β146 HC3 substitution; however, the role of His β146 HC3 residue in eliciting the Root effect is controversial (Fago et al., 1993).

The previously published data on *G. morhua* were integrated by additional functional studies on the hemolysate (Fig. 3.10), which contains partially polymerised Hb forms (Verde et al., 2006). Experiments were performed at 5°C and 10°C, in the absence and presence of allosteric effectors. A strong Bohr effect was observed, and enhancement by organophosphates was high. In the whole pH range, the nHill was close to one, reflecting very low levels, or apparent lack of subunit cooperativity.

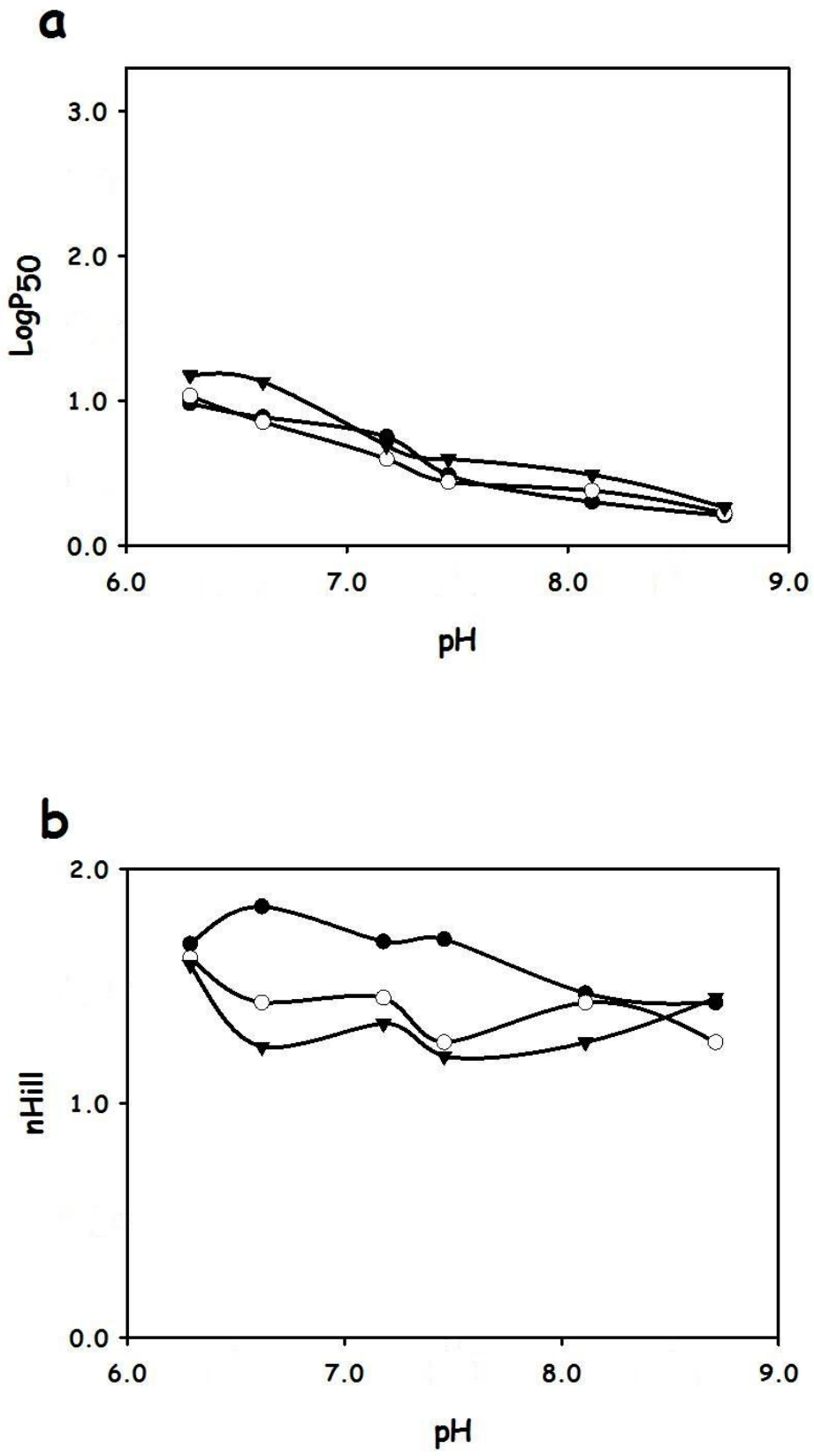

Fig 3.8: a) O_2 equilibrium isotherms (Bohr effect) and b) subunit cooperativity at atmospheric pressure as a function of pH of *L. reticulatus* hemolysate. 100 mM HEPES at 5°C, in the absence of effectors is shown by the open circles, in the presence of 100 mM NaCl by the filled circles, 100mM NaCl, 3mM ATP by the filled triangles.

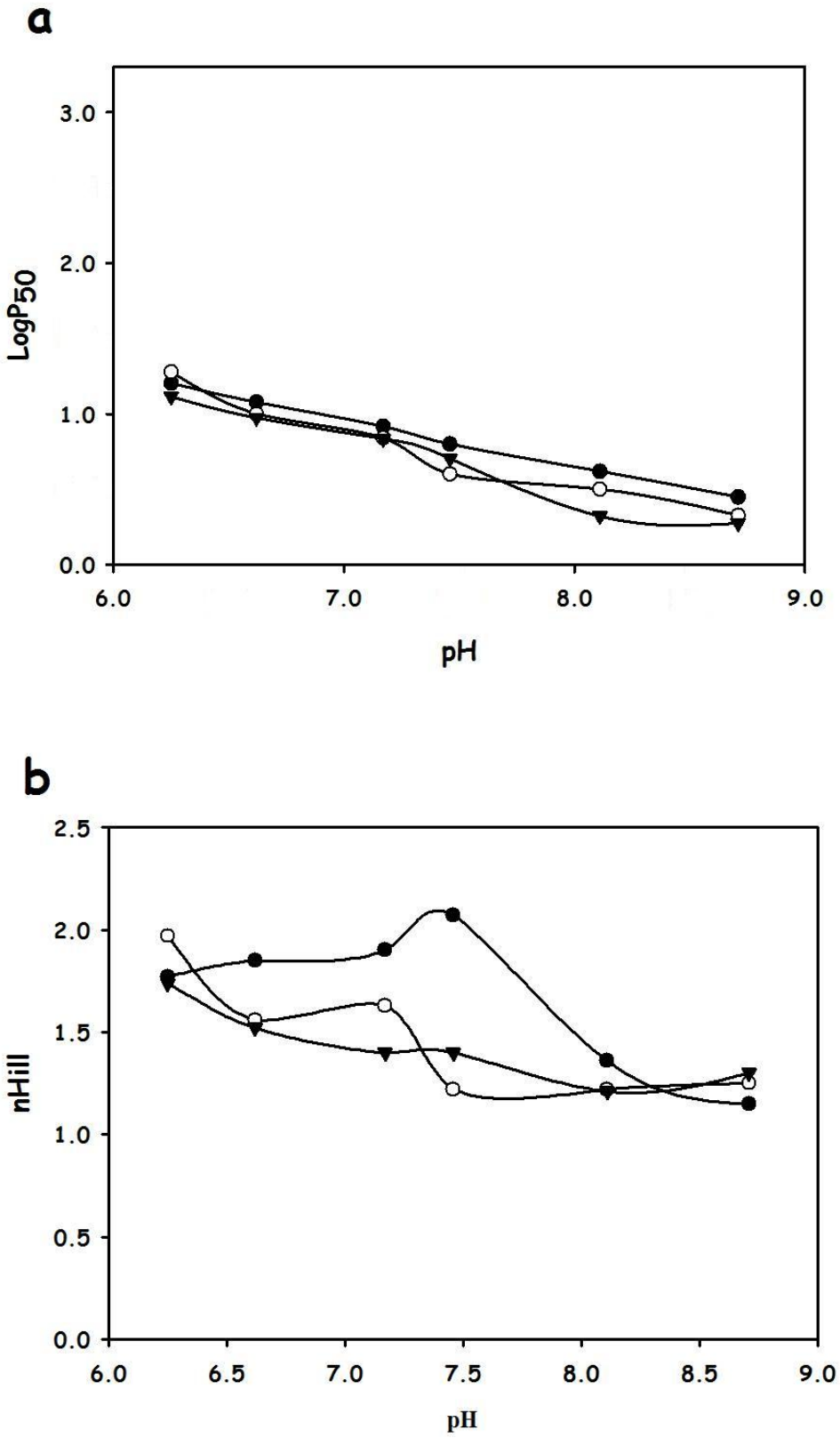

Fig 3.9: a) O_2 equilibrium isotherms (Bohr effect) and b) subunit cooperativity at atmospheric pressure as a function of pH of *L. reticulatus* hemolysate. 100 mM HEPES at 10°C, in the absence of effectors is shown by the open circles, in the presence of 100 mM NaCl by the filled circles, 100mM NaCl, 3mM ATP by the filled triangles.

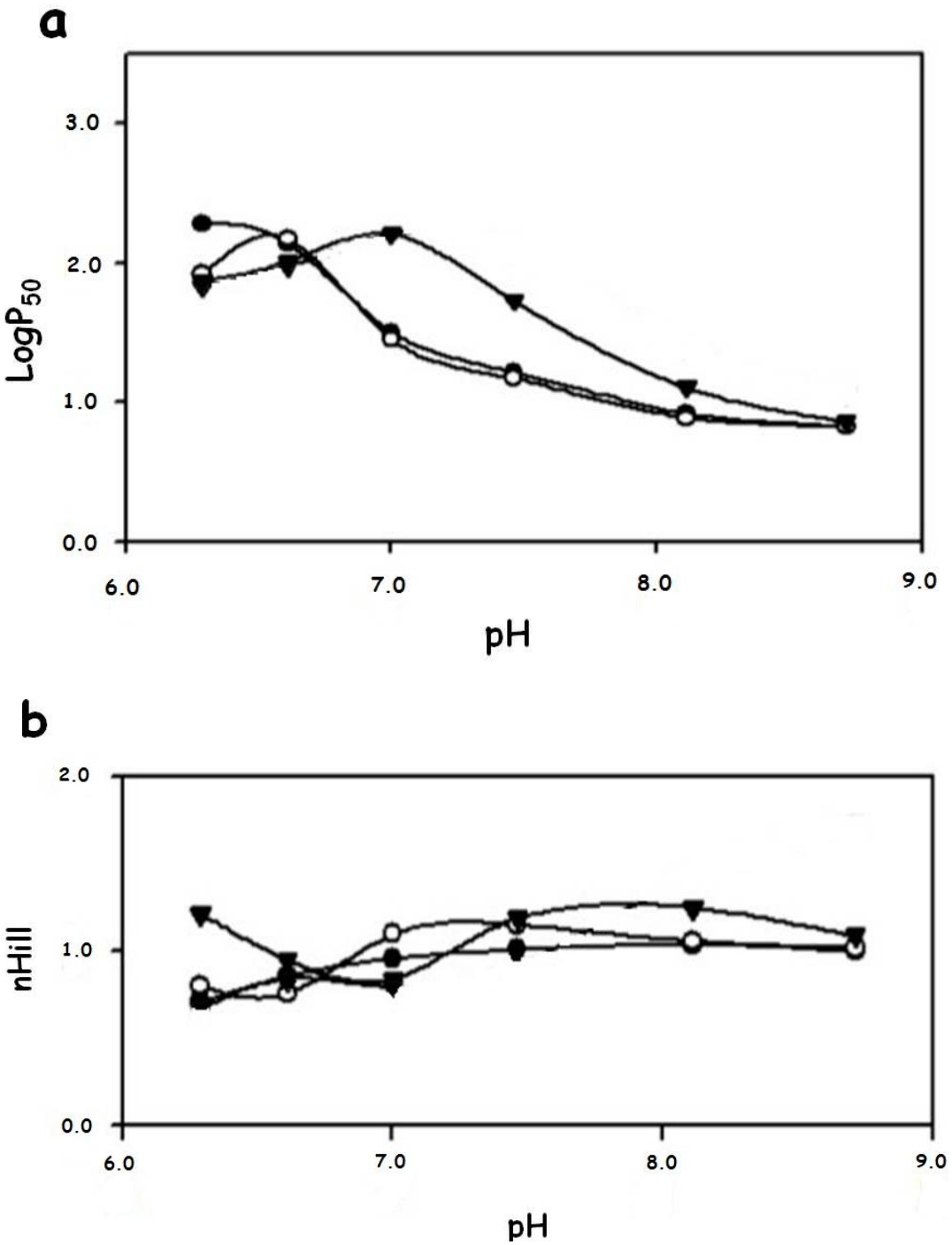

Fig 3.10: a) O_2 equilibrium isotherms (Bohr effect) and b) subunit cooperativity at atmospheric pressure as a function of pH of *G. morhua* hemolysate. 100 mM HEPES at 5°C, in the absence of effectors is shown by the open circles, in the presence of 100 mM NaCl by the filled circles, 100mM NaCl, 3mM ATP by the filled triangles.

3.2 Antarctic fish Cygb

3.2.1 The primary structure

The species *D. mawsoni* and *C. aceratus* have two genes encoding fish Cygbs. Only one Cygb gene from the retina and one from brain of *D mawsoni* and *C. aceratus*, respectively were identified. The amino acid sequences of *C. aceratus* and *D. mawsoni* Cygb were derived from the cDNA sequences obtained by RT-PCR amplification of total RNA using appropriate primers. *In silico* translation of the cDNA sequences provided the Cygb protein sequences. Antarctic fish Cygb sequences together to those of mammalian and other fish species reported in literature were aligned by the CLUSTAL W (1.83) program (Thompson et al., 1994) following standard parameters.

Antarctic fish Cygbs: The two Antarctic fish Cygbs have 179 amino acid residues and differ in only four positions: Arg24 A4 → Lys, Asn52 B15 → Lys, Ile158 H14 → Met, Val165 H21 → Ile. The theoretical molecular weights are 20137.0 Da and 20118.8 Da for *C. aceratus* and *D. mawsoni* Cygbs, respectively. Their sequence identity is 98% (Fig. 3.11).

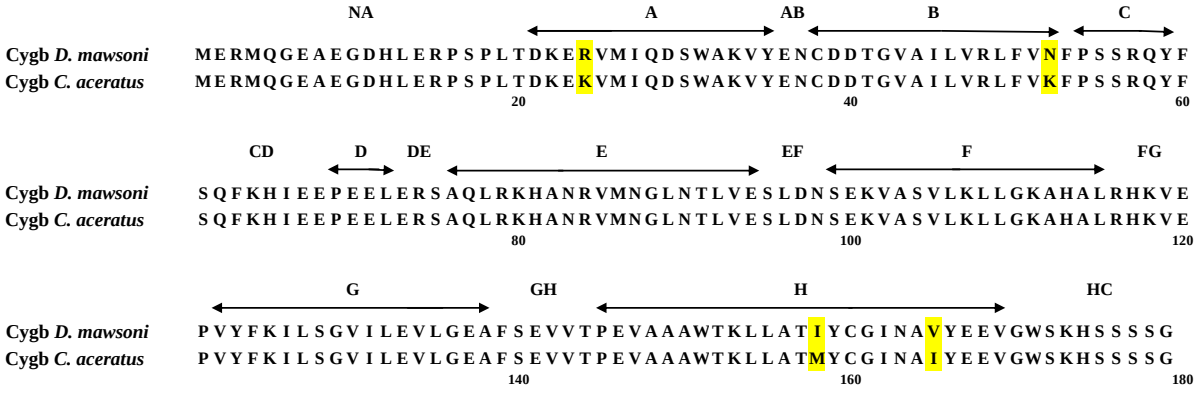

Fig. 3.11: Amino-acid sequences of *D. mawsoni* and *C. aceratus* Cygb. In yellow are indicated the differences.

Human Cygb – Antarctic fish Cygbs: When compared to mammalian counterparts in a multiple sequence alignment (Fig 3.12), Antarctic fish Cygbs display some striking peculiarities. Apart from these differences, conservation is high in the rest of the Cygb sequences between mammals and fish, with Antarctic fish Cygbs about 55% identical to human.

The residues suggested to be essential for the function, Leu46 B10, Phe60 CD1, His81 E7, Arg84 E10, Val85 E11 and His113 F8, considered equally important in binding the exogenous ligand (de Sanctis et al., 2004; Smagghe et al., 2006; Sugimoto et al., 2004; Roesner et al., 2005; Doorslaer et al., 2004; Fago et al., 2004) are conserved and present in both Antarctic fish Cygbs.

In human Cygb, Cys38 B2 and Cys83 E9 residues (Lechauve et al., 2010) are known to form a disulfide bridge. In Antarctic fish Cygbs, these cysteine residues occupy different position in the primary structure and they are far to form a disulfide bridge (F. J. Luque, personal communication).

Another difference is the length of the C-terminal sequences (CH region). In the Antarctic fish Cygbs this region is shorter.

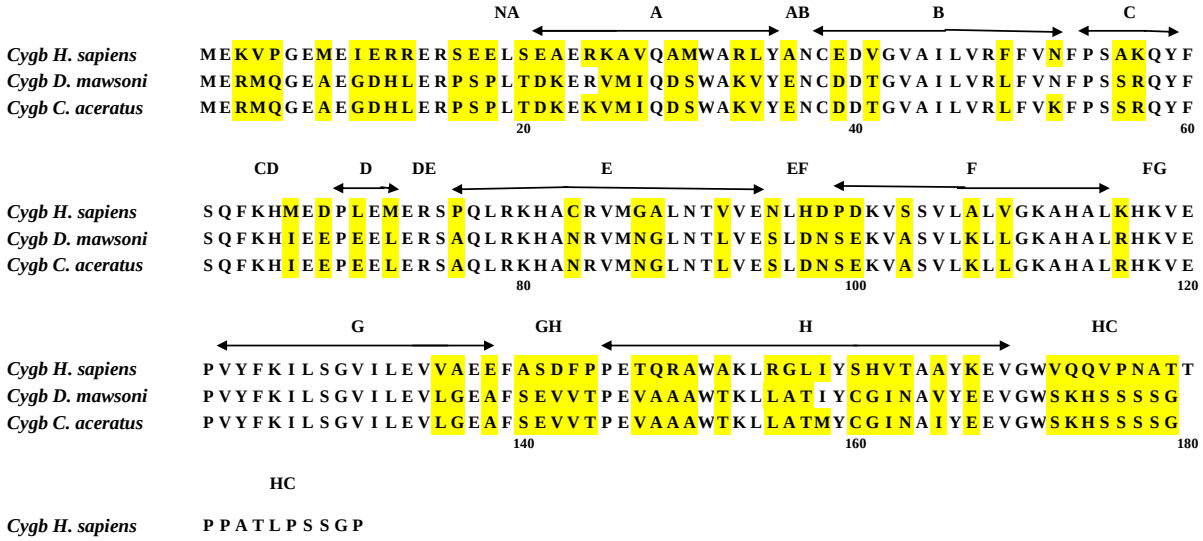

Fig 3.12: Amino-acid sequences of the Antarctic fish and human (de Sanctis et al., 2004) Cygb. The different residues are in yellow.

Temperate fish Cygbs - Antarctic fish Cygbs: The comparison between the temperate fish and the Antarctic fish Cygbs is important to understand possible differences due to cold

adaptation. Some temperate fish Cygb-1 and Cygb-2 sequences are reported in literature and their sequence identity with those of Antarctic fish Cygbs are in table 3.

Table 3

Sequence identity between Antarctic fish Cygbs and temperate fish Cygbs (*T. nigroviridis* Cygb-1 and -2; *O. latipes* Cygb-1 and -2; *D. rerio* Cygb-1 and -2)

	C. aceratus Cygb-1	*D. mawsoni* Cygb-1
T. nigroviridis Cygb-1	72%	72%
T. nigroviridis Cygb-2	56%	58%
O. latipes Cygb-1	75%	77%
O. latipes Cygb-2	55%	56%
D. rerio Cygb-1	48%	49%
D. rerio Cygb-2	60%	61%

C. aceratus and *D. mawsoni* Cygbs can be considered Cygb-1, because they are more closely related to the Cygbs-1 from temperate fish, except to *D. rerio* Cygbs. The sequence alignment is shown in Fig. 3.13.

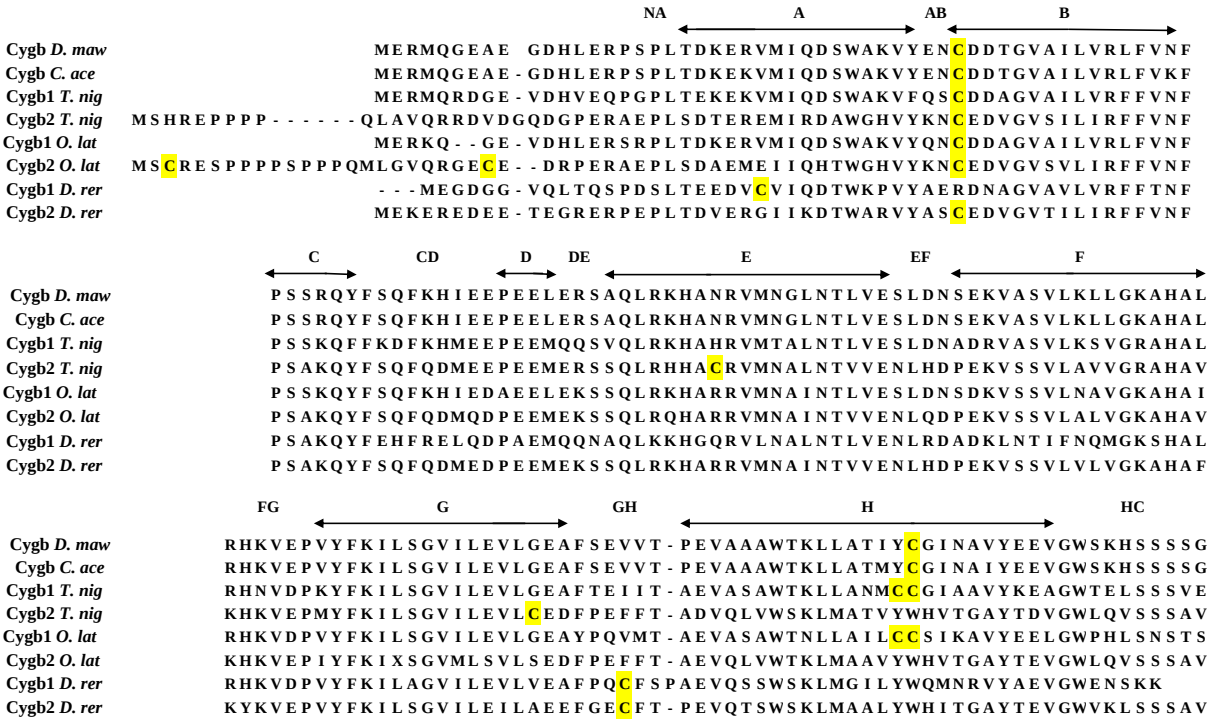

Fig 3.13: Amino-acid sequences of Antarctic fish, *T. nigroviridis* (Fuchs et al., 2005), *O. latipes* (Fuchs et al., 2005), *D. rerio* (Fuchs et al., 2005) Cygbs. The Cys residues are in yellow.

3.2.2 Cloning, expression and purification

Subcloning, expression and purification was carried out in collaboration with the Prof. Sylvia Dewilde during a training time of three months in her laboratories, at the University of Antwerp, Belgium.

C. aceratus and *D. mawsoni* Cygbs were subcloned in pET3a expression vector and over-expressed in *E. coli*, then purified by ion-exchange and gel-filtration chromatographies (data not shown).

The over-expression showed important difference with the human Cygb. The two Antarctic fish Cygbs were over-expressed without haem precursor, δ-Aminolevulinic acid. Moreover, whereas generally the human Cygb is expressed in inclusion bodies end refolded in presence of hemin, the two fish Cygbs are unexpectedly soluble and bind the haem group. The spectra of the lysate showed a high concentration of hexacoordinated ferric-form Cygbs in agreement with the expected result and SDS-PAGE indicating a high amount of protein with the expected weight (about 20 kDa).

Different steps of purification were tried. The more efficient procedure was the following:

- Ammonium sulfate precipitation
- DEAE bulk
- Gel Filtration Chromatography: Sephacryl S-200 column
- Ionic Chromatography: HiTrap DEAE FF column
- Ionic Chromatography: Q Sepharose column
- Ionic Chromatography: Mono Q column (pH 7.6)

The final result was a high amount of protein, as shown in fig. 3.14.

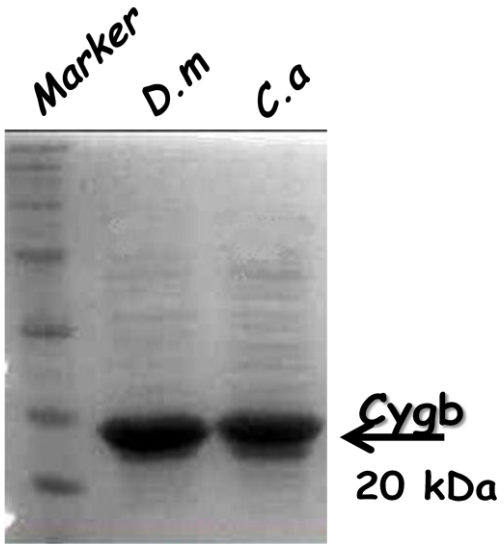

Fig. 3.14: SDS-PAGE of *D. mawsoni* (on left) and *C. aceratus* (on the right) Cygb.

3.2.3 Spectroscopic characterisation of the Fe-coordination

A fast way to observe if the protein is in a penta or hexacoordinated state is an inspection of the electronic absorbance spectra for both haem oxidation states. The pentacoordinate states have the visible-region absorption bands with weak peaks near 500 and 635 nm and a single asymmetric absorbance band near 555 nm when the protein is in the ferrous form. These bands indicate that the haem iron is in the high-spin electronic configuration in both oxidation states (Antonini and Brunori, 1971). On the contrary, His coordination to the sixth axial position converts the haem iron to the low spin electronic configuration in both oxidation states giving rise to stronger visible absorbance in the ferric state, and splitting of the ferrous visible absorbance band into two peaks near 560 and 530 nm (Kakar et al., 2010).

The Antarctic fish Cygbs were in hexacoordinated form in both haem oxidation states. The hexacoordination was observed in different conditions, displaying no dependence on pH and temperature, like the human Cygb. The spectra are shown in Fig. 3.15 for the ferric and ferrous form and in table 4 are indicated the characteristic wavelengths for each spectrum.

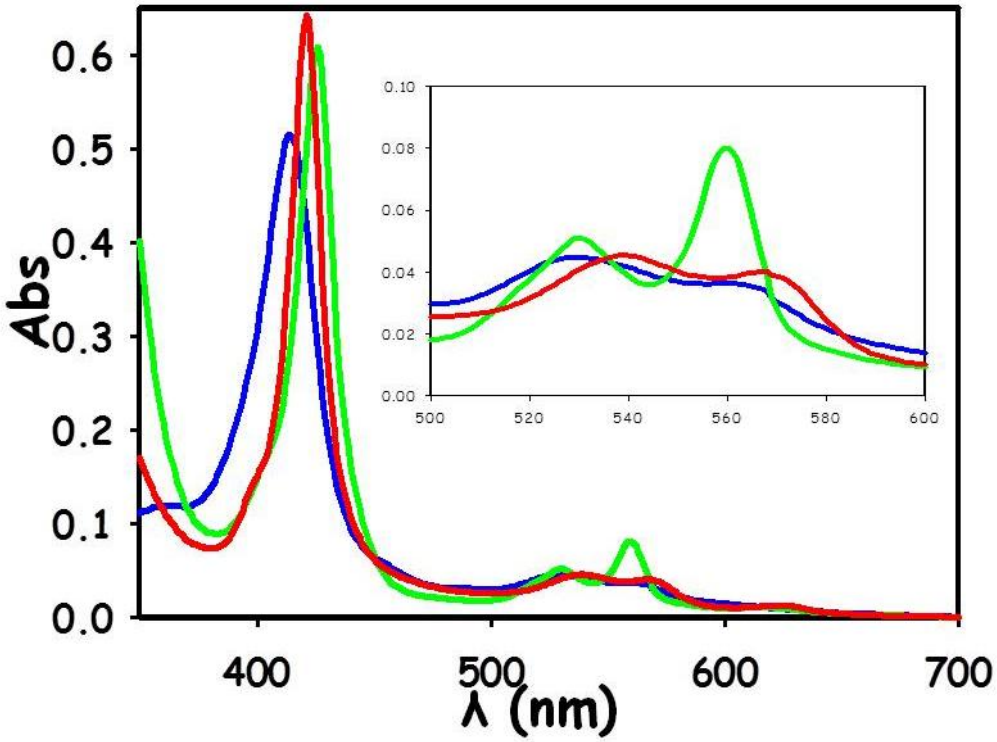

Fig. 3.15: UV-visible absorption spectra of ferric (blue), CO- (red) and deoxy-Cygb (green).

Table 4
Maximum wavelength for hexacoordinated Cygb, for each spectrum.

	λ_1 (nm)	λ_2 (nm)	λ_3 (nm)
Ferric form	560	529	414
Deoxy-form	560	530	426
CO-form	568	540	421

CHAPTER 4

Conclusions

In the biosphere, organisms have succeeded in adapting to a variety of environmental conditions. Extreme marine environments usually combine a range of physical gradients (e.g. in pressure, temperature, pH, salinity) with toxic and/or essential chemicals (O_2, H_2S, CH_4, metals such as Fe, Cu, Mo, Zn, Cd, Pb, etc.) that by far exceed typical oceanic ranges. The cellular macromolecules, proteins and nucleic acids, are very sensitive to environment perturbations; therefore, the study of globins belonging to different species that live in cold but different habitats can be very useful to understand the molecular adaptation to particular environmental and stressful conditions. In fact, despite *L. reticulatus* and *G. morhua* live in the same Arctic region, they showed a different Hb polymerisation behaviour, in response to their different life style.

L. reticulatus was found close to the coasts of Canada (Leim et al., 1966), Greenland (Muus et al., 1990), Iceland, Norway and USA (Robins and Ray 1986) and occurs in soft bottoms at depths between 100-930 m and at 1-4°C. *G. morhua*, an important species for commercial fisheries and aquaculture (cod), lives the regions from Northwest to Northeast Atlantic, coasts of Greenland; around Iceland; coasts of Europe from the Bay of Biscay to the Barents Sea, including the region around Bear Island (Cohen et al., 1990). It is an oceanodromous fish that swims between 0-600 m at temperature not higher than 15°C (Cohen et al., 1990). The different life style of the two species can affect the Hb behaviour, and may explain the different polymerisation mechanisms.

In this study, *in vitro* Hb polymerisation was demonstrated in *L. reticulatus* hemolysate. The high propensity to aggregation and high β sequence identity of these Hbs has been the cause of unsuccessful purification of Hb components from *L. reticulatus*, despite the effort profuse in this direction. A relevant structural property of this Hb is the formation of polymers through disulfide bonds between 5 different Cys residues (Cys α105, Cys β31, Cys β109 and Cys β146). Particularly, several S-S bridges were formed by C-terminal Cys β146, indicating higher reactivity, and/or high flexibility of the domain where this residue is located. This behaviour is similar to that of the human variant Hb Rainier (Carbone et al., 1999), and of *C. kumu* Hb (Fago et al., 1993), where Cys β49 replaces His, commonly found in other fish

Hbs. *In vitro* Hb polymerisation was also demonstrated in *G. morhua*. The aggregates grew only in deoxygenated state at low pH, similarly to that showed previously *in vivo* and shown in the literature (Hárosi et al., 1998). Koldkjær and Berenbrink (2007) showed, in other species of the family Gadidae, reduction of extracellular pH as the primary cause for *in vivo* sickling with a modulation by O_2 pressure. Sickle-cell formation (Koldkjær and Berenbrink, 2007) suggests that this process may be a unique example of Hb plasticity. The relative importance in fish physiology is yet unknown; whether this process occurs *in vivo* is rather difficult to ascertain. Nevertheless, this event deserves further investigation, because of the possible links with SCA. However, the discovery of this unusual process suggests that polymerisation may be a response to stressful environmental conditions, which a migratory species like *G. morhua* may easily experience.

Another important response to stressful environmental conditions of *G. morhua* is the high number of globin genes (four α and five β) that suggests a response to challenges in temperature or to chronic hypoxia by altering their level expression (Borza et al., 2009). This polymorphism was recently related to a different temperature effect of O_2 affinity in Arctic and non-Arctic fish Hbs. Example of co-evolution of structural and regulatory adaptation with a relationship between temperature and functional molecular variation (Star et al., 2011).

In the process of cold adaptation, the evolutionary trend of Antarctic fish has led to unique specialisations, including modification of haematological characteristics, e.g. decreased amounts and multiplicity of Hbs. As the extreme of this trend, an important example of such peculiarity is the colourless blood of the icefish modern notothenioid family Channichthyidae (Ruud, 1954; di Prisco et al., 2002). During this thesis a cytoglobin (Cugb) from an icefish (*C. aceratus*) and a red-blooded fish (*D. mawsoni*) has been cloned, expressed and purified. Since Hb and Mb are key proteins in NO homeostasis (Barouch et al., 2002), the icefish, as natural knockout for Hb/Mb, represents a unique example to investigate whether these disadaptive losses may have evolved cardiac modifications (Hendgen-Cotta et al., 2008). When Mb acts as a nitrite reductase, deoxygenated Mb generates NO from circulating nitrite in cardiac muscle cells under hypoxic stress, where it suppresses the production of radical oxygen species (ROS) in mitochondria, protecting the muscle cells from damage. Excess NO is reconverted to nitrate by oxy Mb acting as a dioxygenase (Hendgen-Cotta et al., 2008). The hearts of Mb-knockout mice do not recover from experimentally imposed ischemia; these mice show no evidence of nitrite-induced reduction in the damage to heart tissue caused by blood-vessel blockage (Cossins and Berenbrink, 2008).

These observations and this study on Antarctic Cygbs may help to better understand icefish physiology and the compensatory adaptations evolved in the cardiovascular system of these natural knockouts. A possible function of Cygb studied is its involvement in NO metabolism, acting as NO dioxygenase, therefore, the recent discovery of Cygb in red-blooded *D. mawsoni* and in the icefish *C. aceratus* suggested a crucial biological function and potentially important implications in the physiology and pathology of their tissues. Moreover, the comparison started in this thesis between two species with different adaptation will be crucial to understand the historical origin of Cygbs.

References

Andersen Ø., Wetten O.F., De Rosa M.C., Andre C., Carelli Alinovi C., Colafranceschi M., Brix O. and Colosimo A. (2009) Haemoglobin polymorphisms affect the oxygen-binding properties in Atlantic cod populations. Proc Biol Sci 276, 833-841.

Antonini E. and Brunori M. (1971) Hemoglobin and myoglobin in their reactions with ligands, North-Holland Publishing Company, Amsterdam.

Aprelev A., Rotter M.A., Etzion Z., Bookchin R.M., Briehl R.W. and Ferrone F.A. (2005) The effects of erythrocyte membranes on the nucleation of sickle hemoglobin. Biophys J 88, 2815-2822.

Balushkin A.V. (1992) Classification, phylogenetic relationships, and origins of the families of the suborder Notothenioidei (Perciformes). J Ichthyol 32, 90-110.

Bargelloni L., Ritchie P.A., Patarnello T., Battaglia B., Lambert D.M. and Meyer A. (1994) Molecular evolution at subzero temperatures: mitochondrial and nuclear phylogenies of fishes from Antarctica (suborder Notothenioidei), and the evolution of antifreeze glycopeptides. Mol Biol Evol 11, 854-863.

Barouch L.A., Harrison R.W., Skaf M.W., Rosas G.O., Cappola T.P., Kobeissi, Z.A., Hobai, I.A., Lemmon, C.A., Burnett, A.L., O'Rourke, B., Rodriguez, E.R., Huang, P.L., Lima, J.A., Berkowitz, D.E., Hare, J.M., 2002. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. Nature 416, 337-339.

Berenbrink M. (2006) Evolution of vertebrate haemoglobins: Histidine side chains, specific buffer value and Bohr effect. Respir Physiol Neurobiol 154, 165-184.

Berenbrink M., Koldkjaer P., Kepp O. and Cossins A.R. (2005) Evolution of oxygen secretion in fishes and the emergence of a complex physiological system. Science 307, 1752-1757.

Berne B.J. and Pecora R. (2000) Dynamic light scattering: with applications to chemistry, biology, and physics. Dover Editions, NY.

Blank M., Kiger L., Thielebein A., Gerlach F., Hankeln T., Marden M.C. and Burmester T. (2011) Oxygen supply from the bird's eye perspective: globin E is a respiratory protein in the chicken retina. J Biol Chem 286, 26507-26515.

Borza T., Stone C., Gamperl A.K. and Bowman S. (2009) Atlantic cod (Gadus morhua) hemoglobin genes: multiplicity and polymorphism. BMC Genet 10, 51-64.

Brittain T. (2005) Root effect hemoglobins. J Inorg Biochem 99, 120-129.

Brunori M. (1999) Hemoglobin is an honorary enzyme. Trends Biochem Sci 24, 158-161.

Brunori M. (2001) Nitric oxide moves myoglobin centre stage. Trends Biochem Sci 26, 209-210.

Burmester T., Ebner B., Weich B. and Hankeln T. (2002) Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. Mol Biol Evol 19, 416-421.

Burmester T., Haberkamp M., Mitz S., Roesner A., Schmidt M., Ebner B., Gerlach F., Fuchs C. and Hankeln T. (2004) Neuroglobin and cytoglobin: genes, proteins and evolution. IUBMB Life 56, 703-707.

Burmester T., Weich B., Reinhardt S. and Hankeln T. (2000) A vertebrate globin expressed in the brain. Nature 407, 520-523.

Camardella L., Caruso C., D'Avino R., di Prisco G., Rutigliano B., Tamburrini M., Fermi G. and Perutz M.F. (1992) Hemoglobin of the Antarctic fish Pagothenia bernacchii. Amino acid sequence, oxygen equilibria and crystal structure of its carbonmonoxy derivative. J Mol Biol 224, 449-460.

Carbone V., Salzano A.M., Pagano L., Viola A., Buffardi S., De Rosa C. and Pucci P. (1999) Hb Rainier (β145(HC2)Tyr → Cys) in Italy. Characterisation of the amino acid substitution and the DNA mutation. Hemoglobin 23, 111-124.

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

Christoph G.W., Hofrichter J. and Eaton W.A. (2005) Understanding the shape of sickled red cells. Biophys J 88, 1371-1376.

Cohen D.M., Inada T., Iwamoto T. and Scialabba N. (1990) FAO Species Catalogue. Gadiform fishes of the world (Order Gadiformes). An annotated and illustrated catalogue of cods, hakes, grenadiers and other gadiform fishes known to date. FAO Fish Synop 10.

Cossins A. and Berenbrink M. (2008) Physiology: myoglobin's new clothes. Nature 454, 416-417.

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

Dayton P.K., Mordida B. J. and Bacon F. (1994) Polar marine communities. Amer Zool 34, 90-99.

de Sanctis D., Dewilde S., Pesce A., Moens L., Ascenzi P., Hankeln T., Burmester T. and Bolognesi M. (2004) Cristal structure of Cytoglobin: the fourth globin type discovered in man displays haem hexa-coordination. J Mol Biol 336, 917-927.

Detrich H.W. III, Johnson K.A. and Marchese-Ragona S.P. (1989) Polymerization of Antarctic fish tubulins at low temperatures: energetic aspects. Biochemistry 28, 10085-10093.

Detrich H.W. III, Parker S.K., Williams R.C.J., Nogales E. and Downing K.H. (2000) Cold adaptation of microtubule assembly and dynamics. Structural interpretation of primary sequence changes present in the alpha- and beta-tubulins of antarctic fishes. J Biol Chem 275, 37038-37047.

di Prisco G., Cocca E., Parker S. and Detrich H.W. (2002) Tracking the evolutionary loss of haemoglobin expression by the white-blooded Antarctic icefishes. Gene 295, 185-191.

di Prisco G., Eastman J.T., Giordano D., Parisi E. and Verde, C. (2007) Biogeography and adaptation of Notothenioid fish: hemoglobin function and globin-gene evolution. Gene 398, 143-155.

Dykes G.W., Crepeau R.H. and Edelstein S.J. (1979) Three-dimensional reconstruction of 14-filament fibers of hemoglobin S. J Mol Biol 130, 451-472.

Doorslaer S.V., Vinck E., Trandafir F., Ioanitescu I., Dewilde S. and Moens L. (2004) Tracing the structure-function relationship of neuroglobin and cytoglobin using resonance Raman and electron paramagnetic resonance spectroscopy. IUBMB Life 56, 665-670.

Duff S., Wittenberg J. and Hill R. (1997) Expression, purification, and properties of recombinant barley (Hordeum sp.) hemoglobin. Optical spectra and reactions with gaseous ligands. J Biol Chem 272, 16746-16752.

Eastman J.T. (1993) Antarctic fish biology: evolution in a unique environment. Academic Press, San Diego, CA.

Eastman J.T. (1997) Comparison of the Antarctic and Arctic fish faunas. Cybium 21, 335-352.

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

Eaton W.A. and Hofrichter J. (1990) Sickle cell hemoglobin polymerization. Advan Protein Chem 40, 263-279.

Embury S.H. (2004) The not-so-simple process of sickle cell vasoocclusion. Microcirculation 11, 101-113.

Fago A., Hundahl C., Dewilde S., Gilany K. and Moens L. (2004) Allosteric regulation and temperature dependence of oxygen binding in human neuroglobin and cytoglobin. J Biol Chem 279, 44417-44426.

Fago A., Romano M., Tamburrini M., Coletta M., D'Avino R. and di Prisco, G. (1993) A polymerising Root-effect fish hemoglobin with high subunit heterogeneity. Correlation with primary structure. Eur J Biochem 218, 829-835.

Fermi G., Perutz M.F., Shaanan B. and Fourme R. (1984) The crystal structure of human deoxyhaemoglobin at 1.74 A resolution. J Mol Biol 175, 159-174.

Ferrone F.A., (2004) Polymerization and sickle cell disease: a molecular view. Microcirculation 11, 115-128.

Flögel U., Merx M.W., Godecke A., Decking U.K. and Schrader J. (2001) Myoglobin: A scavenger of bioactive NO. Proc Natl Acad Sci U S A 98, 735-740.

Fordel E., Geuens E., Dewilde S., Rottiers P., Carmeliet P., Grooten J. and Moens L: (2004) Cytoglobin expression is upregulated in all tissues upon hypoxia: an *in vitro* and *in vivo* study by quantitative real-time PCR. Biochem Biophys Res Commun 319, 342-348.

Fordel E., Thijs L., Martinet W., Lenjou M., Laufs T., Van Bockstaele D., Moens L. and Dewilde S. (2006) Neuroglobin and cytoglobin overexpression protects human SH-SY5Y neuroblastoma cells against oxidative stress-induced cell death. Neurosci Lett 410, 146-151.

Fuchs C., Burmester T. and Hankeln T. (2006) The amphibian globin gene repertoire as revealed by the Xenopus genome. Cytogenet Genome Res 112, 296-306.

Fuchs C., Luckhardt A., Gerlach F., Burmester T. and Hankeln T. (2005) Duplicated cytoglobin genes in teleost fishes. Biochem Biophys Res Commun 337, 216-223.

Galkin O. and Vekilov G. (2004) Mechanisms of homogeneous nucleation of polymers of sickle cell anemia hemoglobin in deoxy state. J Mol Biol 336, 43-59.

Gamperl A.K., Busby C.D., Hori T.S.F., Afonso L.O.B. and Hall, J.R. (2009) Hemoglobin genotype has minimal influence on the physiological response of juvenile Atlantic cod (Gadus morhua) to environmental challenges. Physiol Biochem Zool 82, 483-494.

Gardner A.M., Cook M.R. and Gardner P.R. (2010) Nitric-oxide dioxygenase function of human cytoglobin with cellular reductants and in rat hepatocytes. J Biol Chem. 285, 23850-23857.

Geuens E., Brouns I., Flamez D., Dewilde S., Timmermans J.P., Moens L. (2003) A globin in the nucleus! J Biol Chem 278, 30417-30420.

Grove T.J., Hendrickson J.W. and Sidell B.D. (2004) Two species of Antarctic icefishes (Genus *Champsocephalus*) share a common genetic lesion leading to the loss of myoglobin expression. Polar Biol 27, 579-585.

Guo X., Philipsen S. and Tan-Un K.C. (2007) Study of the hypoxia-dependent regulation of human CYGB gene. Biochem Biophys Res Commun 364,145-150.

Hamdane D., Kiger L., Dewilde S., Green B.N., Pesce A., Uzan J., Burmester T., Hankeln T., Bolognesi M., Moens L. and Marden M.C. (2003) The redox state of the cell regulates the ligand binding affinity of human neuroglobin and cytoglobin. J Biol Chem 278, 51713-51721.

Hardison R.C. (1996) A brief history of hemoglobins: plant, animal, protist, and bacteria. Proc Natl Acad Sci U S A 93, 5675-5679.

Hardison R. (1998) Hemoglobins from bacteria to man: evolution of different patterns of gene expression. J Exp Biol 201, 1099-1117.

Hargrove M. (2000) A flash photolysis method to characterize hexacoordinate hemoglobin kinetics. Biophys. J. 79, 2733-2738.

Hárosi F.I., von Herbing I.H. and Van Keuren J.R. (1998) Sickling of anoxic red blood cells in fish. Biol Bull 195, 5-11.

Harrington D.J., Adachi K. and Royer W.E.Jr (1997) The high resolution crystal structure of deoxyhemoglobin S. J Mol Biol 272, 398-407.

Hendgen-Cotta U.B., Merx M.W., Shiva S., Schmitz J., Becher S., Klare J.P., Steinhoff H.J., Goedecke A., Schrader J., Gladwin M.T., Kelm M. and Rassaf T. (2008) Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury. Proc Natl Acad Sci U S A 105, 10256-10261.

Hillery C.A. and Panepinto J.A. (2004) Pathophysiology of stroke in sickle cell disease. Microcirculation 11, 195-208.

Hoffbrand V., Moss P. and Pettit J. (2006) Essential Haematology, 5 ed. Wiley-Blackwell.

Hofmann G.E., Buckley B.A., Airaksinen S., Keen J. and Somero G.N. (2000) The Antarctic fish *Trematomus bernacchii* lacks heat-inducible heat shock protein synthesis. J Exp Biol 203, 2331-2339.

Hundahl C., Allen G.C., Nyengaard J.R., Dewilde S., Carter B.D., Kelsen J. and Hay-Schmidt, A. (2008a) Neuroglobin in the rat brain: localization. Neuroendocrinology 88, 173-182.

Hundahl C., Kelsen J., Dewilde S., Hay-Schmidt A. (2008b) Neuroglobin in the rat brain (II): co-localisation with neurotransmitters. Neuroendocrinology 88, 183-198.

Hundahl C., Stoltenberg M., Fago A., Weber R.E., Dewilde S., Fordel E. and Danscher G. (2005) Effects of short-term hypoxia on neuroglobin levels and localization in mouse brain tissues. Neuropathol Appl Neurobiol 31, 610-617.

Ingram V.M. (1957) Gene mutations in human haemoglobin: the chemical difference between normal and sickle cell hemeoglobin. Nature 180, 326-328.

Kakar S., Hoffman F.G., Storz J.F., Fabian M. and Hargrove M.S. (2010) Structure and reactivity of hexacoordinate hemoglobins. Biophys Chem 152, 1-14.

Kennett J.P. (1977) Cenozoic evolution of Antarctic glaciation, the circum-Antarctic ocean and their impact on global paleoceanography. J Geophys Res 82, 3843-3876.

Koldkjær P. and Berenbrink M. (2007) *In vivo* red blood cell sickling and mechanism of recovery in whiting, *Merlangius merlangus*. J Exp Biol 210, 3451-3460.

Kugelstadt D., Haberkamp M., Hankeln T. and Burmester T. (2004) Neuroglobin, cytoglobin, and a novel, eye-specific globin from chicken. Biochem Biophys Res Commun 325, 719-725.

Imai K. (1999) The haemoglobin enzyme. Nature 401, 437-439.

Lechauve C., Chauvierre C., Dewilde S., Moens L., Green B.N., Marden M.C., Ce´lier C. and Kiger, L. (2010) Cytoglobin conformations and disulfide bond formation. FEBS J 277, 2696-2704.

Lecointre G. and Ozouf-Costaz C. (2004) Les poissons à antigel del'océan austral. Pour La Science 320, 48-54.

Leim A.H. and Scott W.B. (1966) Fishes of the Atlantic coast of Canada. Bull Fish Res Board Can 155, 485.

Li D., Chen X.Q., Li W.J., Yang Y.H., Wang J.Z. and Yu A.C. (2007) Cytoglobin up-regulated by hydrogen peroxide plays a protective role in oxidative stress. Neurochem Res 32, 1375-1380.

Mammen P.P., Shelton J.M., Goetsch S.C., Williams S.C., Richardson J.A., Garry M.G. and Garry D.J. (2002) Neuroglobin, a novel member of the globin family, is expressed in focal regions of the brain. J Histochem Cytochem 50, 1591-1598.

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

Mazzarella L., Bonomi G., Lubrano M.C., Merlino A., Riccio A., Vergara A., Vitagliano L., Verde C. and di Prisco G. (2006a) Minimal structural requirements for root effect: crystal structure of the cathodic hemoglobin isolated from the antarctic fish *Trematomus newnesi*. Proteins 62, 316-321.

Mazzarella L., Vergara A., Vitagliano L., Merlino A., Bonomi G., Scala S., Verde C. and di Prisco G. (2006b) High-resolution crystal structure of deoxy hemeoglobin from

Trematomus bernacchii at different pH values: the role of histidine residues in modulating the strength of the Root effect. Proteins 65, 490-498.

McMahon T.J., Moon R.E., Luschinger B.P., Carraway M.S., Stone A.E., Stolp B.W., Gow A.J., Pawloski J.R., Watke P., Singel D.J., Piantadosi C.A. and Stamler J.S. (2002) Nitric oxide in the human respiratory cycle. Nat Med 8, 711-717.

Monod J., Wyman J. and Changeux J.P. (1965) On the nature of allosteric transitions: a plausible model. J Mol Biol 12, 88-118.

Moran K., Backman J., Brinkhuis H., Clemens S.C., Cronin T., Dickens G.R., Eynaud F., Gattacceca J., Jakobsson M., Jordan R.W., Kaminski M., King J., Koc N., Krylov A., Martinez N., Matthiessen J., McInroy D., Moore T.C., Onodera J., O'Regan M., Mylvaganam S.E., Bonaventura C., Bonaventura J. and Getzoff, E.D. (1996) Structural basis for the Root effect in hemeoglobin. Nat Struct Biol 3, 275-283.

Muus B., Salomonsen F. and Vibe C. (1990) Grønlands fauna (Fisk, Fugle, Pattedyr). Gyldendalske Boghandel, Nordisk Forlag A/S København, p. 464.

Nagai K., Luisi B., Shih D., Miyazaki G., Imai K., Poyart C., De Young A., Kwiatkowsky L., Noble R.W., Lin S.H. and Yu N.T. (1987) Distal residues in the oxygen binding site of haemoglobin studied by protein engineering. Nature 329, 858-860.

Nagel R.L. and Steinberg M.H. (2001) Genetics of the βS gene: origins, epidemiology, and epistasis in sickle cell anemia. In Disorders of Hemoglobin: Genetics, Pathophysiology, and Clinical Management, New York, NY: Cambridge Univ Press pp. 711-755.

Pälike H., Rea B., Rio D., Sakamoto T., Smith D.C., Stein R., St John K., Suto I., Suzuki N., Takahashi K., Watanabe M., Yamamoto M., Farrell J., Frank M., Kubik P., Jokat W. and Kristoffersen Y. (2006) The Cenozoic palaeoenvironment of the Arctic Ocean. Nature 441, 579-581.

Near T. (2004) Estimating divergence times of notothenioid fishes using a fossil-calibrated molecular clock. Antarctic Sci 16, 37-44.

Pan W., Galkin O., Filobelo L., Nagel R.L. and Vekilov, P.G. (2007) Metastable mesoscopic clusters in solutions of sickle-cell hemoglobin. Biophys J 92, 267-277.

Perlmutter M.A. and Plotnick, R.E. (2003) Hemispheric asymmetry of the marine stratigraphic record: conceptual proof of a unipolar ice cap. In Climate controls on stratigraphy (eds C.B. Cecil and N.T. Edgar), Society of Economic Paleontologists and Mineralogists Special Pubblication n.77, pp. 51-66, SEPM, USA.

Perutz M.F. (1990) Haemoglobin. Molecular inventiveness. Nature 348, 583-584.

Perutz M.F. (1998) The stereochemical mechanism of the cooperative effects in hemeoglobin revisited. Annu Rev Biophys Biomol Struct 27, 1-34.

Perutz M.F. and Brunori M. (1982) Stereochemistry of cooperative effects in fish and amphibian hemeoglobins. Nature 299, 421-426.

Perutz M.F., Fermi G., Luisi B., Shanan B. and Liddington R.C. (1987) Stereochemistry of cooperative mechanisms in hemoglobin. Acc Chem Res 20, 309-321.

Perutz M.F., Kendrew J.C. and Watson H.C. (1965) Structure and function of hemeoglobin. II. Some relations between polypeptide chain configuration and amino acid sequence. J Mol Biol 13, 669-678.

Pesce A., Bolognesi M., Bocedi A., Ascenzi P., Dewilde S., Moens L., Hankeln T. and Burmester T. (2002) Neuroglobin and cytoglobin. Fresh blood for the vertebrate globin family. EMBO Rep. 3, 1146-51.

Pisano E., Ozouf-Costaz C. and Prirodina V. (1998) Chromosome diversification in Antarctic fish (Notothenioidei) In Fishes of Antarctica. A biological overview. Springer, Berlin Heidelberg New York, pp. 275-285.

Reuss S., Saaler-Reinhardt S., Weich B., Wystub S., Reuss M.H., Burmester T. and Hankeln T. (2002) Expression analysis of neuroglobin mRNA in rodent tissues. Neuroscience 115, 645-656.

Riggs A. (1988) The Bohr effect. Annu Rev Physiol 50, 181-204.

Ritchie P.A., Lavoué S. and Lecointre G. (1997) Molecular phylogenetics and evolution of Antarctic notothenioid fishes. Comp Biochem Physiol 4, 1009-1027.

Robins C.R. and Ray G.C. (1986) A field guide to Atlantic coast fishes of North America. Houghton Mifflin Company, Boston, USA p. 354.

Roesner A., Fuchs C., Hankeln T. and Burmester T. (2005) A globin gene of ancient evolutionary origin in lower vertebrates: evidence for two distinct globin families in animals. Mol Biol Evol 22, 12-20.

Roesner A., Hankeln T. and Burmester T. (2006) Hypoxia induces a complex response of globin expression in zebrafish (Danio rerio). J Exp Biol 209, 2129-2137.

Rohling E.J., Grant K., Hemleben C., Siddall M., Hoogakker B.A.A., Bolshaw M. and Kucera M. (2008) High rates of sea-level rise during the last interglacial period. Nature Geoscience 1, 38-42.

Routhieaux J., Sarcone S. and Stegenga K. (2005) Neurocognitive sequelae of sickle cell disease: current issues and future directions. J Pediatr Oncol Nurs 22, 160-167.

Ruud J.T. (1954) Vertebrates without erythrocytes and blood pigment. Nature 173, 848-850.

Sambrook J., Fritsch E.F. and Maniatis T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor, NY.

Samuel R.E., Salmon E.D. and Briehl R.W. (1990) Nucleation and growth of fibers and gel formation in sickle-cell hemoglobin. Nature 345, 833-835.

Schmidt M., Giessl A., Laufs T., Hankeln T., Wolfrum U. and Burmester T. (2003) How does the eye breathe? Evidence for neuroglobin-mediated oxygen supply in the mammalian retina. J Biol Chem 278, 1932-1935.

Scott N. and Lecomte J. (2000) Cloning, expression, purification, and preliminary characterization of a putative hemoglobin from the cyanobacterium Synechocystis sp. PCC 6803. Protein Sci 9, 587-597.

Severinghaus J.P., Sowers T., Brook E.J., Alley R.B. and Bender M.L. (1998) Timing of abrupt climate change at the end of the Younger Dryas interval from thermally fractionated polar ice. Nature 391, 141-146.

Sidell B.D. and O'Brien K.M. (2006) When bad things happen to good fish: the loss of hemoglobin and myoglobin expression in Antarctic icefishes. J Exp Biol 209, 1791-1802.

Statius van Eps L.W., (1999) Sickle cell disease. In: Atlas of Diseases of the Kidney. Wiley-Blackwell, Philadelphia, Pennsylvania, p. 22.

Smagghe B.J., Sarath G., Ross E., Hilbert J.L. and Hargrove M.S. (2006) Slow ligand binding kinetics dominate ferrous hexacoordinate hemoglobin reactivities and reveal differences between plants and other species. Biochemistry 45, 561-570.

Star B., Nederbragt A.J., Jentoft S., Grimholt U., Malmstrøm M., Gregers T.F., Rounge T.B., Paulsen J., Solbakken M.H., Sharma A., Wetten O.F., Lanzén A., Winer R., Knight J., Vogel J.-H., Aken B., Andersen Ø., Lagesen K., Tooming-Klunderud A., Edvardsen R.B., Tina K.G., Espelund M., Nepal C., Previti C., Karlsen B.O., Moum T., Skage M., Berg P.R., Gjøen T., Kuhl H., Thorsen J., Malde K., Reinhardt R., Du L., Johansen S.D., Searle S., Lien S., Nilsen F., Jonassen I., Omholt S.W., Stenseth N.C. and Jakobsen K.S. (2011) The genome sequence of Atlantic cod reveals a unique immune system. Nature 477, 207-210.

Sugimoto H., Makino M., Sawai H., Kawada N., Yoshizato K. and Shiro Y. (2004) Structural basis of human cytoglobin for ligand binding. J Mol Biol 339, 873-885.

Sun Y., Jin K., Mao X., Zhu Y. and Greenberg D.A. (2001) Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. Proc Natl Acad Sci U S A 98, 15306-15311.

Tamburrini M., Condò S.G., di Prisco G. and 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. and di Prisco G. (1996) The unique hemoglobin system of Pleuragramma antarcticum, an Antarctic migratory teleost. Structure and function of the three components. J Biol Chem 271, 23780-23785.

Thompson J.D., Higgins D.G., Gibson T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22, 4673-4680.

Tyrrell H.J.V. and Harris K.R. (1984) Diffusion in liquids: a theoretical and experimental study. Butterworth Publishers, Stoneham, MA.

Verde C., Carratore V., Riccio A., Tamburrini M., Parisi E. and 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. and di Prisco G. (2006) The oxygen-transport system in three species of the boreal fish family Gadidae. Molecular phylogeny of hemoglobin. J Biol Chem 281, 22073-22084.

Weber R.E., Jensen F.B. and Cox R.P. (1987) Analysis of teleost hemoglobin by Adair and Monod-Wyman-Changeux models, effect of nucleoside triphosphates and pH on oxygenation of tench haemoglobin. J Comp Physiol B 157, 145‑152.

Wetten O.F, Nederbragt A.J., Wilson R.C., Jakobsen K.S., Edvardsen R.B. and Andersen Ø. (2010) Genomic organization and gene expression of themultiple globins in Atlantic cod: conservation of globin-flanking genes in chordates infers the origin of the vertebrate globin clusters. BMC Evol Biol 10, 315-325.

Wishner B.C., Hanson J.C., Ringle W.M. and Love W.E. (1976) Crystal structure of sickle-cell deoxy hemoglobin. In Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease, U.S. Department of Health, Education, and Welfare pp. 1-31.

Wittenberg B.A. and Wittenberg J.B. (1989) Transport of oxygen in muscle. Annu Rev Physiol 51, 857-878.

Wittenberg J.B. and Wittenberg B.A. (2003) Myoglobin function reassessed. J Exp Biol 206, 2011-2020.

Wittenberg J.B. and Wittenberg D.K. (1974) The choroid rete mirabile. I. Oxygen secretion and structure: comparison with the swimbladder rete mirabile. Biol Bull 146, 116-136.

Yokoyama T., Chong K.T., Miyazaki G., Morimoto H., Shih D.T.B., Unzai S., Tame J.R.H. and Park S.-Y. (2004) Novel mechanisms of pH sensitivity in tuna hemoglobin: a structural explanation of the root effect. J Biol Chem 279, 28632-28640.

Zennadi R., Chien A., Xu K., Batchvarova M. and Telen M.J. (2008) Sickle red cells induce adhesion of lymphocytes and monocytes to endothelium. Blood 112, 3474-3483.

Zermann D.H., Lindner H., Huschke T., Schubert J., (1997) Diagnostic value of natural fill cystometry in neurogenic bladder in children. Eur Urol 32, 223-228.

List of Publications

1. **A Riccio,** G Mangiapia, D Giordano, A Flagiello, R Tedesco, S Bruno, A Vergara, L Mazzarella, G di Prisco, P Pucci, L Paduano and C Verde (2011) Polymerization of hemoglobins in arctic fish: *Lycodes reticulatus* and *Gadus morhua*, IUBMB Life 63(5), 346-354.
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Communications presented at National and International Conferences

1. Russo R, Coppola D, Giordano D, **Riccio A**, Barbiero G, Vergara A, Mazzarella L, di Prisco G, Verde C (2008) The structure and function of the hemoglobins of the non Antarctic notothenioid fish *Eleginops maclovinus* Workshop CAREX "Identification of model ecosystems in estreme environments Sant Feliu de Guixol, Spain.
2. Giordano D, Howes B, Russo R, **Riccio A**, Hui Bon Hoa G, Coletta M, Smulevich G, di Prisco G, Verde C (2009) Structural characterisation of 2-on-2 hemoglobin in the Antarctic psychrophilic bacterium Pseudoalteromonas haloplanktis TAC125. X SCAR International Biology Symposium, Sapporo, Japan.
3. **Riccio A**, Mangiapia G, Giordano D, Bruno S, Fiume I, Pocsfalvi G, Vergara A, Viappiani C, Paduano L, Mazzarella L, di Prisco G, Verde C, (2010). Hemoglobin-polymerisation processes in the Arctic fish, Proteine 2010, Parma, Italy.
4. **Riccio A**, Vergara A, Paduano L, Giordano D, Mangiapia G, Mazzarella L, di Prisco G, Verde C (2010). The Arctic Fish Hemoglobins, International Polar Year Science Conference, Oslo, Norway.
5. **Riccio A**, Mangiapia G, Giordano D, Flagiello A, Bruno S, Vergara A, Padano L, Pucci P, Mozzarella L, di Prisco G, Verde C (2010). The Arctic fish hemoglobins and

their polymerisation process, XVIth International Conference on Oxygen Binding and Sensing Proteins, Antwerp, Belgium.

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Research Communication

Polymerization of Hemoglobins in Arctic Fish: *Lycodes reticulatus* and *Gadus morhua*

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Summary

***In vitro*, and possibly *in vivo*, hemoglobin polymerization and red blood cell sickling appear to be widespread in codfish. In this article, we show that the hemoglobins of the two Arctic fish *Lycodes reticulatus* and *Gadus morhua* also have the tendency to polymerize, as monitored by dynamic light scattering experiments. The elucidation of the primary structure of the single hemoglobin of the zoarcid *L. reticulatus* shows the presence of a large number of cysteyl residues in α and β chains. Their role in eliciting the ability to produce polymers was also addressed by MALDI-TOF and TOF-TOF mass spectrometry. The *G. morhua* globins are also rich in Cys, but unlike in *L. reticulatus*, polymerization does not seem to be disulfide driven. The widespread occurrence of the polymerization phenomenon displayed by hemoglobins of Arctic fish supports the hypothesis that this feature may be a response to stressful environmental conditions.**

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Keywords Arctic; fish; hemoglobin; polymerization.

INTRODUCTION

Fish hemoglobins (Hbs) have been extensively studied not only for their structural and functional properties but also because they offer the possibility to investigate functional differentiation and molecular adaptation in organisms living in a large variety of environmental conditions. Fish Hbs, functioning at the interface between the organism and the environment, are especially interesting because gills are in contact with a medium endowed with higher oxygen and lower carbon-dioxide tensions compared to the alveoli of mammalian lungs, where the carbon-dioxide tension is higher and the oxygen tension lower than in the atmosphere. Moreover, in the liquid medium, fishes experience temporal and spatial variations in oxygen availability, salinity, ionic composition, pH, and temperature; hence their molecular processes rely on rapid responses to external stimuli. The basic molecular events associated with these processes involve protein-structural modifications (*1*). Many of the functional differences observed in fish Hbs may be interpreted in terms of substitutions of amino-acid residues, although others are due to changes in the composition and redox properties of the medium in which a given protein works. Changes in concentration of Hb within the red blood cell (RBC) provide another strategy for environment adaptation (*2*).

In vitro, and possibly *in vivo*, Hb polymerization and RBC sickling appear to be widespread in codfish (*3–5*). In a recent study *in vivo*, Koldkjaer and Berenbrink (*5*), using light and transmission electron microscopy, have demonstrated extensive *in vivo* sickling of RBCs of *Merlangius merlangus* (a gadiform related to the Atlantic cod *Gadus morhua*) after capture stress, without any apparent hemolysis. The authors identify the reduction of extracellular pH below resting values as the primary cause for *in vivo* sickling. The discovery of this unusual process suggests that polymerization may be a response to stressful environmental conditions. However, the molecular mechanisms of Hb polymerization and sickling in cods are yet unknown.

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In man, sickle cell anemia (SCA) is associated with the expression of the abnormal mutant sickle cell Hb (HbS), arising from a single-point mutation in the β chain, where polar Glu in position 6 is replaced by non-polar Val (*6*). This substitution induces polymerization that greatly reduces the solubility of the protein in the deoxy state and causes cell sickling. HbS polymers form an extremely viscous gel, responsible for the peculiar deformation of the RBCs (*7*). Polymerization and gel formation are considered the primary pathogenic events of SCA. HbS is also less stable than HbA when oxygenated; it auto-oxidizes at a faster rate and yields high concentrations of reactive oxygen species (ROS).

The primary structure of the single Hb of *Lycodes reticulatus* Hb (family Zoarcidae), living on the sea floor near the coasts of Northern Europe and North America, is herein described. This Hb was found to polymerize and form high-molecular-mass polymers at physiological pH and low temperature. The polymerization process was investigated by dynamic light scattering (DLS), in comparison with that of Hbs of the Atlantic cod *G. morhua* (family Gadidae) (*8*). *G. morhua* is widely distributed not only along the shelf areas of the Arctic basin but also at lower latitudes (*9*).

Unlike in *G. morhua*, polymerization of *L. reticulatus* Hb does not seem to be pH dependent. Hence, the role of the large number of cysteyl residues in α and β chains in eliciting the ability to produce polymers was addressed. It was demonstrated by MALDI-TOF and TOF-TOF mass spectrometry (MS) that the cysteyl residues of this Hb induce polymerization by interchain disulfide bonds, which may function as antioxidants providing protection against ROS, often produced during aerobic metabolism in environments characterized by fluctuating oxygen and pH values (*10*).

Similar to the single Hb of *L. reticulatus*, the *G. morhua* Hbs are also rich in Cys; however, their polymerization does not seem to be disulfide driven. The potential role of His in driving the pH-dependent polymerization observed in *G. morhua* is discussed.

We suggest that the unusual Hb polymerization in these two Arctic fish is an example of the Hb phenotypic plasticity, required in species experiencing variable environments.

EXPERIMENTAL PROCEDURES

Materials

CO was purchased from SON, Società Ossigeno Napoli spa. Sodium dithionite, dithiothreitol (DTT) and Tris-hydroxymethyl-methylamine (Tris) were from Sigma-Aldrich (Steinheim, Germany). The other chemicals were from Merck AG (Darmstadt, Germany), were analytical or reagent grade and were used without further purification.

Specimens, Hemolysates, Amino-Acid Sequencing

Adult *G. morhua* and *L. reticulatus* were collected by bottom and midwater trawling from the R/V *Jan Mayen* (*L. reticulatus*: Greenland, 72°00′N, 21°01′W; *G. morhua*: Svalbard, 78°13′N). Blood was taken by heparinized syringes from the caudal vein. Saline-washed RBCs were kept frozen at −80 °C until use.

Hemolysates were prepared by addition of approximately five volumes of 10 mM Tris-HCl pH 7.6 and stripped of organophosphates with a Sephadex-G25 column (GE-Healthcare Bio-Sciences). All steps were carried out at 0−4 °C (*11*).

Separation of *L. reticulatus* globins was carried out by reverse-phase high-performance liquid chromatography (RP-HPLC) of stripped hemolysate as described (*8*). Addition of 100 mM DTT avoided polymerization. Alkylation of sulfhydryl groups with 4-vinylpyridine, deacetylation of the α-chain N terminus and tryptic digestion were carried out as described (*12–14*). Tryptic peptides were purified by RP-HPLC with a μBondapak C_{18} column (0.39 × 30 cm; Waters Associates) as described (*15*). Sequencing was performed with an Applied Biosystems Procise 492 automatic sequencer, equipped with on-line detection of phenylthiohydantoin amino acids.

Cloning and Sequence Analysis of Globin cDNA

Total RNA was isolated from the spleen of *L. reticulatus* using TRI Reagent (Sigma) (*16*). The cDNA of the β^2 globin was amplified by PCR using oligonucleotides designed on the N-terminal regions as direct primers and at the adaptor primer as the reverse primer. Primer sequences are available from the authors upon request. Amplifications of cDNA were performed with 2.5 units Taq DNA polymerase, 5 pmol each of the 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. Standard molecular biology techniques (*17*) were used in the isolation, restriction, and sequence analysis of plasmid DNA.

Mass Spectrometry

Mass mapping of the α and β^1 chains of *L. reticulatus* was carried out by overnight trypsin digestion of the native protein in 50 mM ammonium bicarbonate buffer pH 8.0 at 37 °C. For disulfide bridges assignments, Hb aggregates purified by gel filtration were concentrated and digested overnight with trypsin in the same buffer used for chromatography (10 mM ammonium acetate pH 7.3) at 37 °C.

In both cases, the peptide mixtures were directly analyzed by MALDI-TOF and TOF-TOF MS on an AB Sciex 4800 MALDI TOF-TOF mass spectrometer. Tandem MS analyses (MS/MS) were carried out on selected signals to confirm amino-acid sequences.

UV–Visible Spectroscopy

UV–visible electronic absorption spectra were acquired from 700 to 250 nm in a Cary 300 UV–visible spectrophotometer (Varian).

Gel Filtration

DTT was added to CO-hemolysates of *L. reticulatus* and *G. morhua* (see below) in 10 mM Tris-HCl pH 7.6, at final concentration of 100 mM. The Hb concentration in the hemolysate was 0.06 mM on a heme basis. Gel filtration was carried out by fast protein liquid chromatography (AKTA-FPLC) with a Superose 6 column (GE-Healthcare). Elution was performed at 4 °C, in 10 mM Tris-HCl pH 7.6, 200 mM NaCl.

Oxygen Binding

Hemolysate stripping was carried out as described (*15*). Oxygen equilibria were measured in 100 mM MES/HEPES in the pH range 6.3–8.7, at 5 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. To obtain stepwise oxygen saturation, a modified gas-diffusion chamber was used, coupled to cascaded Wösthoff pumps for mixing pure nitrogen with air (*18*). Values of pH were measured with a radiometer BMS Mk2 thermostated electrode. Sensitivity to chloride was assessed by adding NaCl to a final concentration of 100 mM. The effect of ATP was measured at a final ligand concentration of 3 mM, in excess with respect to tetrameric Hb. Oxygen affinity (measured as p_{50}) and cooperativity (n_{Hill}) were calculated by linear fitting of the Hill plot. For each experiment, one aliquot of CO-hemolysate was thawed, converted to the oxy form by exposure to light and oxygen, and immediately used; no oxidation to MetHb was spectrophotometrically detectable.

Dynamic Light Scattering

DLS experiments were performed with 0.06 mM (on a heme basis) *L. reticulatus* and *G. morhua* hemolysates filtered through 0.22-μm Millipore filters, in 100 mM Tris-HCl/MES in the pH range 6.6–9.0 at 4 °C. CO-hemolysates (800 μL) were flushed with CO and sodium dithionite was added at a final concentration of 1 mM. Deoxy hemolysates were prepared by photolysis of the CO-hemolysates. Samples were then placed in a dry box filled with nitrogen following addition of few crystals of sodium dithionite. The cuvettes were sealed with rubber caps. DLS was performed with a setup of a Photocor compact goniometer, a SMD 6000 Laser Quantum 50 mW light source operating at 5,325 Å, and a PMT and correlator from Correlator.com. All measurements were performed at 4.00 ± 0.2 °C in a thermostated bath.

In DLS, the intensity autocorrelation function $g^{(2)}(t)$ is measured and related to the electric-field autocorrelation $g^{(1)}(t)$ by the Siegert relation (*19*):

$$g^{(2)}(t) = 1 + \beta\left|g^{(1)}(t)\right|^2 \tag{1}$$

where $\beta(\leq 1)$ is the coherence factor, which accounts for the deviation from ideal correlation and depends on the experimental geometry. The parameter $g^{(1)}(t)$ can be written as the Laplace transform of the distribution of the relaxation rate Γ used to calculate the translational diffusion coefficient D

$$g^{(1)}(t) = \int_{-\infty}^{+\infty} \tau A(\tau) \exp\left(-\frac{t}{\tau}\right)\, d\ln\ \tau \tag{2}$$

where $\tau = 1/\Gamma$. Laplace transforms were performed using a variation of CONTIN algorithm incorporated in Precision Deconvolve software. From the relaxation rates, the z-average of the diffusion coefficient D may be obtained as (*19*)

$$D = \frac{\Gamma}{q^2} \tag{3}$$

where $q = 4\pi n_0/\lambda \sin(\theta/2)$ is the modulus of the scattering vector, n_0 is the refractive index of the solution, λ is the incident wavelength, and θ represents the scattering angle. If the solutions are quite dilute, the Stokes–Einstein equation, which rigorously holds at infinite dilution for spherical species diffusing in a continuum medium, may be used to evaluate the hydrodynamic radius R_{H} of the aggregates.

$$R_{\mathrm{H}} = \frac{kT}{6\pi\eta D} \tag{4}$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the medium viscosity. We note that R_{H} in Eq. (4) for not spherical particles represents the radius of equivalent spherical aggregates with the same diffusion coefficient (*20*). The number of tetramers in each aggregate was obtained by dividing the volume of aggregates (calculated by the formula $V = (4/3)\pi R_{\mathrm{H}}^3$) by the tetramer volume.

RESULTS AND DISCUSSION

The primary structure of Hb of L. reticulatus. Cellulose acetate electrophoresis of the fresh hemolysate of *L. reticulatus* revealed a single Hb. However, the frozen CO-hemolysate, once thawed, showed multiple bands in Blue Native PAGE (data not shown), suggesting formation of polymers during freezing. All attempts to purify non-polymerized Hb to homogeneity were unsuccessful. The RP-HPLC profile of the CO-hemolysate in the polymerized form, in the absence of DTT, indicated the presence of the α chain and of a broad unresolved peak corresponding to the β chains, as established by MS and partial amino-acid sequencing. In the presence of 100 mM DTT, RP-HPLC showed three well-separated globins, namely the α chain and two different β chains in equal amounts exhibiting slight microheterogeneity (as established by amino-acid sequencing and MS, see below). The amino-acid sequences of the α and β chains, in comparison with other sequences, are reported in Fig. 1. The primary structure of the α chain was established by alignment of tryptic peptides and

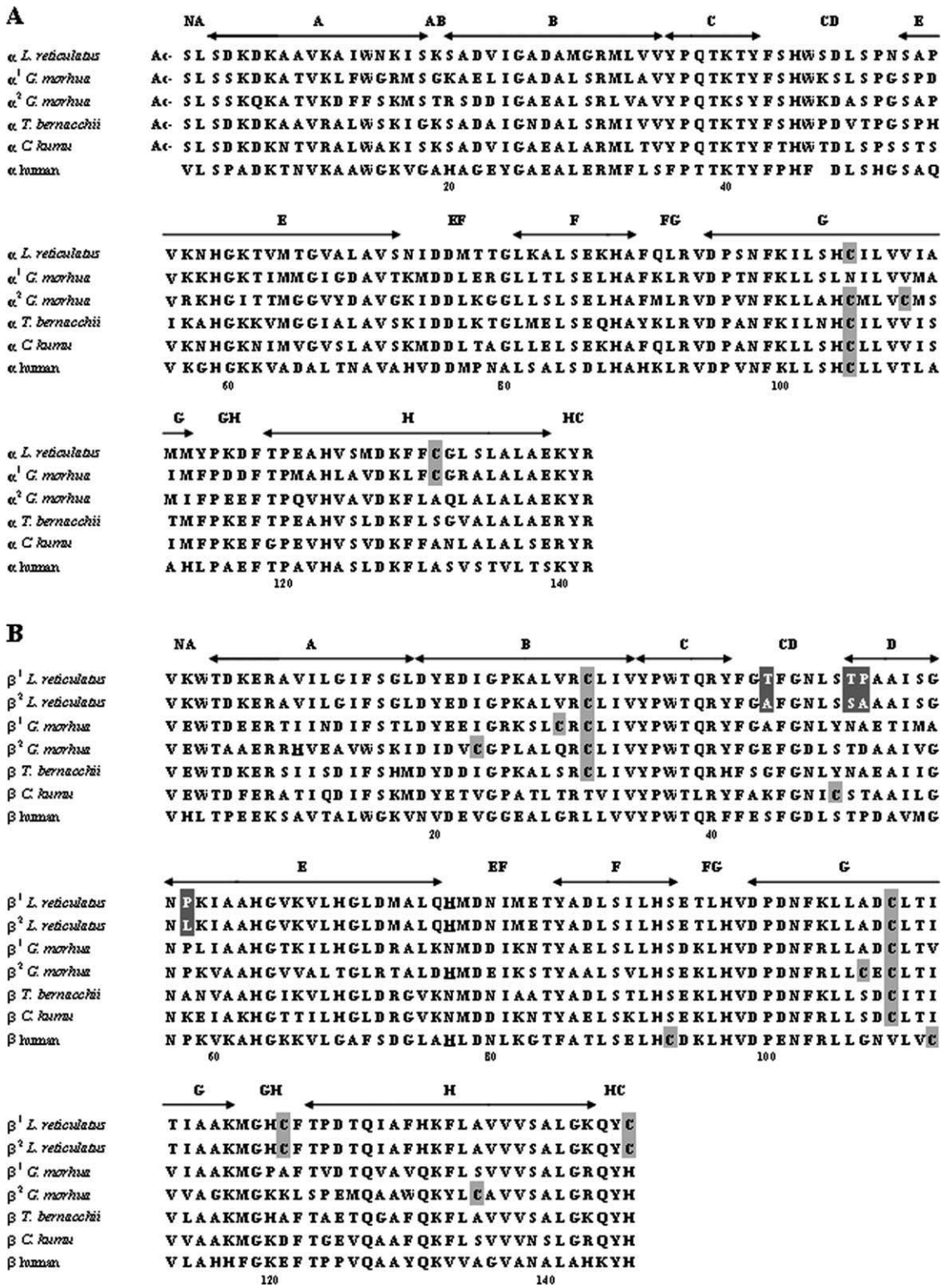

Figure 1. Amino-acid sequence of the (A) α and (B) β chains of *L. reticulatus* (this work) and Arctic *G. morhua* (*8*), Antarctic *T. bernacchii* (*21*) and temperate *C. kumu* (*23*). Cys residues are in light grey boxes. HisA7 and HisEF1 in the β^2 chain of *G. morhua* are underlined. The differences between the two β chains of *L. reticulatus* are indicated with white letters in dark-grey boxes. The helical (A–H) and non-helical (NA, A, CD, EF, FG, GH, and HC) regions, as established for mammalian Hb, are indicated; in α chains, helix D is lacking.

homology with fish globins and confirmed by mass mapping of its tryptic peptides (see below). DNA sequencing was utilized for the β^2 chain and MS for the amino-acid sequencing of the β^1 chain. The latter was digested with trypsin and the peptide mixture directly analyzed by MALDI-TOF and TOF-TOF MS. The accurate mass values of the tryptic peptides were mapped onto the anticipated amino-acid sequence of the β^2 chain used as template. MS/MS analyses were carried out on the selected signals displaying mass differences from the β^2 peptides, leading to the definition of their sequences. The N terminus of the α chain was not available to Edman degradation because of the presence of a blocking acetyl group. The molecular masses were 15,663.3 $\pm$ 0.3 Da for the α and 16,121.5 $\pm$ 0.3 and 16,067.4 $\pm$ 0.6 Da for the β^1 and β^2 chains, respectively, in perfect agreement with the theoretical values calculated based on the primary structures. The two β chains differ in only four positions in a restricted region of the sequence. For the sake of simplicity, we choose to refer to a "single" Hb, and not to two Hbs, in the assumption that this "microheterogeneity" defines a genetic variant and not a functionally distinct Hb. The globins revealed several non-conservative substitutions with respect to other vertebrate globins. Among the functionally important residues suggested to be involved in the molecular mechanism of the Bohr and Root effects in fish Hbs (*21*), Ser β93 F9, Glu β94 FG1, and Gln β144 HC1 are conserved in the β chains, whereas His β146 HC3 is replaced by Cys. In human HbA, the main Bohr groups are N-terminal Val α1 NA1 and C-terminal His β146 HC3, which account for about 30% and 50–65% of Bohr effect, respectively (*22*). In position α NA1, fish Hbs have acetyl-Ser, therefore, the decreased Bohr effect observed in *L. reticulatus* with respect to other fish Hbs (see below) may be due to the His $\rightarrow$ Cys β146 HC3 substitution; however, the role of His β146 HC3 residue in eliciting the Root effect is controversial (*23*). Of the Asp α48 CD6/His α55 E3 and His β69 E13/Asp β72 E16 pairs, supposed to contribute to the Root effect in fish Hbs (*24, 25*), only the latter is conserved. In the $\alpha_1\beta_2$ "dovetailed" switch region in HbA, formed by Pro α44 CD2, Thr α38 C3, Thr α41 C6, and His β97 FG4, Pro α44 CD2 is replaced by Ser and Thr α38 C3 by Gln. Val β60 E4, considered to be invariant in vertebrates, including most teleosts, is replaced by Ile. Val β67 E11, usually present at the distal side of the heme, is replaced by Ile. This substitution may produce functional subunit heterogeneity, as reported in Hb of temperate *Chelidonichthys kumu* (*23*) and in cathodic Hb of Antarctic *T. newnesi* (*26*). In HbA mutants, the bulky side chain of Ile β67 E11 blocks the access of oxygen to the β chain, significantly lowering the association (and equilibrium) constant in both the T (*27*) and R states (*28*). In deoxy HbA, Val β67 E11 overlaps the ligand binding site and is considered to play a key role in controlling the oxygen affinity. The α and β chains of *L. reticulatus* contain several Cys residues often absent in other teleosts, in positions α105 G11, α131 H13, β31 B13, β109 G11, β121 GH4, and β146 CH3.

The previously published (*8*) amino-acid sequences of the two α and the two β chains constituting the three Hbs of *G. morhua* are also reported in Fig. 1. Similar to *L. reticulatus*, they are unusually rich in Cys. Despite the general trend toward reduction in His content in teleost Hbs (*29*), the β^2 chain of *G. morhua* contains two extra His residues, His β10 A7 and His β77 EF1 (*8*). These residues are absent in most fish Hbs with the exception of *L. reticulatus* β globins, which have His β77 EF1.

Oxygen Binding

Functional studies were only performed on the polymerized form of *L. reticulatus* Hb, because it was impossible to obtain non-polymerized Hb unless DTT (which interferes with the measurements) was present. The oxygen-binding experiments were performed at 5 and 10 °C (data not shown), in the absence and presence of allosteric physiological effectors, e.g., chloride and organophosphates (ATP). The Bohr effect was low, and it was not significantly enhanced by the effectors. In the whole pH range and in the presence of the effectors, the Hill coefficient (n_{Hill}) was close to 1.5, reflecting low levels or apparent lack of subunit cooperativity. Therefore, a leading role of polymerization in the lack of Bohr effect in *L. reticulatus* Hb cannot be uniquivocally deduced, because Cys in place of His at the C terminus of this Hb may also substantially decrease such effect.

The previously published data on *G. morhua* were integrated by additional functional studies on the hemolysate, which contains partially polymerized Hb forms (*8*). Experiments were performed at 5 and 10 °C, in the absence and presence of allosteric effectors. A strong Bohr effect was observed, and enhancement by organophosphates was high. In the whole pH range, the Hill coefficient (n_{Hill}) was close to one, reflecting very low levels, or apparent lack of subunit cooperativity.

The Polymerization Process

Gel filtration of the CO-hemolysate of *L. reticulatus* in 10 mM Tris-HCl pH 7.6 in the absence of DTT revealed multiple large peaks, again suggesting formation of polymers (data not shown). The first three fractions contained higher-molecular-mass components, whose spectral features excluded reoxidation of the iron. The last small fraction, having identical elution volume to that of HbA, contained non-polymerized Hb. The results suggest formation of polymers of different molecular size. Polymerization essentially appeared to depend upon formation of intermolecular disulfide bonds, because the first three fractions disappeared on addition of DTT and were replaced by the tetramer. The RP-HPLC of the tetramer obtained from gel filtration resembled the globin pattern of the hemolysate in the presence of DTT, with three well-separated peaks of α chain and the two β chains in equal amounts.

In contrast, gel filtration of the CO-hemolysate of *G. morhua* at pH 7.6 in the absence of DTT revealed a much lower amount of polymers (data not shown). The high-molecular-mass compo-

Table 1
Mass signals of S-S bridged peptides and Cys residues involved in tryptic hydrolysis of high-molecular-weight aggregates of *L. reticulatus* Hb

MH^+	Peptide pair	Cys residues involved
4490.2	β^2 (31–59) + β (105–117)	β^2 Cys 31-β Cys109
2621.4	β (31–40) + β (105–117)	β Cys31-β Cys109
1688.8	β (31–40) + β (144–146)	β Cys31-β Cys146
1755.9	β (105–117) + β (144–146)	β Cys109-β Cys146
3075.5	β (105–117) + β (118–132)	β Cys109-β Cys121
3598.7	α (101–128) + β (144–146)	α Cys105-β Cys146
3462.5	β (118–132) + β (118–132)	β Cys121-β Cys121

nents did not decrease upon addition of 120 mM DTT. In the first extensive study of the oxygen-transport system of three Arctic species of the family Gadidae, namely the Arctic cod *Arctogadus glacialis*, the polar cod *Boreogadus saida*, and the Atlantic cod *G. morhua* (*8*), these fish have identical multiplicity of Hbs. The ion-xchange chromatography of the three hemolysates yielded similar elution patterns, showing one broad band, indicative of unresolved Hbs. Many procedures were attempted to purify the different components to homogeneity, but they were unsuccessful, with the exception of the third component. Hence, concentration-dependent equilibria between dimers or pH-dependent aggregation between tetramers were hypothesized (*8*).

Mass Spectrometry of L. reticulatus *Globins*

The chemical nature of the oligomers of *L. reticulatus* Hb was investigated by mass mapping of their tryptic peptides. The high-molecular mass, DTT-reduced aggregates were isolated by gel filtration and directly digested with trypsin. The peptide mixture was analyzed by MALDI-TOF MS, revealing the occurrence of peptides belonging to both α and β globin chains. A number of mass signals in the spectra could not be associated to any linear peptide within the amino-acid sequence of the globins and were tentatively interpreted as disulfide-containing fragments. Based on their unique mass values, these signals were identified as S-S bridged peptides and their assignments are listed in Table 1, together with the Cys pairs involved in the crosslinks. Selected signals were submitted to MS/MS analyses to confirm the assignments. Mass-spectral analyses confirmed the hypothesis that the Hb oligomeric species of *L. reticulatus* were essentially formed by intermolecular S-S bridges. Further support to this hypothesis is also provided by homology modeling, indicating that the distances between pairs of Cys residues are incompatible to form intramolecular S-S bridges (L. Boechi, personal communication). As expected, the vast majority of the Cys residues involved in disulfide-bridge formation belong to the β globins, suggesting higher reactivity of these residues than those of the α chain, a well-known behavior similar to human globins. A single Cys of the α chain, Cys α105 G11 was indeed found involved in an S-S bridge with Cys β146 CH3. The almost identical sequences of the two β chains impaired to ascertain which chain was involved in each bridge, with the exception of the peptide pair associated with the mass value at m/z 4,490.2 (Table 1). This signal corresponds to the β^2 peptide 31-59 joined to the β^1 (or β^2) fragment 105-117, as the two β globins showed different sequences in the 44–58 region. Many S-S bridges were formed by C-terminal Cys β146 CH3 of the β chain. This behavior is similar to that found in the human variant Hb Rainier, where β C-terminal Tyr is substituted by Cys, leading to an intramolecular disulfide with Cys β93 F9 (*30*).

Dynamic Light Scattering

Globin association in the hemolysates of *L. reticulatus* and *G. morhua* as a function of coordination state (CO and deoxy), pH (6.6–9.0) and addition of 120 mM DTT (final concentration) and at 4 °C, was also investigated by DLS. HbA was used as control of non-aggregating globin, with a hydrodynamic radius $R_H = 3.5 \pm 0.2$ nm. The hemolysates showed multimodal distributions of three–four aggregates, named **I**, **II**, **III,** and **IV** according to increasing size.

At pH 7.6, the *L. reticulatus* CO-hemolysate in the absence of DTT (Fig. 2A) showed three aggregates of increasing size (**II** at 8.5 ± 0.8, **III** at 34 ± 5, and **IV** at 85 ± 12 nm). Addition of DTT (Fig. 2B) led, within 15 min, to formation of an additional species (**I**) and a significant variation of the aggregation size (**I** at 3.3 ± 0.5, **II** at 14 ± 1, **III** at 33 ± 5, and **IV** 174 ± 90 nm). Upon DTT removal, almost instantaneous disappearance of aggregate **I** and the slow return to the initial aggregation distribution occurred. The additional diffusing particle **I** can be confidently associated to the single Hb tetramer of *L. reticulatus* (3.3 nm) (*31*), produced by DTT-induced reduction of disulfide bridges. Because of technical limitations (at higher concentrations multiple scattering occurs making analysis of the results unreliable), it was not possible to investigate the effect of concentration on aggregation.

Indeed, the large number of Cys residues in α and β chains of the *L. reticulatus* hemolysate elicits the ability to produce polymers. DLS experiments not only confirmed the significant role of intermolecular disulfide bridges in the aggregation behavior of the hemolysate of *L. reticulatus* but also defined the multimodal aggregate distribution (Table 2). R_H in both deoxy and CO-hemolysates were quite invariant on pH variation (from 6.6 to 9.0), suggesting no crucial involvement of protonable groups in the aggregation mechanism hemolysate.

The hemolysate of *G. morhua* exhibited modulation of the aggregation behavior, unlike that of *L. reticulatus*. At pH 7.6, in the absence of DTT, the CO-hemolysate of *G. morhua* (Fig. 2C) showed three aggregates (**I** at 3.4 ± 0.3, **II** 88.0 ± 5.0, **III** 421 ± 12 nm). As expected, at higher Hb concentration, the relative population **II/I** and **III/I** increased. The number of aggregates and their R_H were insensitive to DTT (Table 2), suggest-

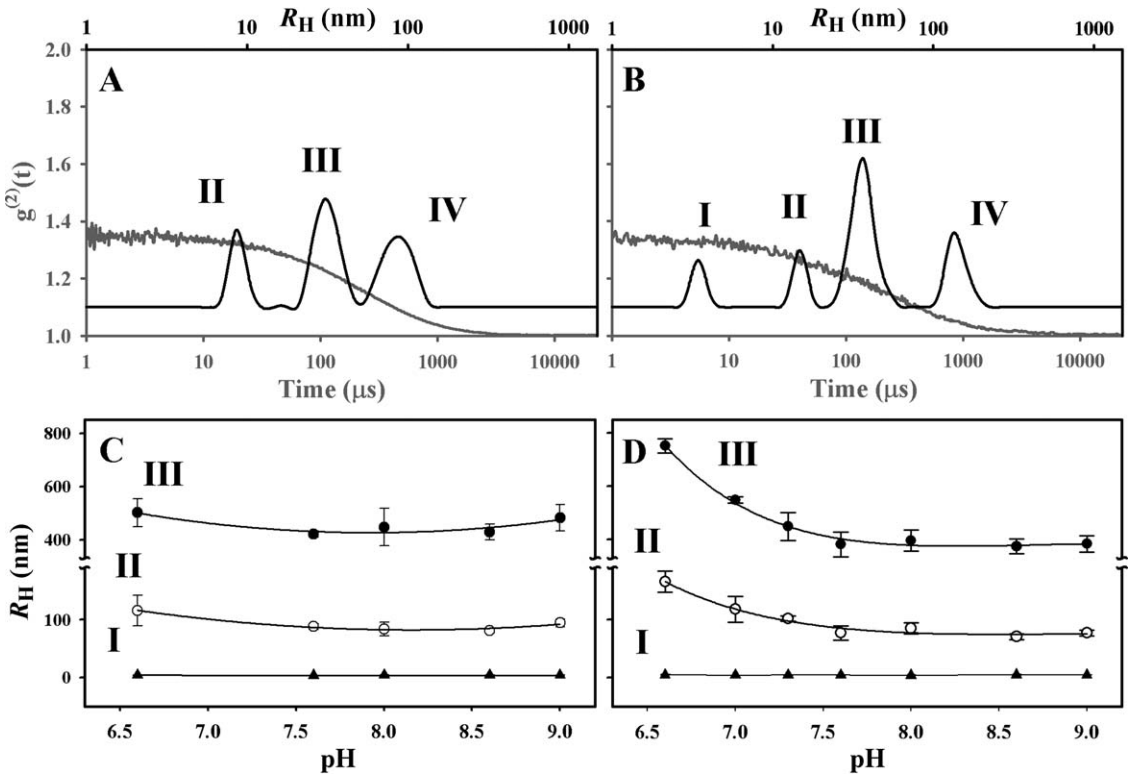

Figure 2. DLS characterization of the hemolysates of *L. reticulatus* (A, B) and *G. morhua* (C, D). (A) CO and (B) deoxy state of *L. reticulatus* Hb with the distribution of aggregates as a function of hydrodynamic radius, R_H, (in black), and examples of the correlation function, $g^{(2)}(t)$, as a function of time for both states (in gray). (C) CO and (D) deoxy state of *G. morhua* Hb with R_H as a function of pH for each multimodal distribution of aggregates (**I**, **II**, and **III**).

ing that involvement of Cys in the aggregation mechanism is not crucial, despite their high content, which is comparable in number (but not in position) to that of the *L. reticulatus* sequence.

In contrast to *L. reticulatus*, the *G. morhua* hemolysate showed dependence of aggregation behavior to pH, particularly in the deoxy state (Fig. 2D). As pH decreased, the *G. morhua* population **I** distribution decreased in favor of the larger aggregates **II** and **III**, whose R_H increased (Table 2). This evidence suggests a significant role of protonable groups in the aggregation mechanism. Indeed, despite the general trend of reduction in His content in teleost Hbs, suggested to be an important step in the evolution of the oxygen-transport system (*29, 31*), the analysis of the amino-acid sequences of *G. morhua* globins (*8*) indicates that one of the two β globins contains two extra His residues (His β7 A7 and His β77 EF1) located on the surface of the protein (*5, 32*).

CONCLUDING REMARKS

Pathological aggregation of proteins is generating increasing interest, and many studies are aimed at the molecular mechanisms underlying the role of point mutations in the primary structure in driving aggregation. Fish appear to be useful models for studying polymerization-related phenomena in RBCs and provide advantages for links with the physiology and biochemistry of human sickling disease. By studying the structure and function of polymerizing fish Hbs, we can better understand this important group of vertebrates, and we can learn more about the lethal pathology of human RBC sickling.

In this study, *in vitro* Hb polymerization was demonstrated in *L. reticulatus*. The structural properties of this Hb include formation of polymers through disulfide bonds. Several S-S bridges were formed by C-terminal Cys β146 CH3, indicating higher reactivity, and/or high flexibility of the domain where this residue is located. This behavior is similar to that of the human variant Hb Rainier (*30*), and of *C. kumu* Hb (*23*), where Cys β49 CD8 replaces His, commonly found in other fish Hbs. Hb polymerization has also been recorded in other teleosts (*33*).

Previous studies (*4, 5*) of Hb polymerization that occurs in RBCs of several fish, sometimes leading to sickle-cell formation (*5*), suggest that this process may be a unique example of Hb plasticity. The relative importance in fish physiology is yet unknown; whether this process occurs *in vivo* is rather difficult to ascertain, but deserves further investigation, because of the possible links with SCA.

Table 2

R_H of each *L. reticulatus* and *G. morhua* aggregate in CO and deoxy state at different pH. In bold, R_H after DTT addition

	L. reticulatus								*G. morhua*					
	CO state				Deoxy state				CO state			Deoxy state		
pH	R_H (nm) I	R_H (nm) II	R_H (nm) III	R_H (nm) IV	R_H (nm) I	R_H (nm) II	R_H (nm) III	R_H (nm) IV	R_H (nm) I	R_H (nm) II	R_H (nm) III	R_H (nm) I	R_H (nm) II	R_H (nm) III
6.6	–	7.8 ± 0.5 (11)	32 ± 5 (7.6×10^2)	93 ± 3 (1.8×10^4)	4.5 ± 0.4 (2)	17 ± 3 (1.1×10^2)	78 ± 7 (1.1×10^4)	385 ± 93 (1.3×10^6)	3.9 ± 0.4 (1)	116 ± 26 (3.6×10^4)	501 ± 51 (2.9×10^6)	4.3 ± 0.6 (1)	166 ± 18 (1.1×10^5)	752 ± 27 (9.9×10^6)
7.0	–	–	–	–	–	–	–	–	–	–	–	3.5 ± 0.1 3.5 ± 0.6 (1)	118 ± 22 121 ± 60 (3.8×10^4)	548 ± 12 507 ± 150 (3.8×10^6)
7.3	–	–	–	–	–	–	–	–	–	–	–	3.9 ± 0.3 (1)	102 ± 4 (2.5×10^4)	449 ± 53 (2.1×10^6)
7.6	– **3.3 ± 0.1**	8.5 ± 0.8 **14 ± 1** (14)	34 ± 5 **33 ± 5** (9.2×10^2)	85 ± 12 **174 ± 90** (1.4×10^4)	4.9 ± 0.6 **3.3 ± 0.6** (3)	20 ± 3 **13 ± 4** (1.8×10^2)	71 ± 7 **49 ± 4** (8.3×10^3)	354 ± 66 **139 ± 4** (1.0×10^6)	3.4 ± 0.3 **3.5 ± 0.1** (1)	88 ± 5 **89 ± 6** (1.6×10^4)	421 ± 12 **453 ± 29** (1.7×10^7)	3.8 ± 0.3 **3.4 ± 0.6 (1)**	77 ± 13 **86 ± 9** (1.1×10^4)	382 ± 47 **400 ± 20** (1.3×10^6)
8.0	–	–	–	–	–	–	–	–	3.5 ± 0.3 (1)	84 ± 12 (1.3×10^4)	447 ± 70 (2.0×10^7)	3.4 ± 0.3 3.5 ± 0.3 (1)	85 ± 10 72 ± 4 (1.4×10^4)	397 ± 40 360 ± 6 (1.4×10^6)
8.6	– **3.6 ± 0.9**	6.9 ± 0.3 **19 ± 5** (8)	23 ± 2 **102 ± 10** (2.8×10^2)	103 ± 12 **269 ± 68** (2.5×10^4)	4.9 ± 0.3 **3.3 ± 0.2** (3)	20 ± 3 **12.7 ± 2** (1.8×10^2)	80 ± 11 **60 ± 10** (1.2×10^4)	289 ± 78 **240 ± 90** (5.6×10^5)	3.6 ± 0.4 **3.2 ± 0.3** (1)	81 ± 4 **86 ± 6** (1.2×10^4)	429 ± 30 **424 ± 50** (1.7×10^7)	4.2 ± 0.6 (1)	71 ± 5 (8.3×10^3)	375 ± 28 (1.2×10^6)
9.0	–	–	–	–	–	–	–	–	**3.6 ± 0.3** (1)	**95 ± 7** 2(2.0×10^4)	**482 ± 50** (2.6×10^6)	**4.0 ± 0.2** (1)	**77 ± 5** (1.0×10^4)	**384 ± 30** (1.3×10^6)

In brackets, number of tetramer in the aggregate (see Experimental procedures for details).

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REFERENCES

1. Perutz, M. F. (1983) Species adaptation in a protein molecule. *Mol. Biol. Evol.* **1**, 1–28.
2. Weber, R. E. and Jensen, F. B. (1988) Functional adaptations in hemoglobins from ectothermic vertebrates. *Annu. Rev. Physiol.* **50**, 161–179.
3. Yoffey, J. M. (1929) A contribution to the study of the comparative histology and physiology of the spleen, with reference chiefly to its cellular constituents. I. In Fishes. *J. Anat.* **63**, 314–344.
4. Hárosi, F. I., von Herbing, I. H., and Van Keuren, J. R. (1998) Sickling of anoxic red blood cells in fish. *Biol. Bull.* **195**, 5–11.
5. Koldkjaer, P. and Berenbrink, M. (2007) *In vivo* red blood cell sickling and mechanism of recovery in whiting *Merlangius merlangus*. *J. Exp. Biol.* **210**, 3451–3460.
6. Ingram, V. M. (1957) Gene mutations in human haemoglobin: the chemical difference between normal and sickle cell haemoglobin. *Nature* **180**, 326–328.
7. Galkin, O. and Vekilov, G. (2004) Mechanisms of homogeneous nucleation of polymers of sickle cell anemia hemoglobin in deoxy state. *J. Mol. Biol.* **336**, 43–59.
8. Verde, C., Balestrieri, M., de Pascale, D., Pagnozzi, D., Lecointre, G., and di Prisco, G. (2006) The oxygen transport system in three species of the boreal fish family Gadidae. *J. Biol. Chem.* **281**, 22073–22084.
9. Andriashev, A. P. (1970) Cryopelagic fishes of the Arctic and Antarctic and their significance in polar ecosystems. In *Antarctic Ecology* (Hodgate, M. W., ed). pp. 97–304, Academic Press, London.
10. Reischl, E., Dafre, A. L., Franco, J. L., and Wilhelm Filho, D. (2007) Distribution, adaptation and physiological meaning of thiols from vertebrate hemoglobins. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **146**, 22–53.
11. Tamburrini, M., Condò, S. G., di Prisco, G., and Giardina, B. (1994) Adaptation to extreme environments: structure-function relationships in Emperor penguin haemoglobin. *J. Mol. Biol.* **237**, 615–621.
12. D'Avino, R. and di Prisco, G. (1989) Hemoglobin from the Antarctic fish *Notothenia coriiceps neglecta*. *Eur. J. Biochem.* **179**, 699–705.
13. Tamburrini, M., Brancaccio, A., Ippoliti, R., and di Prisco, G. (1992) The amino acid sequence and oxygen-binding properties of the single hemoglobin of the cold-adapted Antarctic teleost *Gymnodraco acuticeps*. *Arch. Biochem. Biophys.* **292**, 295–302.
14. Tamburrini, M., D'Avino, R., Fago, A., Carratore, V., Kunzmann, A., and di Prisco, G. (1996) The unique hemoglobin system of *Pleuragramma antarcticum*, an Antarctic migratory teleost. Structure and function of the three components. *J. Biol. Chem.* **271**, 23780–23785.
15. Tamburrini, M., Romano, M., Carratore, V., Kunzmann, A., Coletta, M., and di Prisco, G. (1998) The hemoglobins of the Antarctic fishes *Artedidraco orianae* and *Pogonophryne scotti*. Amino acid sequence, lack of cooperativity, and ligand binding properties. *J. Biol. Chem.* **273**, 32452–32459.
16. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor, NY.
18. Weber, R. E., Jensen, F. B., and Cox, R. P. (1987) Analysis of teleost hemoglobin by Adair and Monod-Wyman-Changeux models, effect of nucleoside triphosphates and pH on oxygenation of tench haemoglobin. *J. Comp. Physiol. B* **157**, 145–152.
19. Berne, B. J. and Pecora, R. (2000) *Dynamic light scattering: with applications to chemistry, biology, and physics*. Dover Editions, NY.
20. Tyrrell, H. J. V. and Harris, K. R. (1984) *Diffusion in liquids: a theoretical and experimental study*. Butterworth Publishers, Stoneham, MA.
21. Camardella, L., Caruso, C., D'Avino, R., di Prisco, G., Rutigliano, B., Tamburrini, M., Fermi, G., and Perutz, M. F. (1992) Hemoglobin of the Antarctic fish *Pagothenia bernacchii*. Amino acid sequence, oxygen equilibria and crystal structure of its carbonmonoxy derivative. *J. Mol. Biol.* **224**, 449–460.
22. Perutz, M. F. and Brunori, M. (1982) Stereochemistry of cooperative effects in fish and amphibian hemoglobins. *Nature* **229**, 421–426.
23. Fago, A., Romano, M., Tamburrini, M., Coletta, M., D'Avino, R., and di Prisco, G. (1993) A polymerising Root-effect fish hemoglobin with high subunit heterogeneity. Correlation with primary structure. *Eur. J. Biochem.* **218**, 829–835.
24. Mazzarella, L., Vergara, A., Vitagliano, L., Merlino, A., Bonomi, G., Scala, S., Verde, C., and di Prisco, G. (2006) High resolution crystal structure of deoxy hemoglobin from *Trematomus bernacchii* at different pH values: the role of histidine residues in modulating the strength of the Root effect. *Proteins: Struct, Funct, Bioinf* **65**, 490–498.
25. Yokoyama, T., Chong, K. T., Miyazaki, G., Morimoto, H., Shih, D. T., Unzai, S., Tame, J. R., and Park, S. Y. (2004) Novel mechanisms of pH sensitivity in tuna hemoglobin: a structural explanation of the Root effect. *J. Biol. Chem.* **279**, 28632–28640.
26. Mazzarella, L., Bonomi, G., Lubrano, M. C., Merlino, A., Riccio, A., Vergara, A., Vitagliano, L., Verde, C., and di Prisco, G. (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.
27. Nagai, K., Luisi, B., Shih, D., Miyazaki, G., Imai, K., Poyart, C., De Young, A., Kwiatkowsky, L., Noble, R. W., Lin, S. H., and Yu, N. T. (1987) Distal residues in the oxygen binding site of haemoglobin studied by protein engineering. *Nature* **329**, 858–860.
28. Mathews, H. J., Rohlfs, R. J., Olson, J. S., Tame, J., Renaud, J. P., and Nagai. K. (1989) The effects of E7 and E11 mutations on the kinetics of ligand binding to R state human haemoglobin. *J. Biol. Chem.* **264**, 16573–16583.
29. Berenbrink, M., Koldkjaer, P., Kepp, O., and Cossins, A. R. (2005) Evolution of oxygen secretion in fishes and the emergence of a complex physiological system. *Science* **307**, 1752–1757.
30. Carbone, V., Salzano, A. M., Pagano, L., Viola, A., Buffardi, S. De Rosa, C., and Pucci, P. (1999) Hb Rainier (β145(HC2)Tyr $\rightarrow$ Cys) in Italy. Characterisation of the amino acid substitution and the DNA mutation. *Hemoglobin* **23**, 111–124.
31. Pan, W., Galkin, O., Filobelo, L., Nagel, R. L., and Vekilov, P. G. (2007) Metastable mesoscopic clusters in solutions of sickle-cell hemoglobin. *Biophys. J.* **92**, 267–277.
32. Berenbrink, M. (2006) Evolution of vertebrate haemoglobins: Histidine side chains, specific buffer value and Bohr effect. *Respir. Physiol. Neurobiol.* **154**, 165–184.
33. Borgese, T. A., Harrington, J. P., Duffy, L., and Bourke, S. (1988) Hemoglobin properties and polymerisation in the marine teleost *Lophius americanus* (Goosefish). *Comp. Biochem. Physiol.* **91B**, 663–670.

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Review

Cold-adapted bacteria and the globin case study in the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125

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ABSTRACT

Environmental oxygen availability may play an important role in the evolution of polar marine organisms, as suggested by the physiological and biochemical strategies adopted by these organisms to acquire, deliver and scavenge oxygen. Stress conditions such as extreme temperatures increase the production of reactive oxygen species (ROS) in cells. Thus, in order to prevent cellular damage, adjustments in antioxidant defences are needed to maintain the steady-state concentration of ROS.

Cold-adapted bacteria are generally acknowledged to achieve their physiological and ecological success in cold environments through structural and functional properties developed in their genomes. A short overview on the molecular adaptations of polar bacteria and in particular on the biological function of oxygen-binding proteins in *Pseudoalteromonas haloplanktis* TAC125, selected as a model, will be provided together with the role of oxygen and oxidative/nitrosative stress in regulating adaptive responses at cellular and molecular levels.

Contents

1. The extreme marine environments

It is currently recognised that, by virtue of their extension, extreme environments are the most important part of the Earth's biosphere. Their study is still limited, but more and more extreme environments are now becoming accessible thanks to the broadening of technological progress and research on extreme adaptations.

The marine environments are generally of various nature and consequently include a wide variety of microorganism communities able to adapt even under the most stressing conditions and to grow at remarkably high rates; microorganisms are a potential treasure of gene resources (Bowler et al., 2009) and possess a great potential for producing new and different bioactive metabolites and enzymes for bio-prospecting studies.

Extreme marine environments usually combine a range of physical gradients (e.g. pressure, temperature, pH, salinity) and toxic and/or essential chemicals (oxygen, H_2S, CH_4, metals such as Fe, Cu, Mo, Zn, Cd, Pb, etc.) that by far exceed typical oceanic ranges. Communities often rely on species-specific interactions to carry out major ecological functions. Studies of interspecies interactions (e.g. genetic exchange) and of novel metabolic pathways are cutting-edge issues that need to be tackled to understand their role in marine environments.

The Antarctic marine habitats are unique natural laboratories for fundamental research on the evolutionary processes that shape biological diversity in extreme environments. The Antarctic biota

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evolved under the influence of a suite of geological and climatic factors, including geographic isolation of the landmass and continental shelves, extreme low temperature and intense seasonality. Isolation and extreme environmental history have forged a unique biota, both on land and in the sea. Unlike deep oceans, polar marine environments are subject to large seasonal variations in sea-ice cover, greatly affecting the biology of organisms (Moline et al., 2008).

In an extreme environment such as Antarctica, one of the most important driving forces in the evolutionary adaptations of marine organisms is the enhanced oxygen solubility in the cold waters of the Southern Ocean (Chen et al., 2008). These environmental conditions may cause the production of high levels of ROS, able to oxidise proteins, DNA and lipids and leading to extensive injury of cellular components and cell death (Fig. 1, adapted from Zhou et al., 2010). ROS capable of such damage include, but are not limited to, the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH·). Intracellular free radicals, i.e., free, small molecules with an unpaired electron, are often ROS. ROS are formed and degraded by all aerobic organisms, either when present in the concentrations required for normal cell function, or in excessive amounts, leading to oxidative stress. A balance between oxidant and antioxidant intracellular systems is hence vital for cell function, regulation, and adaptation to growth conditions. Therefore, cold-adapted organisms must develop an effective and intricate network of defence mechanisms against oxidative stress.

2. The polar bacteria

Temperature-dependent gene expression and *in situ* comparative analyses will significantly progress taking advantage from microbial genomes. Microorganisms have been found in a great variety of icy environments (where they stay viable for very long times), e.g. permafrost, polar oceans, snow, sea ice, glacial ice, cryoconite holes. Examples include ice-covered hypersaline and other lakes (Priscu et al., 1998) and cryptoendolithic communities colonising the pore spaces of exposed rocks in the Dry Valleys (de la Torre et al., 2003) and other Antarctic locations, methanogenic Archaea (Tung et al., 2005) and ultra-small microorganisms found in the deepest part of a 3053-m ice core in Greenland (Miteva and Brenchley, 2005).

Thanks to their short generation times and being most bacteria cultivable, they can be used in several experiments aimed to understand cold responses, since the responses of multiple generations to selective forces (e.g. environmental conditions and their changes) can be followed relatively easily and rapidly in selection experiments.

Currently, the knowledge of polar microorganisms based on ecological and genomic perspectives is in the early phase of an exponential growth. The sequences of some bacterial polar microbial genomes are already in GenBank, accompanied by publications, e.g. the euryachaeota *Methanogenium frigidum* and *Methanococcoides burtonii* (Saunders et al., 2003) from Lake Ace in the Antarctic region of the Vestfold Hills, the γ-proteobacterium *Colwellia psychrerythraea* 34H (Methé et al., 2005) and *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) (Médigue et al., 2005) and the δ-proteobacteria *Desulfotalea psychrophila* (Rabus et al., 2004). Studies on many others are in various stages of completion (Table 1, adapted from Murray and Grzymski, 2007). Recently, the genome of the *Exiguobacterium sibiricum* strain isolated from 3-million-year old permafrost was sequenced and annotated (Rodrigues et al., 2008). Adequate understanding of microbial diversity and genome-linked capabilities will enable us to assess polar-ecosystem structure and function, as well as to establish the effects of climate change.

3. Molecular adaptations in polar microorganisms

Evolution has allowed cold-adapted organisms not simply to survive, but to grow successfully under the extreme conditions of cold habitats, through a variety of structural and physiological adjustments in their genomes. These strategies include synthesis of factors, such as cold-shock proteins (Cavicchioli et al., 2000), molecular chaperones (Watanabe and Yoshida, 2004), compatible solutes (Pegg, 2007) and structural modifications leading to the maintenance of membrane fluidity (Russell, 1998; Chintalapati et al., 2004). In addition to adaptations at the cellular level, a key adaptive strategy is the modification of enzyme kinetics, allowing maintenance of sufficient reaction rates at thermal extremes. Enzyme catalysis is based on increased flexibility in some regions of cold-active-enzyme architecture and high activity with concomitant increase in thermolability (Georlette et al., 2004). However, the adaptations to protein architecture essential to cold-

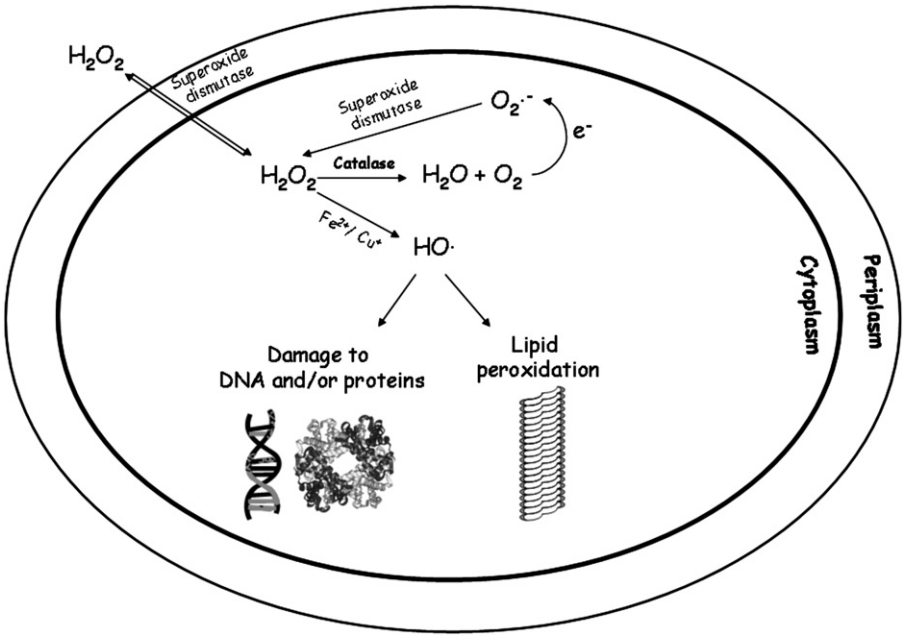

Fig. 1. Oversimplified scheme of oxidative and antioxidative systems in bacterial cells. Adapted from Zhou et al. (2010).

Table 1
Polar bacterial and archaeal genomes. The status of genome sequencing without accession number is still in progress or available by URL, adapted from Murray and Grzymski (2007).

Domain	Group	Species	Strain origin	Status of genome sequencing/ accession number or URL	Reference
Archaea	Euryarchaeota	*Methanogenium frigidum*	Ace Lake, Antarctica	Draft/http://psychro.bioinformatics.unsw.edu.au/blast/mf_blast.php	Saunders et al. (2003)
Archaea	Euryarchaeota	*Methanococcoides burtonii* DSM6242	Ace Lake, Antarctica	Completed/CP000300	Saunders et al. (2003)
Bacteria	γ-Proteobacteria	*Colwellia psychrerythraea* 34H	Arctic marine sediments	Completed/CP000083	Methé et al. (2005)
Bacteria	γ-Proteobacteria	*Shewanella frigidimarina* NCMB400	Sea ice, seawater, Antarctica	Completed/CP000447	
Bacteria	γ-Proteobacteria	*Psychrobacter arcticus* 273-4	Siberian permafrost	Completed/CP000082	
Bacteria	γ-Proteobacteria	*Psychrobacter cryohalolentis* K5	Siberian permafrost	Completed/CP000323, CP000324	
Bacteria	γ-Proteobacteria	*Oleispira antarctica* RB-8	Rod Bay, Ross Sea, Antarctica	In progress	
Bacteria	γ-Proteobacteria	*Pseudoalteromonas haloplanktis* TAC125	Coastal Antarctic seawater, Terre Adélie	Completed/CR954246, CR954247	Médigue et al. (2005)
Bacteria	δ-Proteobacteria	*Desulfotalea psychrophila* LSv54	Arctic marine sediments, Svalbard	Completed/CR522870, CR522871, CR522872	Rabus et al. (2004)
Bacteria	Firmicutes	*Exiguobacterium sibiricum* 255-15	Siberian permafrost	Completed/AADW00000000	Rodrigues et al. (2008)
Bacteria	Bacteriodetes	*Psychroflexus torquis* ATCC 700755	Sea ice algal assemblage, Prydz Bay, Antarctica	Draft/AAPR00000000	
Bacteria	Bacteriodetes	*Polaribacter filamentous* 215	Surface seawater, north of Deadhorse, Alaska	In progress	
Bacteria	Bacteriodetes	*Polaribacter irgensii* 23-P	Nearshore marine waters off Antarctic Peninsula.	Draft/AAOG00000000	
Bacteria	γ-Proteobacteria	*Psychromonas ingrahamii* 37	Sea ice, off Point Barrow in northern Alaska	Completed/CP000510	
Bacteria	Actinobacteria	*Actinobacterium* PHSC20C1 (marine)	Nearshore marine waters of Antarctic Peninsula	Draft/AAOB00000000	

active enzymes are yet not well understood. Nevertheless, the biochemical properties of cold-active enzymes make them attractive for exploitation in biochemical, bioremediation, and industrial processes (Feller and Gerday, 2003).

The comparative analysis of the genome of *M. frigidum* and *M. burtonii* was the first study encompassing psychrophile to hyperthermophile lifestyles (Saunders et al., 2003). Preliminary studies on proteins have revealed the presence in their genome of cold-shock-domain folds and the typical properties of cold-adapted proteins, namely an increased number of glutamyl and threonyl residues.

In *Ph*TAC125, a significant bias towards asparagyl residues was found (Médigue et al., 2005).

E. sibiricum is constitutively adapted to cold with differential gene expression between 4 °C and 28 °C (Rodrigues et al., 2008).

To preserve their function, proteins must reach a balance of structural rigidity and flexibility in their environments. Generally, enzymes isolated from psychrophiles living in perennially cold habitats are endowed with high catalytic efficiency at low temperature and low stability due to enhanced flexibility (Feller and Gerday, 2003).

Among cold-adapted bacteria, the genus *Colwellia*, within γ-proteobacteria, provides an unusual case, i.e. all characterised members are strictly psychrophilic (requiring temperatures of -20 °C to grow on solid media) and live in stably cold environments, including deep sea and Arctic and Antarctic sea ice (Deming and Junge, 2005). Many species produce extracellular polymeric substances relevant to biofilm formation and cryoprotection (Krembs et al., 2002) and enzymes capable of degrading high-molecular-mass organic compounds. Cold-adapted bacteria have developed responses to strong oxidative stress. Indeed marine organisms have been exposed to permanent excess of oxygen, due to its high solubility at cold temperatures, leading to oxygen reserves larger than those available in warmer waters. The apparent benefits of easier oxygen supply are contrasted by the constraints on kinetic effects at low temperature, which impair the functional capacities of molecules, and by increased production of ROS. Therefore, augmented capacities in antioxidative defence are likely to be important components of evolutionary adaptations in a cold and oxygen-rich environment. The genome sequence of *C. psychrerythraea*, an obligately psychrophilic Arctic bacterium, has provided an important opportunity to better understand its potential functions in the marine environment and to gain insight into adaptation (Methé et al., 2005). Environments in which *Colwellia* have been found include ice formations currently under study as models of past ice ages on Earth (Deming, 2002).

C. psychrerythraea (Methé et al., 2005) seems to have faced high oxygen concentration by developing enhanced antioxidant capacity owing to the presence of several genes that encode catalases and superoxide dismutases. In contrast, the genome sequence of *Ph*TAC125 reveals that the bacterium copes with increased oxygen solubility by enhancing production of oxygen-scavenging enzymes and deleting entire metabolic pathways, such as those which generate ROS as side products. The deletion of the ubiquitous molybdopterin-dependent metabolism in the *Ph*TAC125 genome (Médigue et al., 2005) and the number of proteins involved in scavenging chemical groups (see below) can be seen in this perspective. Oxygen-consuming lipid desaturases achieve both protection against oxygen and synthesis of lipids, making the membrane fluid. These characteristics make this bacterium not only a model for the study of adaptation to cold marine conditions but also an attractive tool for biotechnology production of proteins (Médigue et al., 2005). The cold environment of *Ph*TAC125 raises the problem of how this microorganism copes with ROS. High levels of ROS are potentially toxic for the cell, being involved in a large number of pathological mechanisms (Finkel, 2003). ROS may act as signalling molecules during cell differentiation, cell-cycle progression and in response to extracellular stimuli (Sauer et al., 2001). Indeed, low temperatures should favour oxygen solubility and increase the stability of oxygen-derived toxic compounds.

4. The globins of *Ph*TAC125 and their potential role in oxidative stress

*Ph*TAC125 provides an opportunity for studying molecular strategies adopted by cold-adapted bacteria to cope with low temperatures and high oxygen concentration.

The presence of several enzymes involved in scavenging chemical groups affected by ROS (such as peroxiredoxins and peroxidases) and one catalase-encoding gene (katB) with a possible homologue (*PSHAa*1737) (Médigue et al., 2005) makes *Ph*TAC125 a well-adapted microorganism against ROS under cold conditions.

A further sign, which may be related to the peculiar features of cold habitat, may be the synthesis of bacterial hemoglobins and flavohemoglobin, surprisingly versatile proteins serving several biological functions. Interestingly, the *C. psychrerythraea* genome does not possess genes encoding 2-on-2 (2/2) hemoglobins, whereas the *E. sibiricum* 255-15 (Rodrigues et al., 2008) and *Psychromonas ingrahamii* 37 (Riley et al., 2008) genomes contain genes encoding 2/2 hemoglobins. These molecules are bound to fulfil an important physiological role, including protection of the cell from nitrosative and oxidative stress.

Multiple genes encoding 2/2 hemoglobins (annotated as *PSHAa0030, PSHAa0458, PSHAa2217*) and one for flavohemoglobin (*PSHAa2880*) have been discovered in the genome of *Ph*TAC125, suggesting that specific and distinct functions may be associated to these two classes of proteins (Giordano et al., 2007).

The 2/2 hemoglobins are widely distributed in bacteria, unicellular eukaryotes and plants. They are small oxygen-binding hemoproteins, generally shorter than vertebrate hemoglobins (exhibiting the classical 3/3-fold myoglobin-like) because they lack 20–40 amino-acid residues (Pesce et al., 2000), (Fig. 2). The globin fold is based on a 2/2 α-helical sandwich (Pesce et al., 2000). The original phylogenetic analysis of these hemoglobins classifies them into three groups, denoted I, II, and III (Vuletich and Lecomte, 2006). A number of three-dimensional structures of proteins belonging to the three groups have been recently elucidated at atomic resolution by X-ray crystallography and NMR (see Nardini et al., 2007, and references therein); 2/2 hemoglobins belonging to the three groups may coexist in some bacteria, suggesting distinct functions. Such postulated functions, consistent with observed biophysical properties, include long-term ligand or substrate storage, NO detoxification, oxygen/NO sensing, redox reactions, and oxygen delivery under hypoxic conditions (Wittenberg et al., 2002). The high affinity for oxygen suggests that 2/2 hemoglobins function as oxygen scavengers rather than oxygen transporters (Ouellet et al., 2003).

Phylogenetic analyses showed that the 2/2 hemoglobins encoded by the *PSHAa0030* and *PSHAa2217* genes belong to group II, and that encoded by *PSHAa0458* to group I. The *PSHAa*0030 gene encoding the 2/2 hemoglobin hereafter called *Ph*HbO, was cloned, and over-expressed in *Escherichia coli*. The recombinant protein was purified to be structurally and functionally investigated (Giordano et al., 2007). Recombinant *Ph*HbO is a mixture of the ferric and ferrous forms, also showing predominance of hexacoordination in both forms, strongly dependent on pH and temperature (Giordano et al., 2007; Verde et al., 2009; Howes et al., unpublished). In the absence of exogenous ligands, an internal amino-acid residue is able to coordinate the heme iron, either in ferrous or ferric form (Fig. 3). Hexacoordinated hemoglobins are generally observed in bacteria, unicellular eukaryotes, plants, invertebrates and in some tissues of higher vertebrates (Vinogradov and Moens, 2008), but only a few cases have been examined and reported in the literature for bacterial 2/2 hemoglobins, such as the ferrous form of *Mycobacterium leprae* 2/2 hemoglobin (Visca et al., 2002), the ferric form of 2/2 hemoglobins from the cyanobacteria *Synechococcus* sp. PCC 7002 (Scott et al., 2002) and *Synechocystis* sp. PCC 6803 (Falzone et al., 2002), and the ferrous form of 2/2 hemoglobin of *Herbaspirillum seropedicae* (Razzera et al., 2008). Their physiological role is not well understood.

Hexacoordination has also been found in the ferric state (β chains) of several tetrameric hemoglobins (Riccio et al., 2002; Vitagliano et al., 2004; Vergara et al., 2007, 2008; Vitagliano et al., 2008) and in ferric and ferrous states of neuroglobins (Pesce et al., 2004) and cytoglobins (de Sanctis et al., 2004). The occurrence of ferrous (hemochrome) and ferric (hemichrome) oxidation states in members of the hemoglobin superfamily is not uniform suggesting that the functional roles of these oxidation states are multiple, possibly being a tool for modulating ligand-binding or redox properties. According to the evidence of higher peroxidase activity in Antarctic fish hemoglobins, the exchange between hemichrome and pentacoordinated forms may play a distinctive physiological role in Antarctic teleosts (Vergara et al., 2008; Vitagliano et al., 2008).

Hexacoordination may suggest a common physiological mechanism for protecting cells against oxidative chemistry in response to high oxygen concentration. Several roles have been hypothesised for the hexacoordinated neuroglobin and cytoglobin, e.g. oxygen scavenger under hypoxic conditions (Burmester et al., 2000, 2002), terminal oxidases (Sowa et al., 1999), oxygen-sensor proteins (Kriegl et al., 2002), proteins involved in NO metabolism (Smagghe et al., 2008).

A further confirmation of involvement of *Ph*HbO in the protection against the stress induced by high oxygen concentration comes from recent results on genomic mutant strain, in which the *Ph*HbO encoding gene (*PSHAa0030*) was inactivated by insertional mutagenesis (Parrilli et al., 2010). Disk diffusion assays display a hydrogen peroxidase sensitivity of *Ph*TAC125(-30) mutant in comparison with the wild-type. The above results suggest that *Ph*HbO may be endowed of peroxidase activity.

5. The globins of *Ph*TAC125 and their potential role in nitrosative stress

The *Ph*TAC125 genome contains genes putatively involved in the metabolism of NO, namely NO reductase and nitrite reductase, or in

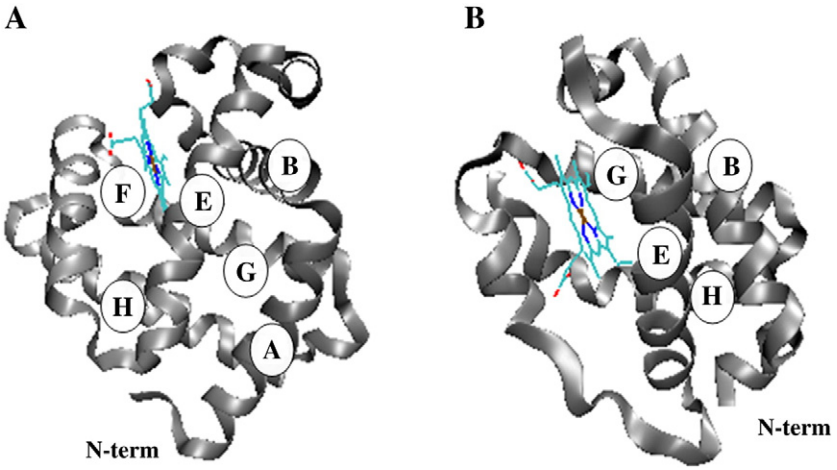

Fig. 2. Comparison between the three-dimensional structure of a 3/3 hemoglobin and a 2/2 hemoglobin. (A) Sperm whale myoglobin (PDB code: 1VXF) is the typical 3/3 hemoglobin where the heme group is surrounded by 3 helices on the proximal site (F, G, H) and 3 helices on the distal site (A, B, E). (B) Example of a 2/2 hemoglobin (PDB code: 1UVY) where the heme pocket is sandwiched between helices B and E on the distal site and helices G and H on the proximal site.

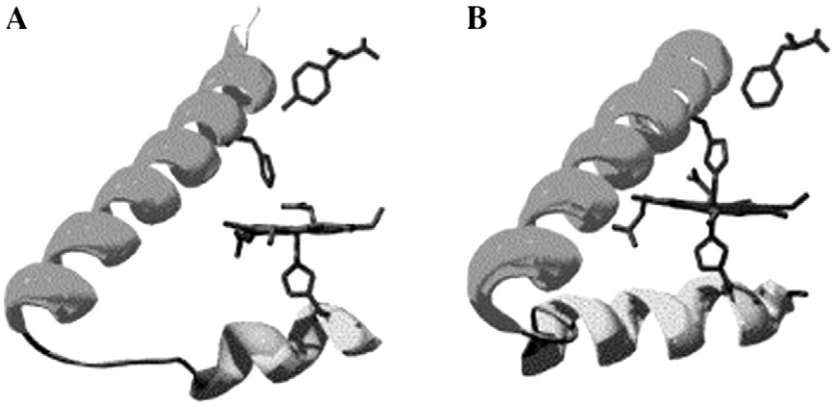

Fig. 3. (A) Heme coordination in a pentacoordinated hemoglobin and (B) in a hexacoordinated hemoglobin where the distal histidyl residue acts as the sixth ligand.

NO scavenging, as mentioned earlier, i.e. encoding flavohemoglobin and 2/2 hemoglobins.

The physiological role fulfilled by *Ph*HbO was also investigated by a genomic approach, taking advantage of the availability of genetic tools evolved by this Antarctic bacterium (Parrilli et al., 2008), combined with the possibility to study the purified protein (Giordano et al., 2007). The mutant strain, in which the *Ph*HbO encoding gene was inactivated (Parrilli et al., 2010), was grown under controlled conditions and its growth behaviour was compared to that of wild-type cells, when oxygen pressure and growth temperature were changed, observing lower duplication speed and poor bacterial growth when *Ph*TAC125 was cultivated in microaerobiosis, especially at higher temperatures (e. g. 15 °C), due to lower oxygen solubility than at 4 °C. The suggested involvement of *Ph*HbO in cellular protection against NO-induced stress was confirmed by the higher sensitivity of the mutant than wild-type cells, to spermidine NONOate, a NO releaser (Parrilli et al., 2010). Bacterial cells have developed mechanisms for NO detoxification, against cytotoxic effects of NO (Poole, 2005). Homeostasis of NO is achieved through balance between its production and consumption (Fig. 4). At high concentrations, NO is not a messenger: it is toxic. Its ability to react with oxygen and ROS leads to production of reactive nitrogen species (RNS) (Poole and Hughes, 2000). In a rich oxygen environment and under cold stress, NO detoxification may require more than one defence mechanism. In the *Ph*TAC125 genome, besides the gene encoding the hexacoordinated *Ph*HbO, there is also a gene encoding a flavohemoglobin, a protein having the heme-containing oxygen-binding domain, and a FAD-containing reductase domain. It is widely recognised as a NO-detoxifying protein (Poole, 2005). Several adaptations have been proposed in protection against NO in bacteria (Nunoshiba et al., 1995), and flavohemoglobin has a role in some of these (Mowat et al., 2009).

A transcriptional analysis of the *Ph*HbO and flavohemoglobin-encoding genes was carried out on *Ph*TAC125 wild type and *Ph*TAC125(-30) mutant grown in all tested conditions. The transcription of the flavohemoglobin encoding gene was observed only in *Ph*TAC125(-30) mutant when grown at 4 °C in microaerobiosis. Since the transcription of flavohemoglobin-encoding genes is usually directly or indirectly induced by NO (Hausladen et al., 1998; Spiro, 2007) the observed flavohemoglobin-gene expression is suggestive of the occurrence of a NO induced stress related to the *Ph*HbO absence (Parrilli et al., 2010).

No data are available on the presence of NO in *Ph*TAC125; however, cellular adaptation aimed at protection against damages caused by NO and NO-derived species has been demonstrated in the phylogenetically related γ-proteobacterium *Escherichia coli* (Nunoshiba et al., 1995).

6. Concluding remarks

Psychrophilic bacteria have successfully coped with the two main physical challenges they had to confront, namely firstly the low thermal energy, which slows down the metabolic flux, and secondly, the viscosity of the medium, significantly increased by low temperatures, strongly contributing to slow down the biochemical reaction rates.

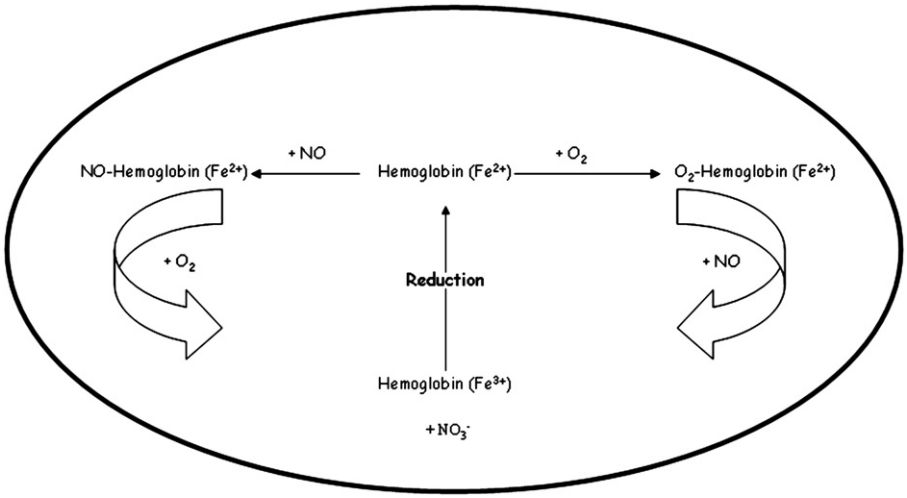

Fig. 4. Oversimplified reaction of scavenging of NO by hemoglobin. Hemoglobin in ferrous form may react with either NO or oxygen. The liganded hemoglobin will react further with the other ligands to produce ferric hemoglobin and nitrate. Flavohemoglobins display a reductase domain to achieve the re-reduction of the heme iron following NO destruction.

Genome analyses indicate that cold adaptation is the result of synergistic changes in the overall genome configuration reflected in the up-regulation and expansion of specific genes rather than the presence of specific genes responsible for psychrophilic genotype and lifestyle. Cold-adapted bacteria require preservation of the flexibility, topology, and interactions of macromolecules such as DNA, RNA and proteins, which are the main targets of these adaptations as they regulate the equilibrium between substrates and products, macromolecular assemblies and appropriate folding. In cold-adapted proteins the adaptive modifications appear to rely on higher flexibility of key parts of the molecule and/or decreased stability, partially compensating the effects of low temperature (Marx et al., 2007). In addition, Antarctic marine bacteria potentially experience the pressure of oxidative stress and the metabolic costs associated with antioxidant defences. Therefore, augmented capacities in antioxidant defence are likely to be important components in evolutionary adaptations in a cold and oxygen-rich environment. Although the number of deposited 2/2 hemoglobin sequences has grown very fast in the last decade, we still possess limited functional information for these proteins. However, more recent data strongly suggest that these proteins are able to perform physiological tasks other than the reversible binding of oxygen typical of the 3/3 hemoglobins. These additional functions may include oxygen scavenging, NO processing, protection against oxidative damage and sulfide binding (Nicoletti et al., 2010).

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References

Bowler, C., Karl, D.M., Colwell, R., 2009. Microbial oceanography in a sea of opportunity. Nature 459, 180–184.
Burmester, T., Weich, B., Reinhardt, S., Hankeln, T., 2000. A vertebrate globin expressed in the brain. Nature 407, 520–523.
Burmester, T., Ebner, B., Weich, B., Hankeln, T., 2002. Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. Mol. Biol. Evol. 19, 416–421.
Cavicchioli, R., Thomas, T., Curmi, P.M., 2000. Cold stress response in Archaea. Extremophiles 4, 321–331.
Chen, Z., Cheng, C.H., Zhang, J., Cao, L., Chen, L., Zhou, L., Jin, Y., Ye, H., Deng, C., Dai, Z., Xu, Q., Hu, P., Sun, S., Shen, Y., Chen, L., 2008. Transcriptomic and genomic evolution under constant cold in Antarctic notothenioid fish. Proc. Natl. Acad. Sci. U.S.A. 105, 12944–12949.
Chintalapati, S., Kiran, M.D., Shivaji, S., 2004. Role of membrane lipid fatty acids in cold adaptation. Cell. Mol. Biol. (Noisy-le-grand) 50, 631–642.
de la Torre, J.R., Goebel, B.M., Friedmann, E.I., Pace, N.R., 2003. Microbial diversity of cryptoendolithic communities from the McMurdo Dry Valleys, Antarctica. Appl. Environ. Microbiol. 69, 3858–3867.
de Sanctis, D., Dewilde, S., Pesce, A., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T., Bolognesi, M., 2004. Crystal structure of cytoglobin: the fourth globin type discovered in man displays heme hexa-coordination. J. Mol. Biol. 336, 917–927.
Deming, J.W., 2002. Psychrophiles and polar regions. Curr. Opin. Microbiol. 5, 301–309.
Deming, J.W., Junge, K., 2005. Colwellia. In: Staley, G.T., Benner, D.J., Krieg, N.R., Garrity, G.M. (Eds.), The Proteobacteria, Part B, Bergey's Manual of Systematic Bacteriology. Springer, New York, pp. 447–454. 2nd., Vol. 2.
Falzone, C.J., Christie Vu, B., Scott, N.L., Lecomte, J.T., 2002. The solution structure of the recombinant hemoglobin from the cyanobacterium *Synechocystis* sp. PCC 6803 in its hemichrome state. J. Mol. Biol. 324, 1015–1029.
Feller, G., Gerday, C., 2003. Psychrophilic enzymes: hot topics in cold adaptation. Nat. Rev. Microbiol. 1, 200–208.
Finkel, T., 2003. Oxidant signals and oxidative stress. Curr. Opin. Cell. Biol. 15, 247–254.
Georlette, D., Blaise, V., Collins, T., D'Amico, S., Gratia, E., Hoyoux, A., Marx, J.C., Sonan, G., Feller, G., Gerday, C., 2004. Some like it cold: biocatalysis at low temperatures. FEMS Microbiol. Rev. 28, 25–42.
Giordano, D., Parrilli, E., Dettaï, A., Russo, R., Barbiero, G., Marino, G., Lecointre, G., di Prisco, G., Tutino, M.L., Verde, C., 2007. The truncated hemoglobins in the Antarctic psychrophilic bacterium *Pseudoalteromonas haloplanktis* TAC125. Gene 398, 69–77.
Hausladen, A., Gow, A.J., Stamler, J.S., 1998. Nitrosative stress: metabolic pathway involving the flavohaemoglobin. Proc. Natl. Acad. Sci. U.S.A. 95, 14100–14105.
Krembs, C., Eicken, H., Junge, K., Deming, J.W., 2002. High concentrations of exopolymeric substances in Arctic winter sea ice: implications for the polar ocean carbon cycle and cryoprotection of diatoms. Deep Sea Res. A 49, 2163–2181.
Kriegl, J.M., Bhattacharyya, A.J., Nienhaus, K., Deng, P., Minkow, O., Nienhaus, G.U., 2002. Ligand binding and protein dynamics in neuroglobin. Proc. Natl. Acad. Sci. USA 99, 7992–7997.
Marx, J.-C., Collins, T., D'Amico, S., Feller, G., Gerday, C., 2007. Cold-adapted enzymes from marine Antarctic microorganisms. Mar. Biotech. 9, 293–304.
Médigue, C., Krin, E., Pascal, G., Barbe, V., Bernsel, A., Bertin, P.N., Cheung, F., Cruveiller, S., D'Amico, S., Duilio, A., Fang, G., Feller, G., Ho, C., Mangenot, S., Marino, G., Nilsson, J., Parrilli, E., Rocha, E.P., Rouy, Z., Sekowska, A., Tutino, M.L., Vallenet, D., von Heijne, G., Danchin, A., 2005. Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125. Genome Res. 15, 1325–1335.
Methé, B.A., Nelson, K.E., Deming, J.W., Momen, B., Melamud, E., Zhang, X., Moult, J., Madupu, R., Nelson, W.C., Dodson, R.J., Brinkac, L.M., Daugherty, S.C., Durkin, A.S., DeBoy, R.T., Kolonay, J.F., Sullivan, S.A., Zhou, L., Davidsen, T.M., Wu, M., Huston, A.L., Lewis, M., Weaver, B., Weidman, J.F., Khouri, H., Utterback, T.R., Feldblyum, T.V., Fraser, C.M., 2005. The psychrophilic lifestyle as revealed by the genome sequence of *Colwellia psychrerythraea* 34H through genomic and proteomic analyses. Proc. Natl. Acad. Sci. USA 102, 10913–10918.
Miteva, V.I., Brenchley, J.E., 2005. Detection and isolation of ultrasmall microorganisms from a 120,000-year-old Greenland glacier iced core. Appl. Environ. Microbiol. 71, 7806–7818.
Moline, M.A., Karnovsky, N.J., Brown, Z., Divoky, G.J., Frazer, T.K., Jacoby, C.A., Torres, J.J., Fraser, W.R., 2008. High latitude changes in ice dynamics and their impact on polar marine ecosystems. Ann. NY Acad. Sci. 1134, 267–319.
Mowat, C.G., Gazur, B., Campbell, L.P., Chapman, S.K., 2009. Flavin-containing heme enzymes. Arch. Biochem. Biophys. 493, 37–52.
Murray, A.E., Grzymski, J.J., 2007. Diversity and genomics of Antarctic marine microorganisms. Phil. Trans. R. Soc. B. 362, 2259–2271.
Nardini, M., Pesce, A., Milani, M., Bolognesi, M., 2007. Protein fold and structure in the truncated (2/2) globin family. Gene 398, 2–11.
Nicoletti, F.P., Comandini, A., Bonamore, A., Boechi, L., Boubeta, F.M., Feis, A., Smulevich, G., Boffi, A., 2010. Sulfide binding properties of truncated hemoglobins. Biochemistry 49, 2269–2278.
Nunoshiba, T., Derojas-Walker, T., Tannenbaum, S.R., Demple, B., 1995. Roles of nitric oxide in inducible resistance of *Escherichia coli* to activated murine macrophages. Infectiv. Immun. 63, 794–798.
Ouellet, H., Juszczak, L., Dantsker, D., Samuni, U., Ouellet, Y.H., Savard, P.Y., Wittenberg, J.B., Wittenberg, B.A., Friedman, J.M., Guertin, M., 2003. Reactions of *Mycobacterium tuberculosis* truncated haemoglobin O with ligands reveal a novel ligand-inclusive hydrogen bond network. Biochemistry 42, 5764–5774.
Parrilli, E., Duilio, A., Tutino, M.L., 2008. Heterologous protein expression in psychrophilic hosts. In: Margesin, R., Schinner, F., Marx, J.C., Gerday, C. (Eds.), Psychrophiles: from Biodiversity to Biotechnology. Springer-Verlag, Berlin Heidelberg, pp. 365–379.
Parrilli, E., Giuliani, M., Giordano, D., Russo, R., Marino, G., Verde, C., Tutino, M.L., 2010. The role of a 2-on 2 haemoglobin in oxidative and nitrosative stress resistance of Antarctic *Pseudoalteromonas haloplanktis* TAC125. Biochimie 92, 1003–1009.
Pegg, D.E., 2007. Principles of cryopreservation. Meth. Mol. Biol. 368, 39–57.
Pesce, A., Couture, M., Dewilde, S., Guertin, M., Yamauchi, K., Ascenzi, P., Moens, L., Bolognesi, M., 2000. A novel two-over-two a-helical sandwich fold is characteristic of the truncated hemoglobin family. EMBO J. 19, 2424–2434.
Pesce, A., Dewilde, S., Nardini, M., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T., Bolognesi, M., 2004. The human brain hexacoordinated neuroglobin three-dimensional structure. Micron 35, 63–65.
Poole, R.K., 2005. Nitric oxide and nitrosative stress tolerance in bacteria. Biochem. Soc. Trans. 33, 176–180.
Poole, R.K., Hughes, M.N., 2000. New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. Mol. Microbiol. 36, 775–783.
Priscu, J.C., Fritsen, C.H., Adams, E.E., Giovannoni, S.J., Paerl, H.W., McKay, C.P., Doran, P.T., Lanoil, B.D., Pinckney, J.L., 1998. Perennial Antarctic lake ice: an oasis for life in a polar desert. Science 280, 2095–2098.
Rabus, R., Ruepp, A., Frickey, T., Rattei, T., Fartmann, B., Stark, M., Bauer, M., Zibat, A., Lombardot, T., Becker, I., Amann, J., Gellner, K., Teeling, H., Leuschner, W.D., Glöckner, F.O., Lupas, A.N., Amann, R., Klenk, H.P., 2004. The genome of *Desulfotalea psychrophila*, a sulfate-reducing bacterium from permanently cold Arctic sediments. Environ. Microbiol. 6, 887–902.
Razzera, G., Vernal, J., Baruh, D., Serpa, V.I., Tavares, C., Lara, F., Souza, E.M., Pedrosa, F.O., Almeida, F.C., Terenzi, H., Valente, A.P., 2008. Spectroscopic characterization of a truncated haemoglobin from the nitrogen-fixing bacterium *Herbaspirillum seropedicae*. J. Biol. Inorg. Chem. 13, 1085–1096.
Riccio, A., Vitagliano, L., di Prisco, G., Zagari, A., Mazzarella, L., 2002. The crystal structure of a tetrameric hemoglobin in a partial hemichrome state. Proc. Natl. Acad. Sci. U.S.A. 99, 9801–9806.
Riley, M., Staley, J.T., Danchin, A., Wang, T.Z., Brettin, T.S., Hauser, L.J., 2008. Genomics of an extreme psychrophile, *Psychromonas ingrahamii*. BMC Genom. 9, 210.

Rodrigues, D., Ivanova, N., He, Z., Huebner, M., Zhou, J., Tiedje, M., 2008. Architecture of thermal adaptation in an *Exiguobacterium sibiricum* strain isolated from 3 million year old permafrost: a genome and transcriptome approach. BMC Genom. 9, 547.
Russell, N.J., 1998. Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications. Adv. Biochem. Eng. Biotechnol. 61, 1–21.
Sauer, H., Wartenberg, M., Hescheler, J., 2001. Reactive oxygen species as intracellular messengers during cell growth and differentiation. Cell. Physiol. Biochem. 11, 173–186.
Saunders, N.F., Thomas, T., Curmi, P.M., Mattick, J.S., Kuczek, E., Slade, R., Davis, J., Franzmann, P.D., Boone, D., Rusterholtz, K., Feldman, R., Gates, C., Bench, S., Sowers, K., Kadner, K., Aerts, A., Dehal, P., Detter, C., Glavina, T., Lucas, S., Richardson, P., Larimer, F., Hauser, L., Land, M., Cavicchioli, R., 2003. Mechanisms of thermal adaptation revealed from the genomes of the Antarctic Archaea *Methanogenium frigidum* and *Methanococcoides burtonii*. Genome Res. 13, 1580–1588.
Scott, N.L., Falzone, C.J., Vuletich, D.A., Zhao, J., Bryant, D.A., Lecomte, J.T., 2002. Truncated hemoglobin from the cyanobacterium *Synechococcus* sp. PCC 7002: evidence for hexacoordination and covalent adduct formation in the ferric recombinant protein. Biochemistry 41, 6902–6910.
Smagghe, B.J., Trent III, J.T., Hargrove, M.S., 2008. NO dioxygenase activity in hemoglobins is ubiquitos in vitro, but limited by reduction in vivo. PloS ONE 3, e2039.
Sowa, A.W., Guy, P.A., Sowa, S., Hill, R.D., 1999. Nonsymbiotic haemoglobins in plants. Acta Biochim. Pol. 46, 431–445.
Spiro, S., 2007. Regulator of bacterial responses to nitric oxide. FEMS Microbiol. Rev. 31, 193–211.
Tung, H.C., Bramall, N.E., Price, P.B., 2005. Microbial origin of excess methane in glacial ice and implications for life on Mars. Proc. Natl. Acad. Sci. USA 102, 18292–18296.
Verde, C., Giordano, D., Russo, R., Riccio, A., Vergara, A., Mazzarella, L., di Prisco, G., 2009. Hemoproteins in the cold. Mar. Gen. 2, 67–73.
Vergara, A., Franzese, M., Merlino, A., Vitagliano, L., di Prisco, G., Verde, C., Lee, H.C., Peisach, J., Mazzarella, L., 2007. Structural characterization of ferric hemoglobins from three Antarctic fish species of the suborder Notothenioidei. Biophys. J. 93, 2822–2829.
Vergara, A., Vitagliano, L., Verde, C., di Prisco, G., Mazzarella, L., 2008. Spectroscopic and crystallographic characterization of bis-histidyl adducts in tetrameric hemoglobins. Meth. Enzymol. 436, 425–444.
Vinogradov, S., Moens, L., 2008. Diversity of globin function: enzymatic, transport, storage, and sensing. J. Biol. Chem. 283, 8773–8777.
Visca, P., Fabozzi, G., Petrucca, A., Ciaccio, C., Coletta, M., De Sanctis, G., Bolognesi, M., Milani, M., Ascenzi, P., 2002. The truncated hemoglobin from *Mycobacterium leprae*. Biochem. Biophys. Res. Commun. 294, 1064–1070.
Vitagliano, L., Bonomi, G., Riccio, A., di Prisco, G., Smulevich, G., Mazzarella, L., 2004. The oxidation process of Antarctic fish hemoglobins. Eur. J. Biochem. 271, 1651–1659.
Vitagliano, L., Vergara, A., Bonomi, G., Merlino, A., Smulevich, G., Howes, B., di Prisco, G., Verde, C., Mazzarella, L., 2008. Spectroscopic and crystallographic analysis of a tetrameric hemoglobin oxidation pathway reveals features of an intermediate R/T state. J. Am. Chem. Soc. 130, 10527–10535.
Vuletich, D.A., Lecomte, J.T., 2006. A phylogenetic and structural analysis of truncated hemoglobins. J. Mol. Evol. 62, 196–210.
Watanabe, Y.H., Yoshida, M., 2004. Trigonal DnaK–DnaJ complex versus free DnaK and DnaJ; heat stress converts the former to the latter and only the latter can do disaggregation in cooperation with ClpB. J. Biol. Chem. 279, 15723–15727.
Wittenberg, J.B., Bolognesi, M., Wittenberg, B.A., Guertin, M., 2002. Truncated hemoglobins: a new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. J. Biol. Chem. 277, 871–874.
Zhou, A., He, Z., Redding-Johanson, A.M., Mukhopadhyay, A., Hemme, C.L., Joachimiak, M.P., Luo, F., Deng, Y., Bender, K.S., He, Q., Keasling, J.D., Stahl, D.A., Fields, M.W., Hazen, T.C., Arkin, A.P., Wall, J.D., Zhou, J., 2010. Hydrogen peroxide-induced oxidative stress responses in *Desulfovibrio vulgaris* Hildenborough. Envir. Microbiol. doi.org/10.1111/j.1462-2920.2010.02234.x.

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Review

Hemoproteins in the cold

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ABSTRACT

This review highlights some aspects of the biochemistry of cold-adapted hemoproteins in fish and bacteria, without claiming to be exhaustive. Heme hexacoordination where the sixth ligand is provided by an internal amino-acid residue, in cold-adapted hemoproteins will be discussed.

Contents

1. Introduction

Few proteins have been studied in such a wide array of organisms as hemoglobin (Hb), and recent discoveries on its structure–function relationship keep stimulating interest. Hbs are very ancient proteins; they probably evolved from enzymes that used to protect the tissues against toxic oxygen levels. Hbs have been found in bacteria, protists, fungi, plants and animals; they serve a wide array of physiological roles, from oxygen transport in vertebrates to catalysis of redox reactions (Gardner et al., 1998; Minning et al., 1999). These different functions suggest the acquisition of new roles, by changes not only in the coding regions, but also in the regulatory elements in the pre-existing structural gene (Hardison, 1998).

Hbs share a common structure comprising 5–8 helices. Thanks to genome sequencing, the evolutionary tree of globins went back to 1800 million years at the time when the oxygen began to accumulate in the atmosphere (Wajcman and Kiger, 2002). It is generally accepted that during the first 2000 million years of existence of the Earth, the oxygen levels in the atmosphere were very low until the advent of the "Great Oxidation" (Holland, 2006). The atmospheric oxygen content reached the present levels about 540 million years ago (mya) (Holland, 2006). At those times, the Hb-like ancestor was likely to have adapted to locally scavenge excessive oxygen concentration and/or, similar to bacterial flavoHbs to be involved in detoxification of nitrogen monoxide (Poole, 2005). The evolution of simple oxygen-binding proteins into multi-subunit proteins, in combination with the

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development of the circulatory system, made the transport of oxygen from the blood to metabolising cells possible on a significant scale (Wajcman and Kiger, 2002).

Thus, the familiar vertebrate Hb, a tetramer of two identical α and β globin chains, developed relatively recent adaptation to widely different environmental conditions (Vinogradov and Moens, 2008). The amino-acid sequences of the α and β globins are about 50% identical, suggesting a common ancestor (Hardison, 1998). The specialised function in higher vertebrates imposes severe structural constraints on the Hb molecule. Hence, it is not surprising that only a small fraction of the residues of the polypeptide chains are allowed to be replaced during evolution. According to the species-adaptation theory of Perutz (1983), the replacement of few key residues may produce functional modulation. The first protein crystal structures of myoglobin (Mb), present in cytoplasm of skeletal and cardiac myocytes, and Hb provided the basis to understand the relationship between changes in amino-acid sequence and protein overall structure (Kendrew et al., 1958; Perutz et al., 1965). During the following four decades, studies of protein structure and function have led to a detailed understanding of these hemoproteins.

The quaternary structure, assembling the four globin subunits, also provided classical source of theories on allosteric conformational transitions (Monod et al., 1965; Perutz et al., 1987). The main concept of the two-state allosteric model of Monod, Wyman and Changeux (MWC) was that the Hb molecule can only exist in two quaternary states, corresponding to a low-affinity structure T (Tense) and a high-affinity structure R (Relaxed) (Monod et al., 1965). According to the MWC model, cooperative oxygen binding arises from a shift in the population from the T to R structure as binding increases. This model further postulates that the heterotropic effects, such as the Bohr effect, are due to shifts of the allosteric equilibrium.

In addition to tetrameric Hbs and monomeric Mbs, four vertebrate hemoproteins have been recently discovered. These are cytoglobin (Cygb) which is widely expressed in vertebrate tissues (Trent and Hargrove, 2002; Burmester et al., 2002), globin E (GbE) (Kugelstadt et al., 2004) in the chicken eye (absent in mammals), globin X (GbX) recently found in fish and amphibians (Roesner et al., 2005) and neuroglobin (Ngb) (Burmester et al., 2000). The latter has received the most attention for its hypothetical role in protecting neurons from several injuries (Greenberg et al., 2008).

Phylogenetic analyses of vertebrate globins suggest a common ancestor, but confirm an ancient evolutionary relationship between GbX and Ngb, suggesting the existence of two distinct globin types in the last common ancestor of Protostomia and Deuterostomia (700 mya) (Roesner et al., 2005) as shown in Fig. 1. In fact, GbX sequences are distinct from vertebrate Hb, Mb, Ngb, and Cygb, but display the highest identity scores with Ngb (26% to 35%). For the first time in vertebrate globins, analysis of the gene structure showed an intron in helix E of Ngb and GbX, supporting the assignment of Ngb and GbX to a gene family different from that including Mb, Hb and Cygb. Only two introns, positioned at B12.2 and G7.0, are present in most vertebrate genes and are phylogenetically ancient ((Wajcman and Kiger, 2002; Roesner et al., 2005).

The variety of recently discovered bacterial Hbs has dramatically changed our view of the globin family. Bacterial Hbs highlight that oxygen transport in vertebrate Hbs is a relatively recent evolutionary acquisition and that the early Hb functions have been enzymatic and oxygen sensing (Vinogradov and Moens, 2008). The bacterial superfamily comprises three families distributed in two structural classes (Fig. 2). Within each family a given globin may occur in a chimeric or in a single-domain structure (Vinogradov and Moens, 2008). The first class, including the two families of flavoHbs and sensor Hbs, respectively involved in nitrosative stress and in adaptive responses to fluctuations of gaseous physiological messengers, displays the "3-on-3" classical Mb-like folding (3/3 Hbs). Historically, the first members of the two families were found to be chimeric. Single-domain flavoHbs are present in eukaryotic globins unlike single-domain sensor globins. The second class includes the third family of "2-on-2" Hbs (2/2 Hbs), and is widely distributed in bacteria, microbial eukaryotes and plants. Currently, there are still some uncertainties about the evolutionary relationship between the three families. The 2/2 Hbs and the sensor globins seem to have kept their original enzymatic functions in prokaryotes, plants and some unicellular eukaryotes. Therefore, the flavoHb family has been the only one able to adapt to different functions more extensively than the other two families (Vinogradov and Moens, 2008).

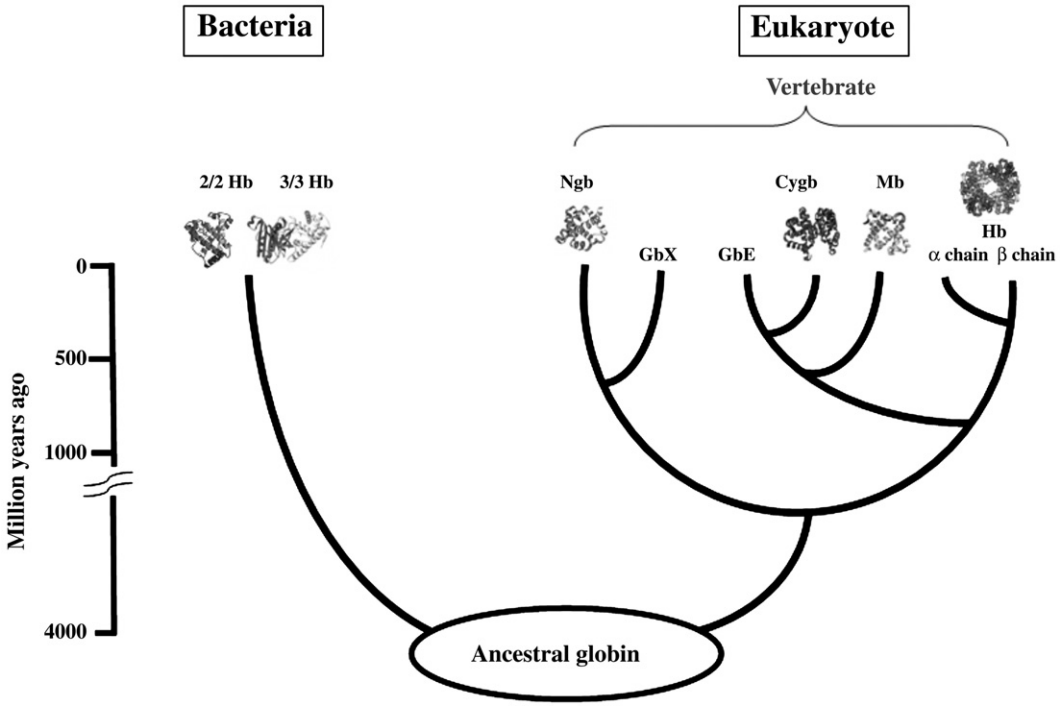

Fig. 1. A simplified phylogenetic tree of vertebrate globins. After Brunori and Vallone, 2007; Vinogradov et al., 2005.

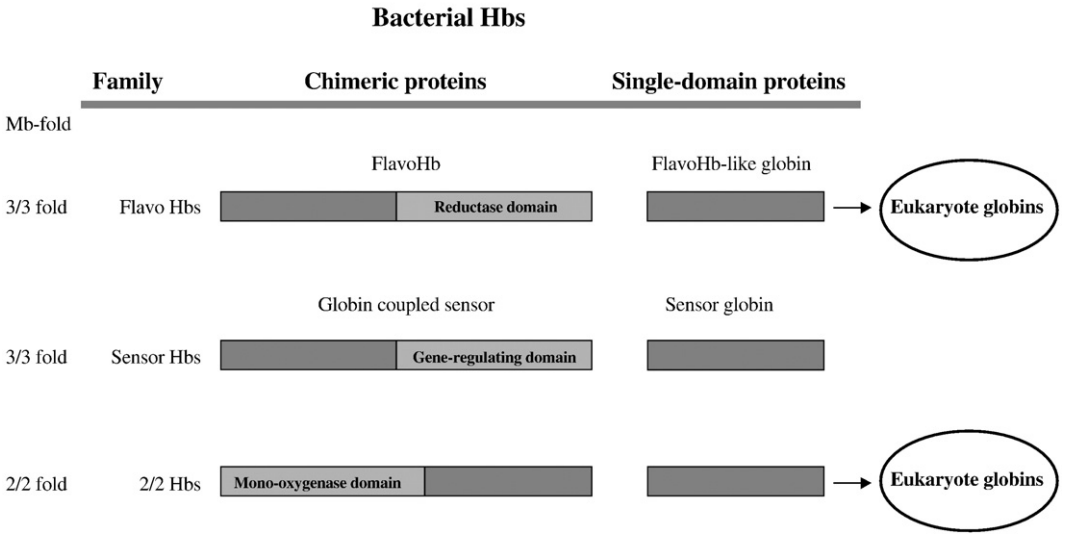

Fig. 2. The three bacterial globin families and their relationships to eukaryotic globins. After Vinogradov and Moens, 2008.

Vinogradov et al. (2005) proposed that all eukaryotic Hbs, including vertebrate α/β globins, Mb, Ngb, and Cygb and invertebrate, bacterial and plant Hbs, emerged from a common ancestor (Fig. 1).

Vertebrate and invertebrate organisms thriving in polar habitats offer opportunities for understanding protein thermal adaptations and their ability to cope with the cold.

In the process of cold adaptation, the evolutionary trend of Notothenioidei, the dominant suborder of Antarctic fish, has led to unique specialisations in many biological features, including hematological parameters and oxygen transport. Decreased amount and multiplicity of Hbs are common features in Antarctic fish. In Channichthyidae, the most phyletically derived notothenioid family, Hb is absent (Ruud, 1954).

For the sake of elaborating unifying principles in cold adaptation, studies on other cold-adapted marine organisms, such as psycrophilic bacteria and invertebrates, ought to be integrated with those on polar fish. Most bacteria can be cultivated in the laboratory, thus it is possible to change growth conditions and investigate how the transcriptome changes in response. Hence, in bacteria it is possible to unravel gene functions and obtain *in vivo* information about how microorganisms adapt to changing environmental conditions.

The recent publication of the genome sequence and annotation of the psychrophilic Antarcticum bacterium *Pseudoalteromonas haloplanktis* TAC125 (Médigue et al., 2005) provides a unique opportunity to explore on "global" ground the cellular strategies adopted by cold-adapted bacteria to cope with the cold. The *P. haloplanktis* TAC125 genome contains multiple genes encoding distinct monomeric Hbs exhibiting a 2/2 α-helical fold, as well as a flavoHb, all bound to fulfil roles other than oxygen transport.

The Antarctic waters are oxygen rich due to increased gas solubility at low temperature; therefore organisms living in such cold environment must face increased levels of reactive oxygen species (ROS).

Recently, Chen et al. (2008) have reported genome-wide studies of the transcriptional and genomic changes associated with cold adaptation in Antarctic notothenioid fish. Their results strongly suggest that evolution in the cold has produced dramatic genomic expansions and/or upregulations of specific protein gene families. Many of their up-regulated genes are involved in the antioxidant function, suggesting that augmented defenses against oxidative stress are important forces in driving the evolutionary adaptations in a cold and oxygen-rich environment.

Cold-adapted bacteria are generally acknowledged to achieve their physiological and ecological success in cold environments through structural and functional properties developed in their genomes. The genome sequence reveals that *P. haloplanktis* TAC125 copes with increased oxygen solubility at low temperature by enhancing production of oxygen-scavenging enzymes and deleting entire metabolic pathways, such as those which generate ROS as side products (Médigue et al., 2005).

This review highlights some aspects of the biochemistry of cold-adapted hemoproteins in fish and bacteria, without claiming to be exhaustive. Heme hexacoordination where the sixth ligand is provided by an internal amino-acid residue, in hemoproteins will be discussed.

2. Hemoproteins in cold-adapted organisms

2.1. Hbs in polar fish

Fish Hbs, similar to other vertebrate Hbs, are tetrameric proteins consisting of two α and two β subunits, each of which contains one oxygen-binding heme group. These subunits are paired in two dimers, $\alpha_1\beta_1$ and $\alpha_2\beta_2$.

Within different species, the transport of oxygen can be modulated by changes in the Hb structure and allosteric-ligand concentration (ATP for most teleost fish), and by changes in the expression of multiple Hbs likely to display different functional features. During evolution, complex and sophisticated molecular mechanisms, e.g. modulation by pH, carbon dioxide, organophosphates and temperature, have been developed to regulate oxygen transport by Hb.

Unlike most mammals, including humans, fish often exhibit Hb multiplicity, usually interpreted as a sign of phylogenetic diversification and molecular adaptation, which results from gene-related heterogeneity and gene duplication events (Dettaï et al., 2008). Oxygen-affinity differences in erythrocytes can also be the result of sequential expression of different Hb chains, variable concentrations of allosteric effectors, and differential response of Hbs to effectors (di Prisco et al., 2007; Verde et al., 2006, 2008).

The capacity of fish to colonise a large variety of habitats appears to have evolved in parallel with suitable modulation of their Hb system at the molecular/functional level.

Unlike temperate and tropical fish Hbs, Notothenioidei (the dominant fish group in the Southern Ocean) have evolved reduction of Hb concentration, as an adaptation to offset the increased blood viscosity at low temperature, thus reducing the amount of energy needed for blood circulation. In the seven red-blooded Antarctic notothenioid families, the erythrocyte number is one order of magnitude lower than in temperate fish, and is reduced by over

three orders of magnitude in the 16 "icefish" species of the eighth family Channichthyidae (Eastman, 1993), in which Hb is absent.

In comparison with temperate species, Antarctic notothenioids have lost globin multiplicity, leading to the hypothesis that in the Antarctic thermostable environment the need for multiple Hbs may be reduced. A single Hb present in lower amounts than in temperate fish can be regarded as the consequence of a less critical role of the oxygen carrier in Antarctic notothenioids, possibly in keeping with the sluggish mode of life, slower metabolism, as well as with the peculiarity of the cold environment (high stability and constancy of physico-chemical conditions, higher oxygen content).

The oxygen affinity of Hbs of many Antarctic species (which controls oxygen binding at the exchange surface and release to the tissues) is quite low (di Prisco, 1988), as indicated by the values of p_{50} (the oxygen partial pressure required to achieve half-saturation). This feature is probably linked to the high-oxygen concentration in the Antarctic waters. The evolutionary development of an alternative physiology based on Hb-free blood may adequately work in the cold for notothenioids in general. Clearly, the benefits due to this loss include reduced costs for protein synthesis.

2.2. Neuroglobin: the search of function of a vertebrate Hb

Ngb is a monomeric heme-containing globin displaying the classical vertebrate folding 3/3 (Burmester et al., 2000; Pesce et al., 2003; Vallone et al., 2004a). The protein is able to bind oxygen and other ligands, and is transcriptionally induced by hypoxia and ischemia (Brunori and Vallone, 2007). Ngb is mainly expressed in retinal neurons and fibroblast-like cells and plays a neuroprotective role during hypoxic stress (Brunori and Vallone, 2007). Evidence includes the observations that neuronal hypoxia and cerebral ischemia induce Ngb expression; knocking down Ngb expression increases hypoxic neuronal injury *in vitro* and ischemic cerebral injury *in vivo* (Greenberg et al., 2008). However, enhanced expression of Ngb does not seem to be a universal response to all forms of neuronal injury, because some insults do not produce such response (Greenberg et al., 2008).

Although many other roles have been suggested, including scavenging of reactive nitrogen and oxygen species (Brunori et al., 2005) and signal transduction (Wakasugi et al., 2003), the Ngb physiological function is still unknown.

Ngb was originally identified in mammalian species, but then it was also found in fish, e.g. the zebrafish *Danio rerio* (Awenius et al., 2001). Mammalian and fish Ngb proteins share about 50% amino-acid sequence identity. Watanabe and Wakasugi have suggested that zebrafish Ngb is a cell-membrane penetrating globin (Watanabe and Wakasugi, 2008).

Recently, the *Ngb* gene was discovered in red-blooded notothenioid fish species, and in at least 13 of the 16 species of the white-blooded icefish family Channichthyidae (Cheng et al., 2009). The deduced amino-acid sequences of *Ngb* gene cloned from three red-blooded species (*Bovichtus variegatus*, *Dissostichus mawsoni*, and *Gymnodraco acuticeps*) and two icefishes (*Chionodraco myersi*, and *Neopagetopsis ionah*) are well conserved. A nearly full-length α-globin cDNA was also obtained from brain RNA of *D. mawsoni* (Cheng et al., 2009). The finding that icefishes retain the *Ngb* gene despite having lost Hb, and Mb in most species, may potentially have important implications in the physiology and pathology of the brain.

As pointed out by Sidell and O'Brien (2006), being the icefishes natural knockouts, they offer remarkable advantages to answer some questions in comparison with the experimentally produced knockouts for Mb expression in mice (Garry et al., 1998; Gödecke et al., 1999). Since Mb deletion in mice leaves the cardiac function uncompromised, probably the development of multiple mechanisms compensates for its lack (Garry et al., 1998; Gödecke et al., 1999). However, the development of compensatory physiological and circulatory adaptations in icefishes argues that loss of Hb and erythrocytes was probably maladaptive under conditions of physiological stress (Sidell and O'Brien, 2006). Whether the *Ngb* gene is expressed is the next important question. Also, whether the α-globin mRNA in the brain is from nervous tissue or from circulating blood needs to be definitely verified.

2.3. Bacterial 2/2 Hbs

2/2 Hbs are small oxygen-binding hemoproteins, generally shorter than vertebrate Hbs by 20–40 amino-acid residues (Pesce et al., 2000). These Hbs show very low amino-acid sequence homology to vertebrate and non-vertebrate Hbs, with few residues conserved throughout the structure. The globin fold is based on a 2/2 α-helical sandwich (Pesce et al., 2000). Modifications of the classical 3/3 fold occur at helix A (almost entirely deleted in all these Hbs), and in the CD-D and EF-F regions. The original phylogenetic analysis of these Hbs classifies them into three groups, denoted I, II, and III (Wittenberg et al., 2002; Vuletich and Lecomte, 2006). 2/2 Hbs belonging to the different groups may coexist in some bacteria, suggesting distinct functions. Such postulated functions, consistent with observed biophysical properties, include long-term ligand or substrate storage, NO detoxification, oxygen/nitrogen monoxide sensing, redox reactions, and oxygen delivery under hypoxic conditions (Wittenberg et al., 2002; Vuletich and Lecomte, 2006). A number of three-dimensional structures of protein belonging to the three groups have been recently elucidated at atomic resolution by X-ray crystallography and NMR (see Nardini et al., 2007 and the references within).

P. haloplanktis TAC125 is a psychrophilic Antarctic bacterium. The *P. haloplanktis* TAC125 genome contains multiple genes encoding 2/2 Hbs (annotated as *PSHAa0030*, *PSHAa0458*, *PSHAa2217*) and a flavoHb gene (*PSHAa2880*), suggesting that specific and distinct functions may be associated to these two classes of proteins (Giordano et al., 2007).

Phylogenetic analyses showed that two 2/2 globins encoded by the *PSHAa0030* and *PSHAa2217* genes belong to group II, and the third one encoded by *PSHAa0458* to group I.

The *PSHAa0030* gene encoding a group-II 2/2 Hb was cloned and over-expressed in *Escherichia coli*. The native form of the protein was a mixture of the ferric and ferrous forms (Giordano et al., 2007). The function of the protein is unclear but the very high-oxygen affinity makes a role in oxygen transport very unlikely (Giordano et al., unpublished). However, *P. haloplanktis* TAC125 is amenable to genetic approaches, and knockout mutations of the these globins may provide valuable information about their biological function.

3. Hexacoordination in hemoproteins

The coordination of a protein side chain to the distal position of the heme iron is expected to influence both the dynamic and structural features of Hb. It is clear that axial ligand strength is an essential property of the molecule that must be considered capable to influence the kinetics of ligand binding, as well as having alternative functional roles. Crystallographic evidence for endogenous coordination at the sixth coordination site of the heme iron has been reported in both the ferrous (hemochrome) and ferric (hemichrome) oxidation state (Vergara et al., 2008). Usually, the sixth ligand is provided by the imidazole side chain of a His in E7, normally present in the distal site of the heme pocket. The occurrence of hemichrome/hemochrome states in members of the Hb superfamily is not uniform suggesting that the functional roles of these oxidation states are multiple, possibly being a tool for modulating ligand-binding or redox properties.

It is well known that tetrameric Hbs, even under physiological conditions, frequently undergo spontaneous oxidation producing a variety of ferric species. The role of these species and their impact in different biological contexts has been highly debated in the last decades. Over the years, hemichromes in tetramers have been considered as precursors of Hb denaturation, since their formation is accelerated by

denaturing agents (Rifkind et al., 1994). It has been shown that hemichromes can be obtained under non-denaturing as well as physiological conditions (Vergara et al., 2008). Recently, it has also been suggested that hemichromes can be involved in Hb protection from peroxide attack (Feng et al., 2005), given that the hemichrome species of human α subunits complexed with the α-helix-stabilising protein (AHSP) do not exhibit peroxidase activity (Feng et al., 2005).

Structural and spectroscopic evidence has shown endogenous coordination at the sixth coordination site in several tetrameric Hbs isolated from Antarctic notothenioid fish (Riccio et al., 2002; Vitagliano et al., 2004; Vergara et al., 2007, 2008; Vitagliano et al., 2008). Under physiological conditions, the oxidation of Antarctic fish Hbs leads to the formation of an endogenous bis-histidyl complex (β-hemichrome) in the ferric state. The bis-His coordination in the ferrous state has never been observed. Thus, under reduction, the hemichrome species is reversibly converted to the classical penta-coordinated deoxy form both in solution (Vitagliano et al., 2004) and in the crystal state (Merlino et al., 2008).

Another example of bis-His coordination in tetrameric Hbs regards horse met-Hb. Notably, bis-His formation invariably involves the α heme in horse Hb (Robinson et al., 2003; Feng et al., 2005).

In comparison with horse Hb, bis-histidyl adducts in Antarctic fish Hbs exhibit large differences in the quaternary structure rearrangement. Horse Hb develops the bis-His form within the R quaternary structure, whereas Antarctic fish Hbs in the bis-His form adopt a quaternary structure that is intermediate between the R and T states (Vergara et al., 2007, 2008).

According to the evidence of higher peroxidase activity in Antarctic fish Hbs, the exchange between hemichrome and pentacoordinated forms may play a distinctive physiological role in Antarctic teleosts (Vergara et al., unpublished).

Hexacoordinated Hbs are also expressed at low structural complexity and observed in bacteria, unicellular eukaryotes (Wittenberg et al., 2002), plants (Watts et al., 2001), invertebrates (Dewilde et al., 2006) and in some tissues of higher vertebrates. In the absence of exogenous ligands, also Ngb (Pesce et al., 2004) and Cygb (de Sanctis et al., 2004) display hexacoordination with distal His E7 coordinating directly with the heme iron, either in ferrous or ferric forms.

The physiological role of these hexacoordinated Hbs is not well understood. Several roles have been suggested.

Firstly, these proteins may scavenge oxygen under hypoxic conditions and supply it for aerobic respiration (Burmester et al., 2000, 2002). Sun et al. (2001, 2003) demonstrated that Ngb is up-regulated under hypoxic conditions, *in vivo* and *in vitro*, and that it protects neurons against the deleterious effects of the hypoxia and ischemia. Formation and cleavage of a disulfide bond influences the functional characteristics of the protein and the formation of the hexacoordinated form. Under hypoxic conditions, the disulfide bond in Ngb will be reduced, with subsequent release of oxygen counteracting hypoxia. Secondly, they may function as terminal oxidases by oxidising NADH under hypoxic conditions and hence enhance ATP production by glycolysis (Sowa et al., 1999). Thirdly, they might be oxygen-sensor proteins, activating other proteins with regulatory function (Hargrove et al., 2000; Kriegl et al., 2002). Fourthly, they may be involved in nitric oxide metabolism (Smagghe et al., 2008).

Hexacoordination, found in monomeric and dimeric Hbs, shows tendency for bis-histidyl hexacoordination and generally exhibit reversible bis-histidyl coordination of the heme iron while retaining the ability to bind exogenous ligands (Weiland et al., 2004). It has been suggested that bis-His adducts can be involved in nitric oxide NO detoxification by acting as NO scavengers. However, there does not seem to exist a distinguishing predisposition in NO scavenging for hexacoordinated Hbs but any Hb may play this role in the presence of a mechanism for heme iron re-reduction (Smagghe et al., 2008).

Currently, some monomeric and dimeric Hb 3D structures, which show the bis-histidyl endogenous coordination, have been deposited in PDB (Mitchell et al., 1995; Hargrove et al., 2000; Hoy et al., 2004; Pesce et al., 2004; Vallone et al., 2004b; de Sanctis et al., 2004, 2005). However, in some Hbs with lower structural complexity, Tyr B10 has been found to act as the sixth ligand at the iron site in the ferrous (Couture et al., 1999) and ferric states (Das et al., 1999; Milani et al., 2005).

In general, bacterial 2/2 Hbs do not show tendency for hexacoordination but few cases have been examined and are reported in the literature.

The ferrous heme iron atom of deoxygenated *Mycobacterium leprae* 2/2 Hb appears to be hexacoordinated (Visca et al., 2002).

Ferric 2/2 Hb from the cyanobacterium *Synechococcus* sp. PCC 7002 (Scott et al., 2002) shares several physical properties with 2/2 Hb from of *Synechocystis* sp. PCC 6803 (Falzone et al., 2002). Both Hbs readily form a hexacoordinate, low-spin complex in the absence of exogenous ligands. Spectral studies support a bis-histidyl ligation to the heme on the distal side.

The 2/2 Hb of the bacterium *Herbaspirillum seropedicae* undergoes transition from an aquomet form in the ferric state, with equilibrium between high and low spin, to a hexacoordinated low-spin form in the ferrous state (Razzera et al., 2008).

Spectroscopic studies of *P. haloplanktis* TAC125 recombinant 2/2 Hb, encoded by the *PSHAa0030* gene, show a predominance of a six-coordinated species in the ferric and ferrous forms. The hexacoordinate form is strongly dependent on pH and temperature; low temperature favours hexacoordinate low-spin forms (Giordano et al., unpublished).

4. Concluding remarks

Hexacoordinated Hbs are endowed with endogenous coordination of the heme iron. It can be hypothesised that hexacoordinated Hbs are universally distributed over the living world and thus may have essential function(s) in cell metabolism.

Hexacoordinated Hbs in general appear to be of more ancient origin than pentacoordinated Hbs. Familiar erythrocyte Hb and muscle Mb have probably originated from a hexacoordinated Hb (Kundu et al., 2003). The question is "what is the function of these hexacoordinated Hbs?" In all cases, the study of hexacoordinated Hbs needs to demonstrate that cells and tissues are able to express significant Hb-reductase activity, necessary to restore the reduced state requested for oxygen binding (Smagghe et al., 2008).

In higher vertebrates, generally, the endogenous hexacoordinated complex is associated with impaired functions. However, it now appears that hemichrome or hemochrome reversible formation is not exceptional, at least among invertebrate, plant and bacterial globins, and globins expressed in low amounts in some tissues of higher vertebrates.

These findings show that the functional role of the hexacoordinated form is not a single one, possibly playing a specific functional role in regulating the kinetics of small ligand-binding or redox properties binding (Smagghe et al., 2008).

In Antarctic fish Hbs it is still disputed whether hemichromes have a biological function or are merely an evolutionary remnant. Also in all other organisms their specific role is still not clear.

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References

Awenius, C., Hanken, T., Burmester, T., 2001. Neuroglobins from the zebrafish *Danio rerio* and the pufferfish *Tetraodon nigroviridis*. Biochem. Biophys. Res. Commun. 287, 418–421.

Brunori, M., Vallone, B., 2007. Neuroglobin, seven years after. Cell. Mol. Life Sci. 64, 1259–1268.

Brunori, M., Giuffrè, A., Nienhaus, K., Nienhaus, G.U., Scandurra, F.M., Vallone, B., 2005. Neuroglobin, nitric oxide, and oxygen: functional pathways and conformational changes. Proc. Natl. Acad. Sci. U.S.A. 102, 8483–8488.

Burmester, T., Weich, B., Reinhardt, S., Hankeln, T., 2000. A vertebrate globin expressed in the brain. Nature 407, 520–523.

Burmester, T., Ebner, B., Weich, B., Hankeln, T., 2002. Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. Mol. Biol. Evol. 19, 416–421.

Chen, Z., Cheng, C.H., Zhang, J., Cao, L., Chen, L., Zhou, L., Jin, Y., Ye, H., Deng, C., Dai, Z., Xu, Q., Hu, P., Sun, S., Shen, Y., Chen, L., 2008. Transcriptomic and genomic evolution under constant cold in Antarctic notothenioid fish. Proc. Natl. Acad. Sci. U.S.A. 105, 12944–12949.

Cheng, C.H., di Prisco, G., Verde, C., 2009. The "icefish paradox". Which is the task of neuroglobin in Antarctic hemoglobin-less icefish? IUBMB Life 61, 184–188.

Couture, M., Das, T.K., Lee, H.C., Peisach, J., Rousseau, D.L., Wittenberg, B.A., Wittenberg, J.B., Guertin, M., 1999. *Chlamydomonas* chloroplast ferrous hemoglobin. J. Biol. Chem. 274, 6898–6910.

Das, T.K., Lee, H.C., Duff, S.M.G., Hill, R.D., Peisach, J., Rousseau, D.L., Wittenberg, B.A., Wittenberg, J.B., 1999. The heme environment in barley hemoglobin. J. Biol. Chem. 274, 4207–4212.

de Sanctis, D., Dewilde, S., Pesce, A., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T., Bolognesi, M., 2004. Crystal structure of cytoglobin: the fourth globin type discovered in man displays heme hexa-coordination. J. Mol. Biol. 336, 917–927.

de Sanctis, D., Dewilde, S., Vonrhein, C., Pesce, A., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T., Ponassi, M., Nardini, M., Bolognesi, M., 2005. Bishistidyl heme hexacoordination, a key structural property in *Drosophila melanogaster* hemoglobin. J. Biol. Chem. 280, 27222–27229.

Dettaï, A., di Prisco, G., Lecointre, G., Parisi, E., Verde, C., 2008. Inferring evolution of fish proteins: the globin case study. Methods Enzymol. 436, 539–570.

Dewilde, S., Ebner, B., Vinck, E., Gilany, K., Hankeln, T., Burmester, T., Kreiling, J., Reinisch, C., Vanfleteren, J.R., Kiger, L., Marden, M.C., Hundahl, C., Fago, A., Van Doorslaer, S., Moens, L., 2006. The Nerve hemoglobin of the bivalve mollusc *Spisula solidissima*. J. Biol. Chem. 281, 5364–5372.

di Prisco, G., 1988. A study of hemoglobin in Antarctic fishes: purification and characterization of hemoglobins from four species. Comp. Biochem. Physiol. B 90B, 631–637.

di Prisco, G., Eastman, J.T., Giordano, D., Parisi, E., Verde, C., 2007. Biogeography and adaptation of Notothenioid fish: hemoglobin function and globin-gene evolution. Gene 398, 143–155.

Eastman, J.T., 1993. Antarctic Fish Biology: Evolution in a Unique Environment. Academic Press, San Diego, CA.

Falzone, C.J., Christie Vu, B., Scott, N.L., Lecomte, J.T., 2002. The solution structure of the recombinant hemoglobin from the cyanobacterium *Synechocystis* sp. PCC 6803 in its hemichrome state. J. Mol. Biol. 324, 1015–1029.

Feng, L., Zhou, S., Gu, L., Gell, D., Mackay, J., Weiss, M., Gow, A., Shi, Y., 2005. Structure of oxidized α-haemoglobin bound to AHSP reveals a protective mechanism for heme. Nature 435, 697–701.

Gardner, P.R., Gardner, A.M., Martin, L.A., Salzman, A.L., 1998. Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. Proc. Natl. Acad. Sci. U.S.A. 95, 10378–10383.

Garry, D.J., Ordway, G.A., Lorenz, J.N., Radford, N.B., Chin, E.R., Grange, R.W., Bassel-Duby, R., Williams, R.S., 1998. Mice without myoglobin. Nature 395, 905–908.

Giordano, D., Parrilli, E., Dettaï, A., Russo, R., Barbiero, G., Marino, G., Lecointre, G., di Prisco, G., Tutino, M.L., Verde, C., 2007. The truncated hemoglobins in the Antarctic psychrophilic bacterium *Pseudoalteromonas haloplanktis* TAC125. Gene 398, 69–77.

Gödecke, A., Flögel, U., Zanger, K., Ding, Z., Hirchenhain, J., Decking, U.K.M., Schrader, J., 1999. Disruption of myoglobin in mice induces multiple compensatory mechanisms. Proc. Natl. Acad. Sci. U.S.A. 96, 10495–10500.

Greenberg, D.A., Jin, K., Khan, A.A., 2008. Neuroglobin: an endogenous neuroprotectant. Curr. Opin. Pharmacol. 8, 20–24.

Hardison, R., 1998. Hemoglobins from bacteria to man: evolution of different patterns of gene expression. J. Exp. Biol. 201, 1099–1117.

Hargrove, M.S., Brucker, E.A., Stec, B., Sarath, G., Arredondo-Peter, R., Klucas, R.V., Olson, J.S., Phillips, G.N., 2000. Crystal structure of a nonsymbiotic plant hemoglobin. Structure 8, 1005–1014.

Holland, H.D., 2006. The oxygenation of the atmosphere and oceans. Philos. Trans. R. Soc. B361, 903–916.

Hoy, J.A., Kundu, S., Trent III, J.T., Ramaswamy, S., Hargrove, M.S., 2004. The crystal structure of *Synechocystis* hemoglobin with a covalent heme linkage. J. Biol. Chem. 279, 16535–16542.

Kendrew, J.C., Bodo, G., Dintzis, H.M., Parrish, R.G., Wyckoff, H., Phillips, D.C., 1958. A three-dimensional model of the myoglobin molecule obtained by X-ray analysis. Nature 181, 662–666.

Kriegl, J.M., Bhattacharyya, A.J., Nienhaus, K., Deng, P., Minkow, O., Nienhaus, G.U., 2002. Ligand binding and protein dynamics in neuroglobin. Proc. Natl. Acad. Sci. U.S.A. 99, 7992–7997.

Kugelstadt, D., Haberkamp, M., Hankeln, T., Burmester, T., 2004. Neuroglobin, cytoglobin, and a novel, eye-specific globin from chicken. Biochem. Biophys. Res. Commun. 325, 719–725.

Kundu, S., Trent III, J.T., Hargrove, M.S., 2003. Plants, humans and hemoglobins. Trends Plant Sci. 8, 387–393.

Médigue, C., Krin, E., Pascal, G., Barbe, V., Bernsel, A., Bertin, P.N., Cheung, F., Cruveiller, S., D'Amico, S., Duilio, A., Fang, G., Feller, G., Ho, C., Mangenot, S., Marino, G., Nilsson, J., Parrilli, E., Rocha, E.P., Rouy, Z., Sekowska, A., Tutino, M.L., Vallenet, D., von Heijne, G., Danchin, A., 2005. Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125. Genome Res. 15, 1325–1335.

Merlino, A., Verde, C., di Prisco, G., Mazzarella, L., Vergara, A., 2008. Reduction of ferric hemoglobin from *Trematomus bernacchii* in a partial bis-histidyl state produces a deoxy coordination even when encapsulated into the crystal phase. Spectroscopy: Biomed. Appl. 22 (2–3), 143–152.

Milani, M., Pesce, A., Nardini, M., Ouellet, H., Ouellet, Y., Dewilde, S., Bocedi, A., Ascenzi, P., Guertin, M., Moens, L., Friedman, J.M., Wittenberg, J.B., Bolognesi, M., 2005. Structural bases for heme binding and diatomic ligand recognition in truncated hemoglobins. J. Inorg. Biochem. 99, 97–109.

Minning, D.M., Gow, A.J., Bonaventura, J., Braun, R., Dewhirst, M., Goldberg, D.E., Stamler, J.S., 1999. *Ascaris* haemoglobin is a nitric oxide-activated "deoxygenase". Nature 401, 497–502.

Mitchell, D.T., Kitto, G.B., Hackert, M.L., 1995. Structural analysis of monomeric hemichrome and dimeric cyanomet hemoglobins from *Caudina arenicola*. J. Mol. Biol. 251, 421–431.

Monod, J., Wyman, J., Changeux, J.P., 1965. On the nature of allosteric transitions: a plausible model. J. Mol. Biol. 12, 88–118.

Nardini, M., Pesce, A., Milani, M., Bolognesi, M., 2007. Protein fold and structure in the truncated (2/2) globin family. Gene 398, 2–11.

Perutz, M.F., 1983. Species adaptation in a protein molecule. Mol. Biol. Evol. 1, 1–28.

Perutz, M.F., Kendrew, J.C., Watson, H.C., 1965. Structure and function of haemoglobin. II. Some relations between polypeptide chain configuration and amino acid sequence. J. Mol. Biol. 13, 669–678.

Perutz, M.F., Fermi, G., Luisi, B., Shanan, B., Liddington, R.C., 1987. Stereochemistry of cooperative mechanisms in hemoglobin. Acc. Chem. Res. 20, 309–321.

Pesce, A., Couture, M., Dewilde, S., Guertin, M., Yamauchi, K., Ascenzi, P., Moens, L., Bolognesi, M., 2000. A novel two-over-two a-helical sandwich fold is characteristic of the truncated hemoglobin family. EMBO J. 19, 2424–2434.

Pesce, A., Dewilde, S., Nardini, M., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T., Bolognesi, M., 2003. Human brain neuroglobin structure reveals a distinct mode of controlling oxygen affinity. Structure 11, 1087–1095.

Pesce, A., Dewilde, S., Nardini, M., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T., Bolognesi, M., 2004. The human brain hexacoordinated neuroglobin three-dimensional structure. Micron 35, 63–65.

Poole, R.K., 2005. Nitric oxide and nitrosative stress tolerance in bacteria. Biochem. Soc. Trans. 33, 176–180.

Razzera, G., Vernal, J., Baruh, D., Serpa, V.I., Tavares, C., Lara, F., Souza, E.M., Pedrosa, F.O., Almeida, F.C., Terenzi, H., Valente, A.P., 2008. Spectroscopic characterization of a truncated haemoglobin from the nitrogen-fixing bacterium *Herbaspirillum seropedicae*. J. Biol. Inorg. Chem. 13, 1085–1096.

Riccio, A., Vitagliano, L., di Prisco, G., Zagari, A., Mazzarella, L., 2002. The crystal structure of a tetrameric hemoglobin in a partial hemichrome state. Proc. Natl. Acad. Sci. U.S.A. 99, 9801–9806.

Rifkind, J.M., Abugo, O., Levy, A., Heim, J.M., 1994. Detection, formation, and relevance of hemichrome and hemochrome. Methods Enzymol. 231, 449–480.

Robinson, V.L., Smith, B.B., Arnone, A., 2003. A pH-dependent aquomet-to-hemichrome transition in crystalline horse methemoglobin. Biochemistry 42, 10113–10125.

Roesner, A., Fuchs, C., Hankeln, T., Burmester, T., 2005. A globin gene of ancient evolutionary origin in lower vertebrates: evidence for two distinct globin families in animals. Mol. Biol. Evol. 22, 12–20.

Ruud, J.T., 1954. Vertebrates without erythrocytes and blood pigment. Nature 173, 848–850.

Scott, N.L., Falzone, C.J., Vuletich, D.A., Zhao, J., Bryant, D.A., Lecomte, J.T., 2002. Truncated hemoglobin from the cyanobacterium *Synechococcus* sp. PCC 7002: evidence for hexacoordination and covalent adduct formation in the ferric recombinant protein. Biochemistry 41, 6902–6910.

Sidell, B.D., O'Brien, K.M., 2006. When bad things happen to good fish: the loss of hemoglobin and myoglobin expression in Antarctic icefishes. J. Exp. Biol. 209, 1791–1802.

Smagghe, B.J., Trent III, J.T., Hargrove, M.S., 2008. NO dioxygenase activity in hemoglobins is ubiquitos in vitro, but limited by reduction in vivo. PloS ONE 3, e2039.

Sowa, A.W., Guy, P.A., Sowa, S., Hill, R.D., 1999. Nonsymbiotic haemoglobins in plants. Acta Biochim. Pol. 46, 431–445.

Sun, Y., Jin, K., Mao, X.O., Zhu, Y., Greenberg, D.A., 2001. Neuroglobin is upregulated by and protects neurons from hypoxic–ischemic injury. Proc. Natl. Acad. Sci. U.S.A. 98, 15306–15311.

Sun, Y., Jin, K., Peel, A., Mao, X.O., Xie, L., Greenberg, D.A., 2003. Neuroglobin protects the brain from experimental stroke in vivo. Proc. Natl. Acad. Sci. U.S.A. 100, 3497–3500.

Trent III, J.T., Hargrove, M.S., 2002. A ubiquitously expressed human hexacoordinate haemoglobin. J. Biol. Chem. 277, 19538–19545.

Vallone, B., Nienhaus, K., Brunori, M., Nienhaus, G.U., 2004a. The structure of murine neuroglobin: novel pathways for ligand migration and binding. Proteins: Struct. Funct. Bioinf. 56, 85–92.

Vallone, B., Nienhaus, K., Matthes, A., Brunori, M., Nienhaus, G.U., 2004b. The structure of carbonmonoxy neuroglobin reveals a heme-sliding mechanism for control of ligand affinity. Proc. Natl. Acad. Sci. U.S.A. 101, 17351–17356.

Verde, C., Parisi, E., di Prisco, G., 2006. The evolution of thermal adaptation in polar fish. Gene 385, 137–145.
Verde, C., Vergara, A., Mazzarella, L., di Prisco, G., 2008. The hemoglobins of fishes living at polar latitudes — current knowledge on structural adaptations in a changing environment. Curr. Protein Pept. Sci. 9, 578–590.
Vergara, A., Franzese, M., Merlino, A., Vitagliano, L., di Prisco, G., Verde, C., Lee, H.C., Peisach, J., Mazzarella, L., 2007. Structural characterization of ferric hemoglobins from three Antarctic fish species of the suborder Notothenioidei. Biophys. J. 93, 2822–2829.
Vergara, A., Vitagliano, L., Verde, C., di Prisco, G., Mazzarella, L., 2008. Spectroscopic and crystallographic characterization of bis-histidyl adducts in tetrameric hemoglobins. Methods Enzymol. 436, 425–444.
Vinogradov, S., Moens, L., 2008. Diversity of globin function: enzymatic, transport, storage, and sensing. J. Biol. Chem. 283, 8773–8777.
Vinogradov, S., Hoogewijs, D., Bailly, X., Arredondo-Peter, R., Gough, J., Dewilde, S., Moens, L., Vanfleteren, J., 2005. Three globin lineages belonging to two structural classes in genomes from the three kingdoms of life. Proc. Natl. Acad. Sci. U.S.A. 102, 11385–11389.
Visca, P., Fabozzi, G., Petrucca, A., Ciaccio, C., Coletta, M., De Sanctis, G., Bolognesi, M., Milani, M., Ascenzi, P., 2002. The truncated hemoglobin from *Mycobacterium leprae*. Biochem. Biophys. Res. Commun. 294, 1064–1070.
Vitagliano, L., Bonomi, G., Riccio, A., di Prisco, G., Smulevich, G., Mazzarella, L., 2004. The oxidation process of Antarctic fish hemoglobins. Eur. J. Biochem. 271, 1651–1659.
Vitagliano, L., Vergara, A., Bonomi, G., Merlino, A., Smulevich, G., Howes, B., di Prisco, G., Verde, C., Mazzarella, L., 2008. Spectroscopic and crystallographic analysis of a tetrameric hemoglobin oxidation pathway reveals features of an intermediate R/T state. J. Am. Chem. Soc. 130, 10527–10535.
Vuletich, D.A., Lecomte, J.T., 2006. A phylogenetic and structural analysis of truncated hemoglobins. J. Mol. Evol. 62, 196–210.
Wajcman, H., Kiger, L., 2002. Hemoglobin, from microorganisms to man: a single structural motif, multiple functions. C. R. Biol. 325, 1159–1174 (Article in French).
Wakasugi, K., Nakano, T., Morishima, I., 2003. Oxidized human neuroglobin acts as a heterotrimeric Gα protein guanine nucleotide dissociation inhibitor. J. Biol. Chem. 278, 36505–36512.
Watanabe, S., Wakasugi, K., 2008. Zebrafish neuroglobin is a cell-membrane-penetrating globin. Biochemistry 47, 5266–5270.
Watts, R.A., Hunt, P.W., Hvitved, A.N., Hargrove, M.S., Peacock, W.J., Dennis, E.S., 2001. A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. Proc. Natl. Acad. Sci. U.S.A. 98, 10119–10124.
Weiland, T.R., Kundu, S., Trent III, J.T., Hoy, J.A., Hargrove, M.S., 2004. Bis-Histidyl Hexacoordination in hemoglobins facilitates heme reduction kinetics. J. Am. Chem. Soc. 126, 11930–11935.
Wittenberg, J.B., Bolognesi, M., Wittenberg, B.A., Guertin, M., 2002. Truncated hemoglobins: a new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. J. Biol. Chem. 277, 871–874.