STRUCTURAL AND FUNCTIONAL STUDIES OF HEMOPROTEINS FROM POLAR MARINE ORGANISMS

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Ai miei genitori, mia sorella
Sabina e Lorenzo perché non
sarei la persona che sono
oggi senza il loro amore ed
il loro costante sostegno.

INDEX

ABBREVIATIONS

Abs: Absorbance
ACC: Antarctic Circumpolar Current
AFGP: Antifreeze Glycoprotein
APF: Antarctic Polar Front
ATP: Adenosine Triphosphate
2,3-BPG: 2,3-Biphosphoglycerate
CO: Carbon Monoxide
2,3-DPG: 2,3-Diphosphoglycerate
DTT: Dithiothreitol
EDTA: Ethylene-diamino-tetra-acetic acid
FAD: Flavin Adenine Dinucleotide
FlavoHb: Flavohemoglobin
FPLC: Fast Protein Liquid Chromatography
GSNO: *S*-nitrosoglutatione
GTP: Guanosine Triphosphate
HbA: Human hemoglobin
Hb: Hemoglobin
HBOCs: Hemoglobin-based oxygen carriers
HEPES: 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid
Hmp: Flavohemoglobin from *Escherichia coli*
HPLC: High-Performance Liquid Chromatography
IMT: 2-iminothiolane
IPP: Inositol Pentaphosphate
LB: Luria-Bertani
MALDI-TOF: Matrix-Assisted Laser Desorption Ionization-Time Of Flight
MAL-PEG: Maleimido Polyethylene Glycol
Mb: Myoglobin
MES: 2-(N-morpholine)-ethane sulfonic acid
Met-Hb: Met-hemoglobin
MS: Mass Spectrometry
NADH: Nicotinamide Adenine Dinucleotide
nHill: Hill coefficient n
NO: Nitrogen Monoxide
p_{50}: O_2 partial pressure required to achieve half-saturation
PBS: Phosphate Buffered Saline Solution
PCR: Polymerase Chain Reaction
4-PDS: 4,4'dithiodipyridine
PEG: Polyethylene Glycol
PEG-Hb^{oxy}: PEGylated Human HbA in oxygenated form
PEG-*Tb*Hb: PEGylated *Trematomus bernacchii* Hb
Ph-2/2HbO: 2-on-2 hemoglobin from *Pseudoalteromonas haloplanktis* TAC125
*Ph*TAC125: *Pseudoalteromonas haloplanktis* TAC125
PITC: Phenyl-isothiocyanate
pO_2: O_2 Partial Pressure
RNS: Reactive Nitrogen Species
ROS: Reactive Oxygen Species

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SVD: Singular Value Decomposition
TFA: Trifluoracetic Acid
TRIS: 2-Amino-2-(hydroxymethyl)-aminomethane
Y: Partial saturation
ΔG: Activation Free Energy
ΔH: Enthalpy of Activation
ΔS: Entropy of Activation
λ: Wavelength

RIASSUNTO

Le specie che vivono in ambienti estremi, come le regioni antartiche ed artiche, sono esposte a forti pressioni evolutive, tra queste la temperatura costituisce il fattore principale. La bassa temperatura può modificare radicalmente la vita di un organismo, influenzando la velocità dei processi metabolici. Essa può rallentare i processi fisiologici, modificare le interazioni proteina-proteina, ridurre la fluidità delle membrane, aumentare la viscosità dei fluidi biologici.

La maggior parte della conoscenza dell'effetto dell'ambiente sulla fisiologia ed evoluzione dei vertebrati deriva da studi sui pesci, i quali hanno molti meccanismi fisiologici in comune con l'uomo. Il loro corpo è immerso nell'acqua e la stretta relazione fisica con l'ambiente acquatico li rende sentinelle sensibili dei cambiamenti ambientali ed offre l'opportunità di comprendere la relazione organismo-ambiente e la risposta dei loro adattamenti alla temperatura.

In Antartide, negli ultimi 40 milioni di anni, la fauna ittica temperata è stata sostituita dalla fauna endemica moderna, perfettamente adattata alle basse temperature ed oggi completamente isolata dal Fronte Polare Antartico.

Le acque antartiche sono dominate da un gruppo tassonomico, il sottordine dei Nototenioidei, che rappresenta un esempio di radiazione adattativa in un ambiente estremo, avendo colonizzato con successo le nicchie ecologiche disponibili. Questo sottordine comprende 8 famiglie (Eastman, 2005): 5 di esse (Nototeniidi, Batidraconidi, Artedidraconidi, Arpagiferidi e Cannictidi) sono prevalentemente antartiche, mentre le altre 3 (Bovictidi, Pseudafriditi ed Eleginopidi) sono sub-antartiche.

Le specie antartiche sono stenoterme, vivono ad una temperatura compresa tra 2°C e -1.86°C (punto di congelamento delle acque marine) e non sopravvivono a temperature superiori a 4°C. Invece le famiglie sub-antartiche comprendono Nototenioidei atipici, mai venuti a contatto con acque fredde e che presumibilmente raggiunsero le attuali latitudini (Nuova Zelanda, Australia e Sud America) prima dei grandi eventi di glaciazione e di isolamento dell'Antartide e vivono in acque caratterizzate da temperature di circa 5-15°C. Studi filogenetici, basati su morfologia e biologia molecolare, assegnano a queste famiglie sub-antartiche le posizioni più basali nel sottordine dei Nototenioidei.

In Antartide, il processo di adattamento al freddo ha determinato l'evoluzione, negli organismi marini, di meccanismi di resistenza e di adattamento importanti anche per il loro alto potenziale applicativo. Un esempio è la sintesi di proteine antigelo, che possono essere importanti in applicazioni industriali, mediche ed agricole. Inoltre, vivendo in un ambiente stabile, estremamente freddo e ben ossigenato, specializzazioni uniche si sono anche evolute nel sistema di trasporto dell'ossigeno, tra cui la riduzione della concentrazione e della molteplicità delle emoglobine e la bassa affinità di legame per l'ossigeno (di Prisco et al., 2007; Verde et al., 2008). All'estremo di questa strategia adattativa vi è il sangue incolore dei Cannictidi (o *icefish*), che non sintetizzano emoglobine e non producono globuli rossi (Ruud 1954). Questi aspetti adattativi sono molto interessanti anche per lo sviluppo di trattamenti innovativi per malattie e sindromi collegate al sangue, come l'anemia, le emoglobinopatie, la talassemia, o di sostituti del sangue. Inoltre, gli *icefish*, che presentano caratteristiche tipiche di patologie umane (demineralizzazione delle ossa, vista poco sviluppata), possono rappresentare modelli naturali per lo studio e

l'identificazione di elementi genetici coinvolti in processi, quali osteoporosi e cecità (Albertson et al., 2009).

Molte sono le caratteristiche strutturali e funzionali delle emoglobine di pesci antartici descritte in studi precedenti. Essi vivono principalmente sui fondali marini ed hanno una sola emoglobina che rappresenta il 95-97% del totale, spesso accompagnata da un componente minoritario con proprietà funzionali simili. Al contrario, come molti teleostei temperati, i Nototenioidei sub-antartici, come *Cottoperca gobio* (Giordano et al., 2009) e *Bovichtus diacanthus* (Coppola et al., 2010), entrambi appartenenti alla famiglia dei Bovictidi, presentano una maggiore molteplicità di emoglobine, probabilmente in risposta alle fluttuazioni di temperatura nelle acque in cui vivono, molto più elevate che in Antartide.

A. In questo contesto, la prima parte del progetto di dottorato ha riguardato la purificazione e la caratterizzazione strutturale e funzionale delle emoglobine di Nototenioidei sub-antartici. Infatti, la possibilità di studiare la molecola di emoglobina in gruppi correlati filogeneticamente, ma che vivono a latitudini inferiori (anche se non di molto), offre l'opportunità di comprendere meglio i meccanismi dell'adattamento al freddo. Inoltre, la conoscenza della relazione struttura/funzione di queste proteine è alla base non solo della comprensione delle funzioni vitali da esse svolte, ma è essenziale per poter proporre loro potenziali utilizzi nelle biotecnologie.

In particolare, i sistemi di trasporto di due specie sub-antartiche, *Eleginops maclovinus* e *Dissostichus eleginoides*, sono stati analizzati e paragonati con quello di *Trematomus bernacchii* (famiglia dei Nototeniidi), una delle specie antartiche più comuni, la cui caratterizzazione era stata studiata in dettaglio.

E. maclovinus, l'unica specie della famiglia degli Eleginopidi, è importante in quanto costituisce il *sister group* dei Nototenioidei antartici, essendo la specie più vicina, dal punto di vista filogenetico, alle 5 famiglie antartiche. *D. eleginoides*, invece, pur appartenendo alla famiglia dei Nototeniidi, molto rappresentata a sud del Fronte Polare Antartico, vive in acque sub-antartiche e quindi permette di evidenziare ancor di più i caratteri mantenuti e/o perduti dalle specie antartiche.

A differenza di molti Nototenioidei antartici, ma in accordo con la molteplicità di emoglobine riscontrata nella maggior parte delle specie non-antartiche analizzate fino ad oggi, *E. maclovinus* possiede tre emoglobine: una componente maggioritaria (Hb1) e due minoritarie (HbC e Hb2, che hanno rispettivamente la catena α e la catena β in comune con Hb1).

D. eleginoides, come la maggior parte delle specie antartiche e come anche la specie sub-antartica *Pseudaphritis urvillii* (l'unica della famiglia dei Pseudafritidi; Verde at al., 2004), ha invece una componente maggioritaria (Hb1, circa il 95% del totale) ed una minoritaria (Hb2), che hanno la catena β in comune. Questo aspetto sottolinea in *D. eleginoides* la presenza di alcuni caratteri tipici delle specie antartiche, probabilmente in parte giustificata dall'appartenenza ad una famiglia prevalentemente distribuita nelle acque a sud del Fronte Polare Antartico.

In tutte le emoglobine analizzate, il legame con l'ossigeno è modulato da effettori eterotropi, con marcati effetti Bohr e Root. Nelle Hb1 di *E. maclovinus* e *D. eleginoides* l'affinità dell'ossigeno è più alta di quella delle emoglobine dei Nototeniodei antartici.

In *E. maclovinus*, le cinetiche di legame con ligandi esogeni, determinate mediante *laser-flash* fotolisi e *stopped flow*, hanno permesso di determinare le

costanti di associazione e dissociazione di ossigeno e CO che indicano una forte stabilizzazione della forma legata (stato T) dell'emoglobina.

Rispetto ad emoglobine dell'uomo e di altre specie del sottordine, sia antartiche (come *T. bernacchii*, Ito et al., 1995; Mazzarella et al., 2006a) che sub-antartiche (come *P. urvillii*, Verde et al., 2004), studi spettroscopici in *E. maclovinus* non hanno messo in evidenza variazioni significative nelle parti distale e prossimale della cavità dell'eme.

Infine, la struttura tridimensionale della forma carbomonossi dell'Hb1 di *E. maclovinus*, esaminata mediante cristallografia ai raggi X, ha rivelato la presenza di due forme cristalline indipendenti, ortorombica ed esagonale. Dati di diffrazione di entrambe le forme sono stati raccolti ad alta risoluzione (1.45 e 1.49 Å, rispettivamente). La prima di queste è stata analizzata in dettaglio. Essa ha messo in evidenza un tipico comportamento associato ad emoglobine nello stato R, sebbene alcune peculiarità siano state osservate a livello della struttura terziaria e quaternaria. Inoltre, la struttura carbomonossi dell'Hb1 di *E. maclovinus*, anche se in assenza di ATP, mostra che la regione C-terminale della globina β_2, dove la catena laterale di un residuo di tirosina adotta una conformazione tipica dello stato T, potrebbe essere coinvolta nell'interazione con l'effettore allosterico.

B. Lo studio dettagliato della struttura e della funzione delle emoglobine antartiche ed un'attenta analisi delle loro caratteristiche peculiari, costruite nell'arco del processo di adattamento ad ambienti estremi, può trovare applicazioni interessanti.

In particolare, queste emoproteine possono rappresentare un valido modello per la progettazione di sostituti del sangue. È da oltre trent'anni, infatti, che si mira all'elaborazione di prodotti innovativi, la cui somministrazione possa sostituire o fungere da complemento alle trasfusioni di sangue, a cui spesso sono correlati numerosi problemi sia sanitari che pratici.

L'utilizzo dell'emoglobina umana, non modificata e libera nel sangue, ha però molti effetti collaterali come l'alta tendenza a dissociare in dimeri, filtrati poi attraverso i reni causando nefrotossicità, o anche fuoriuscita dai vasi sanguigni, accompagnata da una maggiore attività di *scavenger* del monossido di azoto (NO), inducendo vasocostrizione ed aumento della pressione sanguigna.

Strategie mirate a minimizzare questi effetti hanno previsto soprattutto modifiche della molecola di emoglobina, volte in particolare ad aumentarne le dimensioni. Una delle strategie più promettenti che conducono all'aumento del peso molecolare di questa proteina è la modifica chimica della sua superficie tramite coniugazione con macromolecole polimeriche, come il polietilenglicole (PEG), in modo da favorirne la permanenza nel circolo sistemico.

Tra i principali limiti inerenti all'utilizzo di emoglobine umane o animali modificate con PEG vi sono però la loro alta affinità di legame per l'ossigeno, che limita l'adeguata ossigenazione dei tessuti, ed una maggiore destabilizzazione del tetramero (Caccia et al., 2009).

In questo contesto, le emoglobine antartiche appaiono particolarmente interessanti. La loro bassa affinità di legame per l'ossigeno e la stabilità del tetramero le rendono un ottimo sistema, dotato di effetti collaterali minori.

Come secondo obiettivo del progetto, svolto all'Università di Parma in collaborazione con il Prof. Mozzarelli, il Dott. Bruno ed il Prof. Viappiani, l'emoglobina della specie antartica *T. bernacchii* è stata perciò isolata, sottoposta a modificazione mediante PEG ("PEGilazione") ed i derivati ottenuti sono stati caratterizzati

funzionalmente e paragonati a quelli dell'emoglobina umana. Alcuni effetti non specifici causati dall'aggiunta delle molecole di PEG (aumento dell'affinità per l'ossigeno; diminuzione della cooperatività; aumento della quantità di emoglobina nello stato R, osservata mediante esperimenti di *laser-flash* fotolisi), presenti nelle emoglobine umane, bovine e canine, sono stati confermati anche in questo caso. È stato però osservato che anche dopo la reazione di "PEGilazione" l'emoglobina antartica conserva una bassa affinità di legame per l'ossigeno e mostra un effetto Bohr ancora pronunciato. Inoltre, la reattività per l'NO, misurata mediante *stopped flow*, è circa 10 volte più bassa di quella dell'emoglobina umana "PEGilata".

In conclusione, questi risultati hanno dimostrato che i derivati "PEGilati" di emoglobine antartiche hanno requisiti funzionali potenzialmente interessanti per una loro utilizzazione come sostituti del sangue.

C. Uno dei maggiori limiti riscontrati nello studio dei vertebrati è la mancanza di dati riguardanti le sequenze genomiche. Per questo motivo, è importante integrare gli studi sui pesci polari con quelli su microrganismi, che con i loro genomi di dimensioni ridotte hanno anche un grande potenziale per la scoperta di nuovi prodotti biotecnologici.

La possibilità di sequenziare genomi interi di batteri polari è importante per chiarire le funzioni geniche e le strategie adottate. Questi organismi, infatti, hanno evoluto caratteristiche genotipiche e/o fenotipiche utili per fronteggiare problemi correlati con, ad esempio, ridotta attività enzimatica, scarsa fluidità delle membrane, accresciuta solubilità dei gas, stabilità dei radicali liberi presenti.

Tra i microrganismi, un modello per studi sia di base che applicativi è il batterio Gram-negativo *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125), isolato dalle acque in vicinanza della stazione costiera antartica francese Dumont d'Urville, Terre Adélie. Questo organismo psicrofilo è in grado di vivere in un intervallo di temperatura compreso tra 4 e 25°C e si moltiplica raggiungendo alte densità cellulari. Il genoma di questo batterio è stato sequenziato ed annotato (Médigue et al., 2005), permettendo così di comprendere molte delle caratteristiche fisiologiche e metaboliche evolute in risposta alla bassa temperatura.

In un ambiente estremamente freddo e ricco di ossigeno come l'Antartide, è molto importante sviluppare meccanismi di difesa contro lo stress ossidativo e nitrosativo. La sequenza genomica mostra che *Ph*TAC125 risponde all'aumentata solubilita' dell'ossigeno a bassa temperatura mediante grande attivita' di *scavenging* di questo gas, grazie all'azione di perossidasi e catalasi, e all'eliminazione di meccanismi (come ad esempio la sintesi di molibdopterina) che producono specie reattive dell'ossigeno (ROS). Possiede inoltre diverse diossigenasi e desaturasi lipidiche che permettono di utilizzare l'ossigeno direttamente. Sono anche stati trovati nel genoma diversi geni coinvolti del metabolismo dell'NO, come NO e nitrito reduttasi.

In questo contesto, la presenza di geni globinici nel genoma di questo batterio ci ha spinti a cercare di chiarire il ruolo fisiologico delle globine. Questi geni sembrano ubiquitari: oltre che negli organismi superiori, sono presenti anche in batteri, alghe, lieviti, protozoi e funghi, suggerendo che la famiglia delle globine è molto flessibile per quanto riguarda i ruoli biologici.

In particolare, l'analisi *in silico* del genoma di *Ph*TAC125 (Giordano et al., 2007) ha rivelato la presenza di un gene che codifica una flavoemoglobina (*PSHAa2880*) e di tre geni che codificano emoglobine 2/2 (annotate come *PSHAa0030*, *PSHAa0458*,

PSHAa2217), così denominate a causa della conformazione tridimensionale organizzata in una struttura a "*2-over-2 α-helices"*, che si differenzia dal *folding* tipico delle globine.

Mentre è ormai noto che la flavoemoglobina è coinvolta nella detossificazione da NO, il ruolo delle globine 2/2 non è ancora ben chiaro.

Fino ad oggi, la struttura e la funzione dell'emoglobina 2/2 codificata dal gene *PSHAa0030* (*Ph*-2/2HbO) è stata dettagliatamente studiata e caratterizzata mediante spettroscopia, misure cinetiche e dinamica molecolare (Giordano et al., 2007; Howes et al., 2011; Giordano et al., 2011). Studi preliminari hanno inoltre dimostrato che il batterio psicrofilo in cui questo gene globinico è stato inattivato diventa molto sensibile ad alti livelli di ossigeno, perossido di idrogeno ed agenti nitrosativi (Parrilli et al., 2010a), consentendo una prima ipotesi su di un coinvolgimento della globina nella protezione da stress ossidativo e nitrosativo.

A partire da queste premesse, l'ultimo obiettivo del progetto di dottorato ha riguardato uno studio più dettagliato del ruolo fisiologico di *Ph*-2/2HbO, teso a cercare una possibile conferma di una sua funzione nella detossificazione da NO.

In particolare, la ricerca, svolta all'estero in collaborazione con il Prof. Poole dell'Università di Sheffield, ha previsto il clonaggio e l'espressione del gene *PSHAa0030* in un ceppo mutato di *Escherichia coli*, in cui il gene codificante per la flavoemoglobina è stato reso inattivo (*E. coli hmp*). La sola delezione del gene *hmp* è sufficiente a rendere il batterio estremamente sensibile alla presenza di NO e ad altri agenti nitrosanti (Membrillo-Hernández et al., 1999), e rappresenta per questo un utile approccio per testare *in vivo* l'influenza di globine batteriche nella protezione da stress nitrosativo (Hernández-Urzúa et al., 2003; Fabozzi et al., 2006; Lama et al., 2006).

Inserendo il gene *PSHAa0030*, la capacità di crescita del mutante viene ripristinata, quando nel terreno di coltura sono presenti S-nitrosoglutatione (GSNO, agente nitrosante) e/o DetaNONOate (donatore di NO). Inoltre, mediante elettrodi ad ossigeno ed NO, si è potuto dimostrare che l'espressione della globina nel ceppo *E. coli hmp* è in grado di proteggere la respirazione batterica dall'NO, fornendo una conferma ulteriore che la proteina *Ph*-2/2HbO ha un ruolo protettivo dallo stress nitrosativo.

La conoscenza della sequenza del genoma di *Ph*TAC125 e la disponibilita' di numerosi strumenti genetici per poterlo manipolare (Duilio et al. 2004), rende questo batterio un organismo ideale come fonte di esoenzimi psicrofili di potenziale interesse biotecnologico e come ospite per la produzione di proteine ricombinanti a basse temperature (Vigentini et al., 2006; Cusano et al., 2006; Parrilli et al., 2008; Parrilli et al., 2010b). Inoltre, una volta modificato opportunamente mediante ingegneria genetica, acquista importanti capacità degradative e di conseguenza ampie potenzialità nel biorisanamento (Siani et al., 2006; Papa et al., 2009; Parrilli et al., 2010c).

La presenza di geni globinici, probabilmente coinvolti nella protezione da stress ossidativo e nitrosativo, appare molto interessante, anche perché va nella direzione di superare il concetto tradizionale di considerare l'emoglobina esclusivamente come trasportatore di ossigeno. La loro presenza in batteri, alghe, lieviti, protozoi e funghi, suggerisce in realtà che le globine possono ricoprire un ampio spettro di ruoli biologici. Questa caratteristica ci consente di considerare aspetti molto interessanti anche a livello applicativo. Nel corso dei processi fermentativi, ad esempio, la grande richiesta di ossigeno è un'esigenza che diventa sempre più pronunciata al crescere

della scala del processo e rappresenta uno dei principali limiti alla produzione di proteine ricombinanti e di determinati metaboliti desiderati. La presenza di globine batteriche ricombinanti nei processi industriali si è dimostrata utile per promuovere la crescita della cellula ospite persino in condizioni di carenza di ossigeno, sia in termini di velocità che di densità cellulare (Bollinger et al., 2001), alleviando gli effetti negativi che la limitazione della quantità di ossigeno ha sulla crescita e sulla produzione dei prodotti richiesti. Inoltre, il coinvolgimento delle globine batteriche nella protezione da NO è di grande interesse per la microbiologia clinica e per industrie farmaceutiche, in quanto il numero di patogeni resistenti agli antibiotici è ormai in largo aumento ed i geni globinici potrebbero diventare nuovi geni *target* per terapie innovative.

ABSTRACT

Antarctica, more than any other habitat on Earth, represents a unique natural laboratory for fundamental research on the processes that have produced biological diversity in extreme environments and, at the same time, offers potential biotechnological opportunities.

One of the most interesting models, within vertebrates, to study the biological responses to cold is provided by Notothenioidei, a group of related species constituting the dominant suborder of teleosts living in Antarctica. To preserve biological activity, five high-Antarctic families of this suborder, living in a stable, extremely cold, and well-oxygenated marine environment, have evolved unique specialisations, including modification of hematological features, having reduced hemoglobin concentration and multiplicity. On the contrary, the three remaining small basal sub-Antarctic families exhibit high hemoglobin multiplicity, probably as a response to temperature differences and fluctuations of temperate waters, and provide an excellent opportunity for understanding the processes involved in cold adaptation.

In this thesis the structure and function of the oxygen-transport system of two sub-Antarctic notothenioids, *Eleginops maclovinus* (family Eleginopidae) and *Dissostichus eleginoides* (family Nototheniidae), were described and compared with respect to high-Antarctic species, and in particular to the hemoglobin of *Trematomus bernacchii* (family Nototheniidae). In contrast to high-Antarctic notothenioids, the hemoglobins of *E. maclovinus* and *D. eleginoides* show high oxygen affinity and cooperativity, and marked Root effect. In addition, in the major component of *E. maclovinus*, a strong stabilization of the low affinity T quaternary state and some peculiar features at the level of the tertiary and quaternary structures have been identified.

The study of these fish and their adaptations is also interesting in the production of new treatments for blood-related diseases and syndromes, including anaemia, hemoglobinopathies and thalassemias or in the development of hemoglobin-based oxygen carriers, a novel pharmaceutical class used in surgery or emergency medicine. Conjugation of human and animal hemoglobins with polyethylene glycol (PEG) has been widely explored as one the most promising strategies to develop blood substitutes. In fact, PEGylation of human hemoglobin led to products with significantly different oxygen-binding properties with respect to the unmodified tetramer and high NO dioxygenase reactivity, known causes of toxicity.

In this context, hemoglobins from Notothenioidei are particularly interesting as they show peculiar features, namely exceptionally low oxygen affinity, little or no dissociation of the tetramer into dimers, absence of cysteine β93, that make them potentially less sensitive to the undesirable effects of PEGylation. The action of PEGylation on properties of the oxygen-transport system of Antarctic fish, and in particular of *T. bernacchii* hemoglobin, was investigated and compared with that of PEGylated human hemoglobin, confirming that these PEGylated Antarctic hemoglobins potentially meet the functional requirements of blood substitutes.

A remarkable restriction to the research on Antarctic fish is the current lack of genomic sequence data. In contrast, this is not a problem in microorganisms with their small genomes, and which represent a remarkable source for the discovery of new potential biotechnological products.

The Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) displays many features that make it an organism of choice for fundamental, environmental and biotechnological studies. The genome sequence shows that *Ph*TAC125 copes with the high oxygen solubility, due to cold temperature, by increasing production of oxygen-scavenging enzymes and deleting entire metabolic pathways which generate reactive oxygen species as side products. The presence of multiple genes encoding 2/2 hemoglobins (annotated as *PSHAa0030*, *PSHAa0458*, *PSHAa2217*) and a flavohemoglobin gene (*PSHAa2880*), that seem be involved in the protection of the cells from nitrosative and oxidative stress, can also be seen in this perspective. While the flavohemoglobin is widely recognised to be involved in the NO detoxification, the role of the 2/2 hemoglobins is still not clear. Recent *in vivo* results demonstrated that the inactivation of the gene *PSHAa0030* encoding the globin *Ph*-2/2HbO makes the mutant bacterial strain sensitive to high oxygen levels, hydrogen peroxide, and nitrosating agents. On this basis, the physiological role of the globin *Ph*-2/2HbO was investigated by *in vivo* and *in vitro* experiments. The results confirm its involvement in the protection of cells against the toxic effects of NO and related reactive nitrogen species.

CHAPTER I

INTRODUCTION

1.1 Antarctic marine organisms and their evolutionary adaptations

The cellular macromolecules, e.g. proteins and nucleic acids, are very sensitive to environment perturbations caused by fluctuations in temperature, hydrostatic pressure, O_2 availability. Certainly, the temperature is one of the most critical factors affecting cell physiology, influencing strongly metabolic-process rates, protein-protein interactions, membrane fluidity, viscosity of biological fluids.

Antarctica, more than any other habitat on Earth, represents a unique natural laboratory for fundamental research on the processes that have produced biological diversity in extreme environments and, at the same time, offers potential biotechnological opportunities. Polar marine organisms evolved unique properties under the influence of a suite of geological and climatic factors, including geographic isolation, extreme low temperature, intense seasonality, enhanced O_2 solubility.

Indeed, Antarctic shelf has been subject to a series of tectonic and oceanographic events that led to the isolation and cooling of the continent and that began to alter the composition of the Antarctic biota. Key among these events in the formation of the Southern Ocean, which surrounds Antarctica and includes the Weddell and Ross Seas, was the opening of the Drake Passage between southern South America and the Antarctic Peninsula. Recent analyses suggest that its formation occurred possibly approximately 41 million years ago (Scher and Martin, 2006). The Drake Passage led to the development of the Antarctic Circumpolar Current (ACC), the ocean's largest current, and this facilitated the thermal isolation of Antarctica and surrounding water that have driven evolution of a unique Antarctic biota (Eastman, 1993). The Antarctic Polar Front (APF), the northern border of the ACC between 50°S and 60°S, acts as a cold “wall” that prevents mixing of the waters of the Southern Ocean with those of the Indian, Pacific and Atlantic oceans and limits the opportunities for migration of the fauna of the cold-temperate ocean to the south, and vice versa (Coppes and Somero, 2007).

As water temperatures decreased and ice appeared, many groups of organisms became extinct in the Antarctic. With the local elimination of most of the Eocene fauna, competition was reduced and ecological possibilities became available. A taxonomically diverse and cosmopolitan Eocene fauna was replaced by a taxonomically restricted and endemic modern fauna.

One of the most interesting models, within vertebrates, to study the biological responses to cold is provided by fishes, which share most physiological mechanisms with humans. Their bodies, completely submerged in water, have strong physiological relationships with the surrounding environment making them sensitive sentinels of environmental challenge and responses to temperature adaptation.

The Notothenioidei constitutes the dominant suborder of teleosts living in Antarctica (Eastman, 2005). Despite the global diversity of fishes and the large size of the Southern Ocean (10% of the world’s ocean), its current fish fauna consists of only 322 species (Estman, 2005).

Notothenioids opportunistically radiated in this developing ecosystem to fill a variety of niches and became largely dominant on the Antarctic shelf, in diversity,

abundance and biomass (Estman, 2005), as consequence of the ecological success in adapting to the challenging environmental conditions (Clarke and Johnston, 1996).

Eight are the families of this suborder (Eastman, 2005). The five families Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae include genera with an Antarctic distribution, with the exception of some species of Nototheniidae that occur in sub-Antarctic waters, whereas the remaining three small basal families (10 of 11 species of Bovichtidae, and monotypic Pseudaphritidae and Eleginopidae) are sub-Antarctic.

High-Antarctic species of notothenioids are stenothermal (Eastman 1993, 2005) and live between 2°C and -1.86°C (the freezing point of sea water) and appear to have lost the ability to cope with higher temperature. A rise in sea temperatures of only 2°C could potentially compromise the survival of many Antarctic species.

To compensate the effect of low temperature on metabolic processes and to preserve the biological activity, these fishes have altered many of their bio-molecular systems by mutation and selection and have evolved unique specialisations in many biological features, making these species interesting in a wide range of applications.

Some examples of adaptive change in the molecular and cellular machinery include efficient microtubule assembly at low temperature (Detrich *et al*., 1989, 2000; Redeker *et al*., 2004), enzyme-structural constraints (Fields and Somero, 1998; Russell, 2000; Hochachka and Somero, 2002; Collins et al., 2003; D'Amico et al., 2003; Feller and Gerday, 2003; Fields and Houseman, 2004; Johns and Somero, 2004), apparent loss of inducible heat-shock response (Place and Hofmann, 2005; Clark and Peck, 2009), decreased membrane fluidity (Römisch *et al*., 2003), higher levels of ubiquitin-conjugated proteins in tissues (Todgham *et al*., 2007), translocation of the nicotinamide adenine dinucleotide (NADH) 6 dehydrogenase mitochondrial gene (Zhuang and Cheng, 2010), encoding protein sequences different from canonical sequences of the sub-Antarctic species inhabiting temperate waters.

Moreover, the success of the radiation of these high-Antarctic notothenioids has been attributed to the evolution of antifreeze glycoproteins (AFGPs), a key innovation that allowed them to survive and diversify to the climatic cooling (DeVries, 1988; Cheng and DeVries, 1991). AFGPs allow avoiding freezing by binding water, thus preventing growth of ice crystals in the blood and other body fluids. The development of these proteins is also important because of their potential industrial, medical, and agricultural applications in different fields, such as food technology, cold storage of cells lines, tissues, and organs, cryosurgery.

Besides AFGPs, specialised hematological features have been developed in these fishes, having reduced hemoglobin (Hb) concentration and multiplicity (di Prisco et al., 2007; Verde et al., 2008), counterbalancing the increase of the viscosity due to low temperature. Channichthyidae (also called *icefish*), the only known adult vertebrates whose blood is devoid of Hb, represent the extreme of this trend (Ruud 1954). The study of these fish and their adaptations is interesting to produce new treatments for blood-related diseases and syndromes, including anaemia, hemoglobinopathies and thalassemias or in the development of Hb-based O_2 carriers (HBOCs). Moreover, the icefish represent natural mutant models that, with their evolutionary processes, have produced characteristics that imitate human diseases. Comparing the genomes of closely relates species it is possible to find out how they changed, as their bones demineralised, their eyesight deteriorated, revealing genetic elements involved in the parallel human processes, such as osteoporosis, blindness (Albertson et al., 2009).

Sub-Antarctic notothenioids, which comprise sub-Antarctic as well as temperate species, inhabit waters north of the APF (Eastman, 2005). Presumably they migrated and became established in New Zealand, Australia and cold-temperate South American waters before the isolation of Antarctica (Eastman and McCune, 2000) and never experienced near-freezing water temperatures.

The lack of any detectable AFGP coding sequence in the species *Bovichtus variegatus*, *Pseudaphritis urvillii* and *Eleginops maclovinus* is consistent with this hypothesis (Cheng et al., 2003). On the contrary, a reduced AFGP system in *Notothenia angustata* and *N. microlepidota*, with very low blood AFGP concentration and only two to three genes showing some replacements in the functional repeat Thr-Ala-Ala (Cheng et al., 2003), supports the hypothesis that these species had developed cold adaptation before of the migration to temperate latitudes that occurred much later than other sub-Antarctic species. The Patagonian toothfish *Dissostichus eleginoides* is a sub-Antarctic species belonging to the family Nototheniidae, the most diversified family of the suborder in which most of the members have Antarctic distribution. It appears to have no functional AFGP sequences, consistent with its sub-Antarctic distribution. The hypothesis is that this species had the primordial AFGP genotype, lost or mutated following its migration to actual habitats (Cheng et al., 2003).

The availability of species phylogenetically related, living in a wide range of latitudes, is a useful approach to understanding the fish evolutionary history, as well as the molecular bases of cold adaptation. Consequently, high- and sub-Antarctic fish Hbs offer excellent opportunities for comparative approaches, to identify the biological and biochemical characters responsible for thermal adaptation. Moreover, a thorough understanding of the structural and functional properties of these proteins is of vital importance to further investigations into their possible applications.

However, a remarkable restriction to the research on Antarctic fish is the current lack of genomic sequence data. In contrast, this is not a problem in microorganisms with their small genomes, and which represent the greatest potential for the discovery of new potential biotechnology products.

The possibility to sequence whole polar bacterial genomes is important to understand gene functions and increase the information about the strategies adopted to maintain activity and metabolic function, under challenging conditions. Moreover, most bacteria, having short generation times and being cultivable in laboratory, can be used to investigate the transcriptome changes, in response to different growth conditions.

Cold-adapted microorganisms have successfully coped with main physiological challenges, due to the extreme polar conditions, by slow down of the metabolic flux (D'Amico et al., 2006). As in the vertebrate, also here the proteins are the main targets of the adaptive strategies, as they are involved in the most important cell functions, controlling the transport of nutrients and waste products, macromolecular assemblies, appropriate folding, reaction rates, process of transcription and translation. These adaptive modifications appear to depend on higher flexibility of molecular structures and of key parts of the structure of many proteins (D'Amico et al., 2006). In these cold habitats, psychrophiles bacteria have developed, among other peculiarities, cold-active enzymes characterised by increased catalytic efficiency and thermolability. The biochemical properties of these enzymes make them attractive in several biochemical, bioremediation and industrial applications (Feller and Gerday, 2003).

Moreover, the extreme environmental conditions increase the production of reactive O_2 species (ROS) in cells, leading to significant damage to cellular structures. In order to prevent cellular damage, adjustments in antioxidant defenses are needed to maintain the steady-state concentration of ROS and likely represent important components in evolutionary adaptations in cold and O_2-rich environments.

Although other genomes from psychrophilic bacteria have been sequenced, the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) is considered one of the best model organisms for the structural/functional genome analyses. *Ph*TAC125 is a Gram-negative bacterium, isolated in Antarctic coastal sea water, in the vicinity of the French station Dumont d'Urville, Terre Adélie. It is able to grow in a wide temperature range (4-25°C) and to reach very high cell density even in uncontrolled laboratory conditions. Its genome, made of two chromosomes, has been completely sequenced and annotated (Médigue et al., 2005) and provides a interesting opportunity to investigate on "global" ground the cellular strategies adopted to survive the cold.

*Ph*TAC125 displays many features that make it an organism of choice for fundamental, environmental and biotechnological studies (Médigue et al., 2005). Its genome sequence, combined with the availability of genetic tools for host transformation (Duilio et al., 2004), make this bacterium interesting both as a source of cold-active enzymes having biotechnological potential, and as model organism to study protein-secretion mechanisms in marine environments, besides its use as non-conventional host for the recombinant protein production at low temperature (Cusano et al., 2006; Vigentini et al., 2006; Parrilli et al., 2008; Parrilli et al., 2010b). The engineered psychrophilic bacterium shows important degradative capabilities and wide potentiality in bioremediation applications, such as the removal of toxic compounds from chemically contaminated marine environments and cold effluents of industrial processes (Siani et al., 2006; Papa et al., 2009; Parrilli et al., 2010c).

The genome sequence shows that *Ph*TAC125 copes with the high O_2 solubility, due to cold temperature, by increasing production of O_2-scavenging enzymes and deleting entire metabolic pathways which generate ROS as side products. The loss of the ubiquitous molybdopterin-dependent metabolism in the *Ph*TAC125 genome (Médigue et al., 2005) can be seen in this perspective.

1.2 Vertebrate hemoglobins

In humans and most vertebrates, Hb is the main O_2 carrier in the red blood cells. It is a tetrameric protein composed of identical heterodimers, containing an α- and β- chain (**Figure 1A**). Each one of these chains is able to bind one O_2 molecule. This capacity depends on the presence of a prosthetic group, called heme, and responsible for the characteristic red colour of blood. It can reversibily bind O_2 molecules and transport them to various areas of the body. The heme is a organic component and consists of a heterocycling ring, the protoporphyrin, with four pyrrole rings linked by methane bridges, and a central iron atom (Fe2+), the site of O_2 binding, that coordinates the four pyrrole-nitrogen atoms. Four methyl groups, two vinyl groups, and two propionate side chains are bound to the pyrrole rings.

Each protein chain arranges into a globin fold organised into a two layer structure known as a "three-over-three" α-helical sandwich, formed by eight α-helical segments (A-H) and an equal number of non-helical ones placed between them and at the ends of chain. Within the globin fold, the heme is enclosed by helices E, F, G

and H. In deoxy-Hb the central Fe^{2+} atom is pentacoordinated with the four pyrrole N-atoms of protoporphyrin IX and a fifth coordination bond to the side chain of the proximal His in position F8. HisF8 is an invariant residue among globin family members and, combined with other hydrophobic interactions, stabilise the heme group within each subunit. The binding of the O_2 molecule occurs on the distal side of the pentacoordinated heme, establishing a sixth coordination bond to Fe^{2+}. In most vertebrates the binding of the O_2 is stabilised by a hydrogen bond donated by distal HisE7, that together with PheCD1 and HisF8 represent the most conserved residues (**Figure 1B**). Liganded and unliganded Hb have different geometries of heme, planar and domed respectively, and HisF8 follows the movements of the metal in and out of the porphyrin ring.

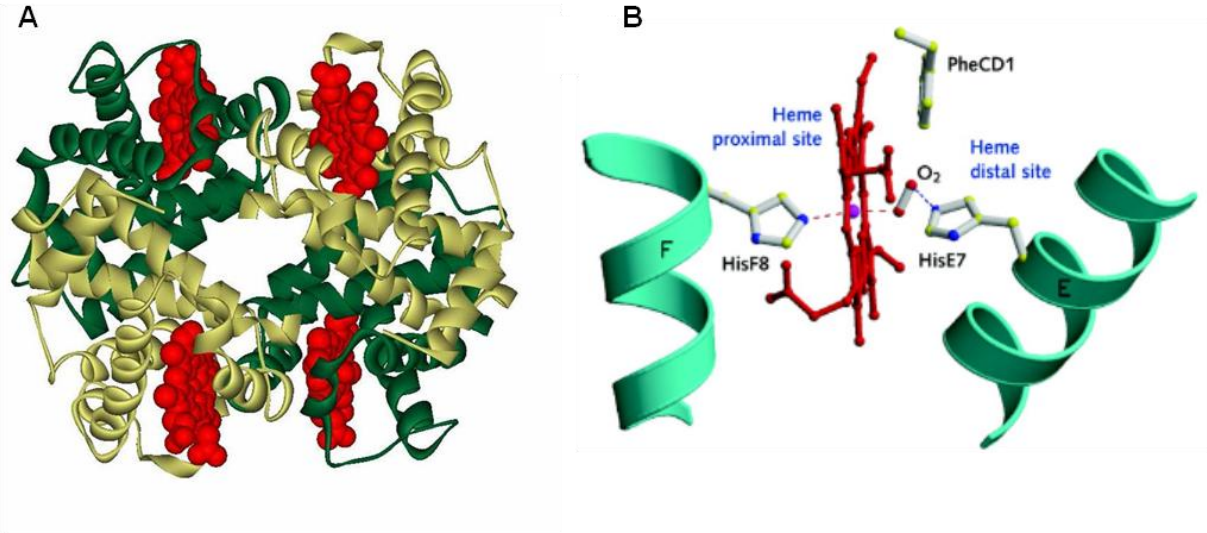

Figure 1. (A) Three-dimensional structure of human oxy-Hb (PDB entry: 2DN1 [Park et al., 2006]). The α subunits in dark green, β subunits in yellow and heme groups in red. **(B)** Heme environment. Taken from Pesce et al., 2002.

The pioneering crystallographic studies of Perutz led to the identification of two distinct Hb quaternary structures, one with low-affinity (called T, tense) and the other with high-affinity (called R, relaxed), associated with the deoxygenated and the oxygenated form of the protein, respectively (Perutz, 1970). On this basis, different theories for the allosteric conformational transitions were proposed. The main concept of the two-state allosteric model of Monod, Wyman and Changeux (MWC) was that Hb can exist only in the two quaternary states T and R (Monod et al., 1965). Although this allosteric transition is still accepted, more recent crystallographic analyses have clearly revealed in Hbs the presence of a larger repertoire of structural states (Schay et al., 2006).

Upon O_2 binding, there are substantial changes in quaternary structure that correspond to the T→R quaternary transition. It involves a rotation of the two $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers relative to each other, so that large conformational changes occur at the interface between the dimers. This rearrangement supplies a pathway for communication between subunits, permitting the cooperative binding of O_2.

As the heme binds or releases O_2 molecules, the overall Hb is subjected to conformational changes which alter their affinity for O_2. The O_2 affinity, expressed as

the gas partial pressure required to achieve half-saturation (p_{50}), is pO_2 dependent and is modulated by allosteric factors, such as protons, chloride ions, carbon dioxide (CO_2), organic phosphate. Organophosphates are different in several organisms, e.g. 2,3-biphosphoglycerate (2,3-BPG) or 2,3-diphosphoglycerate (2,3-DPG) in mammals and frogs, adenosine triphosphate (ATP), guanosine triphosphate (GTP) in teleost fish, and inositol pentaphosphate (IPP) in birds.

The cooperativity, expressed by the Hill coefficient n (nHill), and the variation of O_2 affinity make possible changes in the equilibrium between the R state of Hb, predominantly present in the lungs for optimal O_2 loading, and the T state, predominantly present in the hypoxic tissues for optimal O_2 delivery (Perutz, 1987; Bunn et al., 1986).

The O_2 affinity depends strongly on the pH, and this property is known as alkaline Bohr effect (Riggs, 1988), fundamental characteristic of the Hb that allows an efficient exchange O_2 and CO_2 at both pulmonary and tissue levels.

In many Hbs from teleost fish when the pH is lowered, the O_2 affinity is drastically reduced and the cooperativity is totally lost (indicated by nHill = 1). This feature is known as the Root effect (Brittain, 1987, 2005). In this case, at lower values of the physiological pH range, the Hbs are unable to saturate the ligand sites even at high O_2 pressure. The Root effect originates from an over-stabilisation of the T quaternary structure, dependent on protons (Perutz et al., 1987). The physiological role of Root effect has been linked to the presence of at least one of two anatomical structures that require high O_2 pressure, the *rete mirabile* which provides the gland that inflates the swimbladder with O_2, and the choroid *rete mirabile*, a vascular structure that supplies O_2 to the poorly vascularised retinal tissues (Wittenberg and Wittenberg, 1974).

The study of the molecular bases of the Root effect has been extensively handled by many years. Primary structures and analytical methods have provided useful indications on the physiology and evolution of this effect in fish, but no unequivocal answer to the question of the structural implications.

In recent years, X-ray crystallography succeeded in overtaking one of the classical views, which efforts to correlate all major changes in Hb function with few residue substitutions, thus significantly contributing to address the question in some instances. The extensive structural analysis of the Root effect by X-ray crystallography of Antarctic fish Hbs has been stimulated by the good capacity to crystallize, together with the high sequence identity exhibited by these cold-adapted Hbs. These structural properties by themselves, however, are not sufficient to explain the presence of the Root effect. The current hypothesis is based on overstabilization of the T state, mainly induced by the inter-Asp hydrogen bond at the α_1/β_2 interface (Mazzarella et al., 2006b), possibly modulated by salt bridges involving histidyl residues (Mazzarella et al., 2006a) (see below).

1.2.1 Hemoglobins in suborder Notothenioidei

Fish Hbs have been the subject of several studies because of the large spectrum of their structural and functional features, considered as evolutionary adaptations. The capacity of notothenioids to colonise extreme environments appears happened in parallel with suitable modulation of their Hb system at the molecular and functional level. To preserve biological activity, high-Antarctic families of suborder Notothenioidei, living in a stable, extremely cold, and well-oxygenated

marine environment, have evolved unique hematological features, important for fundamental, environmental and biotechnological studies.

These fishes differ from temperate and tropical species in having highly reduced erythrocyte number, Hb concentration and multiplicity in the blood (di Prisco et al., 2007; Verde et al., 2008), maybe as adaptation to compensate the increased blood viscosity at low temperature. At the extreme of such evolution, the species of the family Channichthyidae are characterised by loss of Hb (Ruud 1954).

The vast majority of high-Antarctic notothenioids, which are mostly bottom dwellers, show a single major Hb (Hb1) (di Prisco et al., 1998), accounting for 95-97% of the total, often accompanied by a functionally similar minor component Hb2 (Verde et al., 2007) and traces of another minor component HbC (di Prisco et al., 1991). The amino-acid sequences and the similar functional properties of these components in a given species, indicate that minor Hbs are vestigial (or may be larval) remains, without physiological importance at least in the adult state (di Prisco et al., 1998).

On the contrary, examining the Hb system of three notothenioids of the family Nototheniidae, *Trematomus newnesi*, *Pagothenia borchgrevinki* (two active criopelagic species) and *Pleuragramma antarcticum* (a pelagic, sluggish but migratory fish), the results show higher Hb multiplicity, probably correlated to the life style that does not resemble that of the sluggish benthic species.

This evidence has led to the hypothesis that in the Antarctic thermostable environment the need of globin multiplicity, commonly exhibit in the fish, may be reduced (Verde et al., 2006a, 2006b).

In comparison with high-Antarctic notothenioids, Hbs of many sub-Antarctic notothenioids, such as *Cottoperca gobio* and *Bovichtus diacanthus* (family Bovichtidae) and *E. maclovinus* (family Eleginopidae) display higher Hb multiplicity (di Prisco et al., 2007; Coppola et al., 2010), probably as a response to temperature differences and fluctuations of temperate waters, much larger than in the Antarctic.

The O_2 affinity of Hbs of many high-Antarctic species is quite low (di Prisco et al., 2007), as indicated by the values of p_{50}. This feature is probably linked to the high O_2 concentration in the cold sea and represents one of the most important aspects that makes Antarctic fish a good model to the study of HBOCs (see below). In contrast, the affinity is higher in Hbs of the sub-Antarctic notothenioids.

In fish, the decreased O_2 affinity and the loss of the cooperativity of Hb at low pH values is strongly dependent on the presence of at least one of the two structures that require high O_2 pressure, as discussed above.

Antarctic fish lack the swimbladder, and only the few species possessing Hbs without a Root effect, as well as those of the family Channichthyidae, are devoid of the choroid *rete* (Eastman, 1988). A general reduction of the Root effect, is noticed during the evolution of the Antarctic notothenioids (di Prisco et al., 2007), corresponding to a variable scenario pertaining to the choroid *rete*. Many high-Antarctic notothenioids have lost the choroid *rete*, although several retain portions of the *rete* and/or small vestigia of the choriocapillaris (Eastman 1988, 1993, 2006). **Figure 2** shows the presence of the choroid *rete* and Root effect in Nototenioidei. The comparison of two phylogenetically related groups of sub- and high-Antarctic species shows that the choroid *rete* is very well developed in sub-Antarctic notothenioids *E. maclovinus*, *P. urvillii*, *C. gobio* and *B. diacanthus*, which are the most basal of the suborder. The Root effect drops to low values in the Artedidraconidae lineage, as well as in one Bathydraconidae (*Gymnodraco*

acuticeps), but it is found at unexpectedly high levels in two species of the latter family (di Prisco et al., 2007) (**Figure 2**).

Because high-Antarctic notothenioids still have Hbs with Root effect also when the choroid *rete* is not present, this function may be subject to neutral selection. The possession of the Root effect may undergo neutral selection pressure in the simultaneous absence of retia mirabilia and presence of high Hb buffer capacity, as in some basal ray-finned fish and in the ancestors of teleosts (Berenbrink et al., 2005; Berenbrink, 2007).

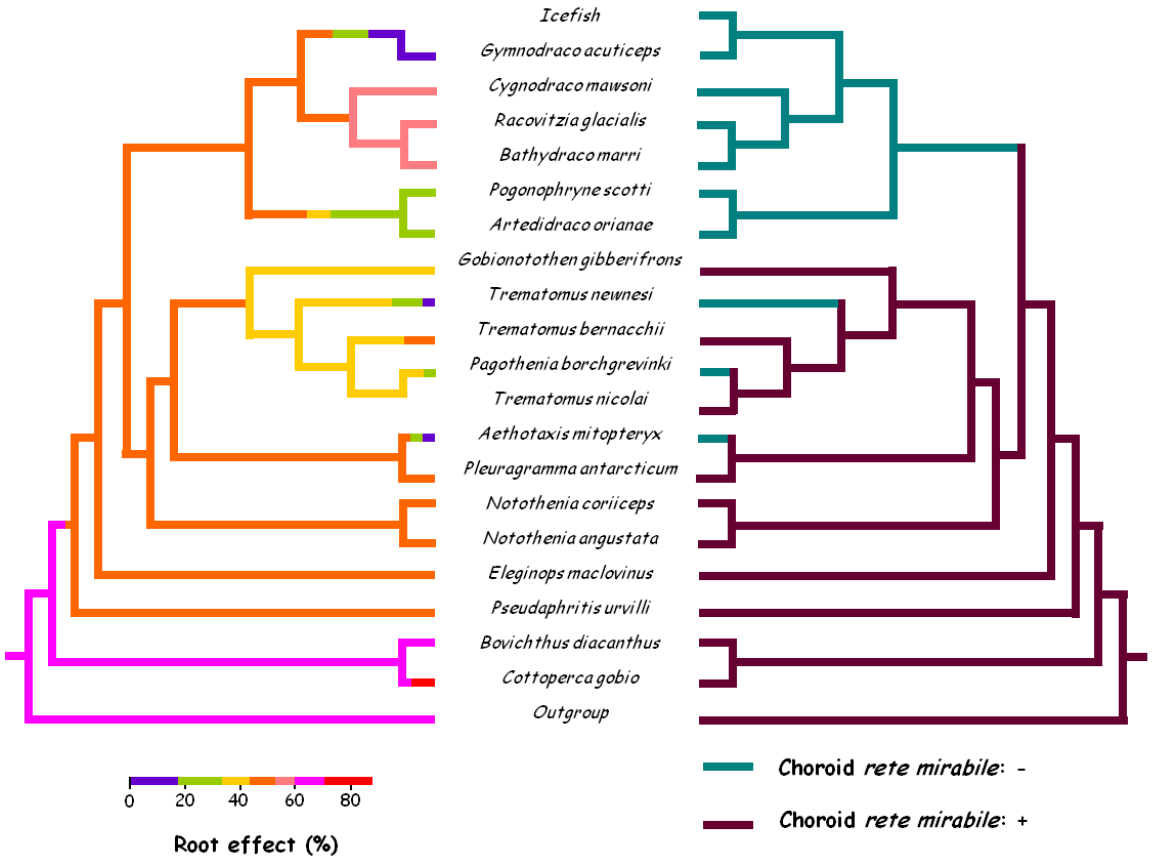

Figure 2. Reconstruction of the evolutionary history of the Root effect. Taken from Coppola et al., 2010.

A partial list of the available X-ray crystal structures of Antarctic fish Hbs is reported in a recent review (Verde et al., 2008). These structural studies strongly suggest that the key region responsible for pH modulation of O_2 affinity and cooperativity includes the aspartic triad at the $\alpha_1\beta_2$ interface (Mazzarella et al., 2006a, 2006b) and the CD corner of the α chain (Mazzarella et al., 2006a; Vergara et al., 2009). At the $\alpha_1\beta_2$ interface, two protons per tetramer are released upon oxygenation due to the breakage of an inter-Asp hydrogen bond between Asp95α and Asp101β (Ito et al., 1995; Mazzarella et al., 2006a, 2006b). This same structural evidence is also common to tuna fish (Yokoyama et al., 2004). At the CDα, the typical switch region in tetrameric Hbs (Baldwin and Chothia, 1979), an important

order-disorder transition takes place upon pH increase due to deprotonation of His55α.

In high-Antarctic Hbs at least three stable quaternary structures have been observed, the classical relaxed state (R) (Camardella et al., 1992; Mazzarella et al., 1999), the tense (T) (Mazzarella et al., 2006a, 2006b) and an R/T intermediate quaternary structure [typical at least of the ferric (Riccio et al., 2002; Vergara et al., 2007) and partially oxidised states (Merlino et al., 2009; Vitagliano et al., 2008)].

1.2.2 Hemoglobin-based blood substitutes

HBOCs are a novel therapeutic class of Hb derivatives administered intravenously, to deliver O_2 to ischemic tissues when the blood transfusion is not available. For such application, unmodified Hb has well known side effects, schematised in **Figure 3**. In fact, unmodified cell-free Hb, shows high tendency to dissociate into dimers, undergoing renal filtration and causing severe nephrotoxicity. Moreover, it extravasates through the endotelium, where it scavenges the vasoactive mediator nitrogen monoxide (NO) and causes a range of toxic effects that include vasoconstriction and blood pressure increase. On this basis, deep researches of possible modifications of the natural Hb tetramer were conducted to reduce toxicity.

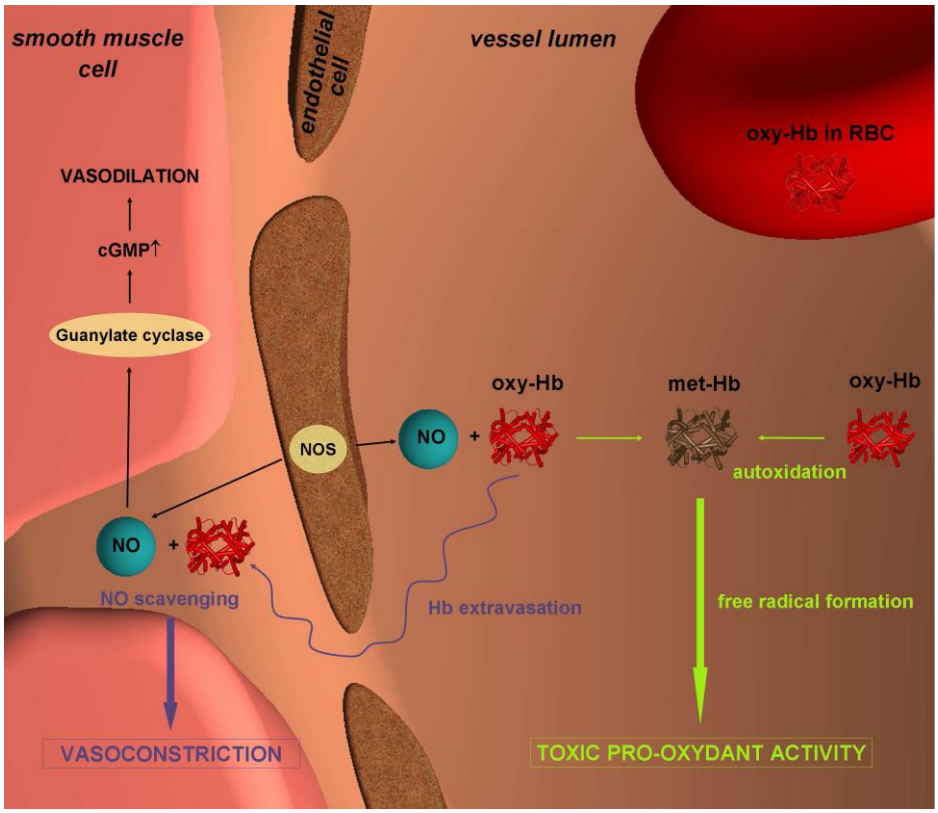

Figure 3. Possible mechanisms of toxicity of cell-free Hb and HBOCs. Taken from Bruno et al., 2010.

Until now, the strategies used to elude these effects mainly aim to increase the molecular size of the natural Hb tetramers to limit the size-dependent vessel extravasation and renal ultrafiltration. Beside some attempts at designing recombinant Hbs with higher molecular weight or lower dimer-tetramer dissociation constants (Bobofchak et al., 2003; Faggiano et al., 2011; Fronticelli et al., 2007; Fronticelli et al., 2009), most products proposed for clinical applications consist of Hb purified from whole blood and chemically modified to achieve intramolecular cross-linking or conjugation with polyethylenglycol (PEG).

Hb conjugation with PEG represents one the most promising strategies, since its hydration shell increases the hydrodynamic radius of the protein (Faggiano et al., 2010; Svergun et al., 2008). Furthermore, PEG itself is an inert polymer showing little or absent toxicity and immunogenicity (Acharya et al., 2007). Its structure can improve the resistance to proteolysis and to the action of antibodies (Veronese et al., 1996; Schiavon et al., 2000) and limit Hb uptake by macrophage CD163 receptor, a Hb scavenger (Schaer et al., 2006).

PEG-decorated human Hbs were the brightest HBOCs to have reached advanced clinical trials but phase III trials, carried out at dosages lower than those required in severe transfusion tests, suggested that some mechanisms of toxicity prevent their application as a replacement of red blood cells.

One of the limits of HBOCs is their higher O_2 affinity compared to Hb in red blood cells, limiting the adequate tissue oxygenation (Acharya et al., 2007; Portöro et al., 2008). Unmodified HbA free in the plasma cannot bind the intraerythrocytic allosteric effector 2,3-BPG, which increases the p_{50} of uncomplexed Hb from 10 Torr to around 26 Torr at 37°C and pH 7.4. Moreover, the concentration of the plasma cell-free Hb is usually low enough to significantly dissociate into dimers, which do not show cooperativity and have a p_{50} close to that of R state. PEGylation itself was shown to destabilise the Hb tetramer and shift the tetramer-dimer equilibrium towards the latter, with loss of cooperativity and a further increase in affinity (Caccia et al., 2009). In particular, the PEGylation of cysteine β93, conserved in 90% of vertebrates (Reischl et al., 2007), seems to be associated with the increased affinity in the obtained products, perturbations of heme pocket and of interface between dimers, autoxidation and heme loss, determining high tetramer dissociation. The higher p_{50} and cooperativity obtained by the PEGylation under anaerobic conditions is noteworthy with the lower accessibility of cysteine β93 in the T state.

These recent failures suffered by PEGylated products in clinical trials (Natanson et al., 2008), have pushed on deeper investigation of new possible strategies focused on the use of non-human Hbs, which differ in terms of PEGylation pattern, O_2-binding properties and sensitivity to allosteric effectors, taking advantage of the low immunogenicity of PEGylated proteins in general (Acharya et al., 2007).

Products consisting of bovine Hb decorated with 10-12 5,000 Da-MW PEG units and investigated as a possible blood substitute showed a p_{50} of 10.2 Torr at 37°C, (Winslow et al., 1998), higher than that of PEGylated Hb but still far from that of human blood (around 26 Torr). TetraPEGylated canine Hb (Acharya, et al., 2007) similarly shows a p_{50} of 10.0 Torr in the same conditions.

In this contest, the Hbs from Antarctic fish are particularly interesting, because of their peculiar features that make them potentially less sensitive to the undesirable effects of PEGylation. In particular, these Hbs show exceptionally low affinity for O_2, little or no dissociation of the tetramer into dimers, even in the ligated form (Giangiacomo et al., 2001), absence of cysteine β93 (in most notothenioids fish),

known to greatly perturb the properties of PEGylated Hbs. The remaining cysteine residues are all buried inside the protein matrix, suggesting that the reaction could be carried out regardless of the quaternary or ligation state.

1.3 Bacterial globins

The traditional view of the exclusive role of Hb as O_2 carrier in vertebrates is now overcome. The discovery of globin genes among prokaryotic and eukaryotic microorganisms, including bacteria, yeasts, algae, protozoa and fungi, suggests that the globin superfamily is exceptionally flexible in terms of biological roles and possible applications. The number of these ‘‘globin-like’’ proteins is currently increasing as different genomes from microorganisms are sequenced and annotated.

Non-vertebrate globins display high variability in primary and tertiary structures, which probably indicate their adaptations to specific functions in the respect to their vertebrate homologs.

Microbial globins can be classified, depending on their domains and structures, in three distinct classes, such as chimeric globins (including the two families of flavoHbs and sensor Hbs) and myoglobin (Mb)-like single-domain globins, which contain a Hb domain with a classical three-over-three (3/3) α-helical sandwich motif (**Figure 4A**), and the 2-on-2 (2/2) Hb, characterized by a novel two-over-two α-helical fold (**Figure 4B**).

Globins from unicellular organisms discovered until now, contain a proximal histidine at the F8 position that coordinates to the heme iron and a highly conserved tyrosine residue in B10 in the distal pocket. The distal histidine E7, important in stabilizing heme-bound O_2 in mammalian Hbs, may be replaced by a variety of different polar or non-polar residues (Egawa and Yeh, 2005).

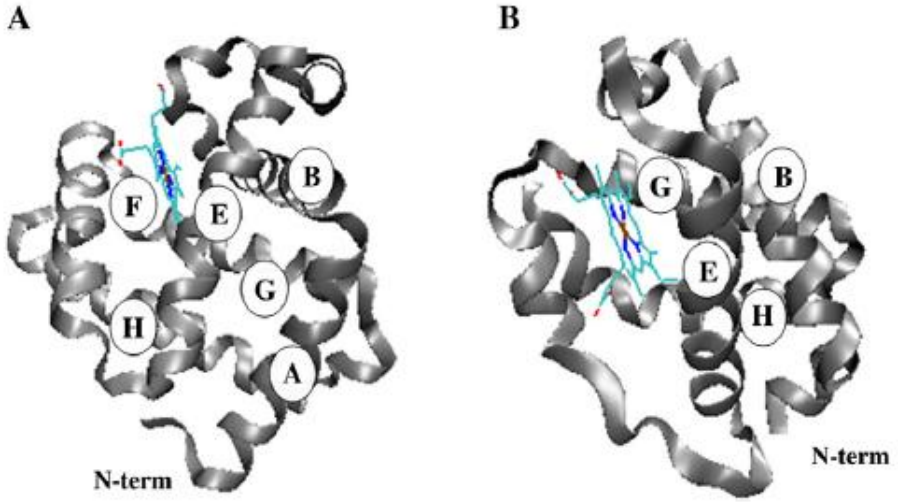

Figure 4. Comparison between the three-dimensional structure of a 3/3 Hb **(A)** (sperm whale Mb; PDB code: 1VXF) and a 2/2 Hb **(B)** (PDB code: 1UVY). Taken from Russo et al., 2010.

Examples of chimeric globins are represented by FlavoHbs, discovered in bacteria, yeasts and fungi, consisting of an N-terminal globin domain fused with C-terminal reductase domain, with binding sites for NAD(P)H and FAD.

The interest in flavoHbs is mostly relative to their role in the NO detoxification.

NO is a signalling and defence molecule of major importance in biological systems. It is involved in many physiological and pathological processes within the mammalian body, both beneficial and dangerous. The abundance of NO in cells, and the large number of NO-sensitive targets, indicate that organisms need to have defence mechanisms against this toxic compost. Several adaptations and reactions have been proposed to participate in protection against NO in bacteria (Fang, 1997; Nunoshiba et al., 1995) and flavoHb is involved in some of these (Mowat et al., 2010).

Several studies on purified native or recombinant flavoHbs from bacteria and yeast have revealed details of their structure, function, and reaction mechanism (Lewis et al., 2008). FlavoHb from *Escherichia coli* (Hmp) is the best characterised member of the family. The *E. coli* flavoHb gene (*hmp*) was the first one to be cloned and sequenced (Vasudevan et al., 1991). It is subject to complex control (reviewed by Poole, 2008), being upregulated in response to NO and nitrosating agents (Poole et al., 1996; Membrillo-Hernández et al., 1998, 1999). The deletion of *hmp* gene alone abolishes the NO-consuming activity (Liu et al., 2000) and is sufficient to render bacteria hypersensitive to NO and other nitrosative stresses (Membrillo-Hernández et al., 1999). It is thus generally believed that flavoHbs provide protection against NO and related reactive nitrogen species. Biochemical studies suggest that, under aerobic conditions, Hmp provides protection of cells against attack by NO and related reactive nitrogen species through an NO dioxygenase reaction (Gardner et al., 1998; Hausladen et al., 1998), converting NO to the relatively innocuous nitrate ion (Poole and Hughes, 2000; Gardner, 2005; Mowat et al., 2010) (**Figure 5A**). Anaerobically, Hmp exhibits lower NO-reductase activity, converting NO to nitrous oxide (Poole and Hughes, 2000; Vinogradov and Moens, 2008) (**Figure 5B**).

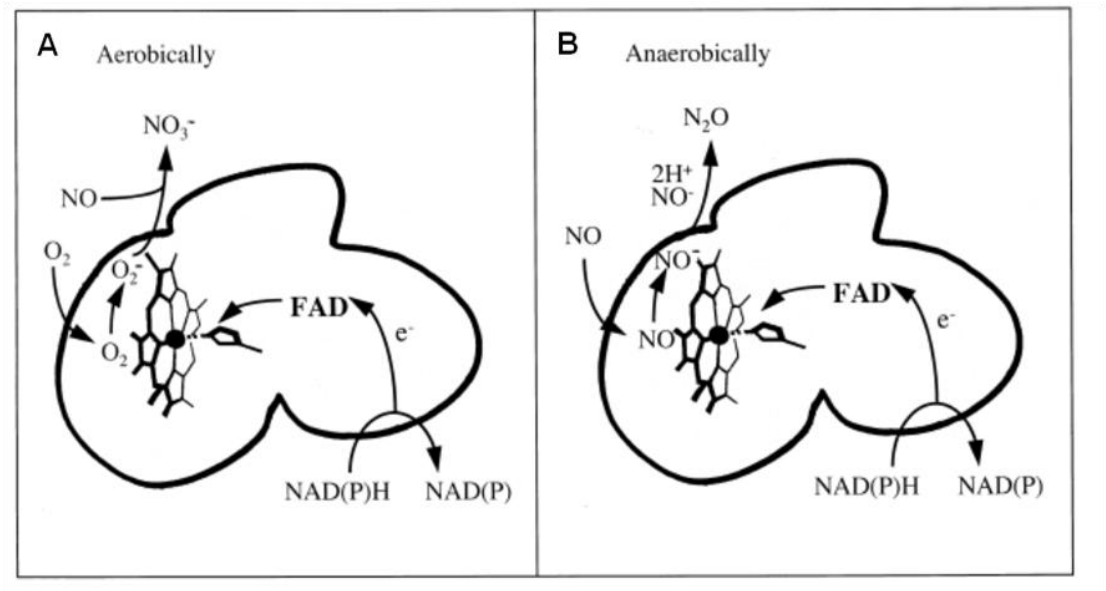

Figure 5. Electron transfer and ligand reduction reactions in Hmp, in aerobic **(A)** and anaerobic **(B)** conditions. Taken from Poole and Hughes, 2000.

Although Hmp is implied in NO detoxification, other physiological functions can be taken into account. In fact, FlavoHbs seem also be involved in the repair of the lipid-membrane-oxidative damage generated during oxidative/nitrosative stress (Bonamore and Boffi, 2008).

Among the single-domain globins, that from *Vitreoscilla stercoraria*, named Vgb, has been the first one to be identified and characterised. Vgb consists of 2 identical subunits and its physiological role is hypothesised to be similar to that of Mb as O_2 store to facilitate transport to the terminal respiratory oxidases.

In accord with this proposal, the expression of *vgb* gene is highly induced under microaerobic conditions (Dikshit et al., 1989; Khosla and Bailey, 1989) and, in a double terminal oxidase mutant in *E. coli*, restores the ability of the bacteria to respire and grow (Dikshit et al., 1992). Recently, it has been shown that when *vgb* gene is expressed in *E. coli* as a chimera with the flavoreductase (Frey et al., 2002; Kaur et al., 2002), it provides protection from nitrosative stress in cells, suggesting NO detoxification activity as an alternative role of this protein (Frey et al., 2002; Kaur et al., 2002). On the basis of these observations, two possible functions have been proposed. When Vgb is in dimeric form, it participates in O_2 transport; in monomeric form, it could associate with a reductase and protect cells from nitrosative stress.

The 2/2 Hbs family is the most recently discovered in the Hb superfamily and is found in eubacteria, cyanobacteria, protozoa and plants but also in pathogenic bacteria. These globins are shorter by 20-40 amino acid residues than the single-domain globins described above, altering the tertiary structure of the protein. It results in only 4 α-helices (B/E and G/H) arranged in antiparallel pairs connected by an extended polypeptide loop, that constitute a unusual 2-on-2 globin fold (Milani et al., 2001; Pesce et al., 2000) (**Figure 4B**). Moreover, the N-terminus of the A helix, that usually confers stability through contact with the EF corner, is almost completely absent and the majority of the heme-proximal F helix is replaced with a polypeptide segment, known as the pre-F loop. Differences in the residues of this loop, affect the orientation of the proximal HisF8, and probably contribute to differences in O_2-binding properties of these globins (Pathania et al., 2002).

On the basis of phylogenetic analysis, the 2/2 Hb class can be further divided into three distinct sub-groups: I (or N), II (or O) and III (or P) (Wittenberg et al., 2002; Vuletich and Lecomte, 2006) and specific structural features distinguish each of the three groups (Nardini et al., 2007). 2/2 Hbs belonging to the three groups may coexist in some bacteria, suggesting distinct functions, possibly related to the physiological response in the defence from ROS and reactive nitrogen species (RNS) (Wu et al., 2003). Examples of supposed functions, consistent with observed biophysical properties, include long-term ligand or substrate storage, NO detoxification, O_2/NO sensing, enzymatic function(s), and O_2 delivery under hypoxic conditions (Wittenberg et al., 2002). The high affinity for O_2 suggests that 2/2 Hbs function as O_2 scavengers rather than O_2 transporters (Wittenberg et al., 2002; Ouellet et al., 2003).

1.3.1 Globins of *Pseudoalteromonas haloplanktis* TAC125 and their involvement in oxidative and nitrosative stress.

In a rich-O_2 environment and cold stress, defence mechanisms involved in oxidative and nitrosative stress are important.

The genome of the Antarctic bacterium *Ph*TAC125 contains several O_2-scavenging enzymes, such as peroxiredoxins and peroxidases and one catalase-encoding gene (katB) with a possible homologue (*PSHAa1737*) (Médigue et al., 2005), indicating a good adaptation against ROS, under cold conditions. Moreover, several genes putatively involved in the metabolism of NO, such as NO reductase and nitrite reductase, have been found.

In this contest, the presence of multiple genes encoding 2/2 Hbs (annotated as *PSHAa0030*, *PSHAa0458*, *PSHAa2217*) and a flavoHb gene (*PSHAa2880*) (Médigue et al., 2005), that seem be involved in the protection of the cells from nitrosative and oxidative stress, is very interesting.

Phylogenetic analyses showed that two 2/2 Hb, encoded by the *PSHAa0030* and *PSHAa2217* genes belong to group II, and the third one encoded by *PSHAa0458* gene to group I (Giordano et al., 2007).

Until now, analyses about the flavoHb and the group II 2/2 Hb encoded by *PSHAa0030* (hereafter named *Ph*-2/2HbO) were carried out.

The recombinant production in *E. coli* of the cold flavoHb, protein widely recognised to be involved in the NO detoxifying (as explained above), was performed using several process designs, differing in bioreactor geometry, O_2 supply and the presence of a nitrosating compound to identify the best production condition (Parrilli et al., 2010d). In all production processes, the recombinant protein, accumulated in inclusion bodies, was solubilised in non-denaturing conditions.

The gene *PSHAa0030*, coding one of three 2/2 Hbs, has been cloned. The recombinant protein was overexpressed in *E. coli* and purified (Giordano et al., 2007) to be structurally and functionally investigated. A detailed characterisation of this protein was performed by Resonance Raman, electronic absorption and electronic paramagnetic resonance spectroscopies, kinetics measurements and computer simulation approaches. The results show that *Ph*-2/2HbO, characterised by the presence of a tryptophyl residue on the bottom of the heme distal pocket in position G8 and two tyrosyl residues (TyrCD1 and TyrB10), displays a hexacoordinated heme structure, both in the ferric and ferrous heme oxidation states, and high structural flexibility, probably linked to the peculiarity of the cold environment (Howes et al., 2011).

Hexacoordinated Hbs are generally observed in bacteria, unicellular eukaryotes, plants, invertebrates, in some tissues of higher vertebrates (Vinogradov and Moens, 2008), and also in the ferric state (β chains) of several tetrameric Hbs (Riccio et al., 2002; Vitagliano et al., 2004; Vergara et al., 2007, 2008; Vitagliano et al., 2008), but only few cases are reported in the literature for bacterial 2/2 Hbs. Examples are the 2/2 Hbs from *Mycobacterium leprae* (Visca et al., 2002), *Synechococcus* sp. PCC 7002 (Scott et al., 2002), *Synechocystis* sp. PCC 6803 (Falzone et al., 2002), and *Herbaspirillum seropedicae* (Razzera et al., 2008).

The presence of hexacoordinated Hbs in the superfamily is not uniform suggesting multiple possible functional roles, probably modulating ligand-binding or redox properties. In accord with the higher peroxidase activity in Antarctic fish Hbs, (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 O_2 concentration.

The physiological role of *Ph*-2/2HbO was investigated using a genomic approach, by the construction of a mutant strain in which the *PSHAa0030* gene was inactivated (Parrilli et al., 2010a). The inactivation of the *Ph*-2/2HbO gene made the

mutant bacterial strain sensitive to high O_2 levels, hydrogen peroxide, and nitrosating agents (Parrilli et al., 2010a), suggesting the involvement of the protein in the protection from oxidative and nitrosative stress. Moreover, it has been found that the transcription of the flavoHb-encoding gene occurs only in the mutant grown in microaerobiosis at 4°C, thus suggesting that the occurrence of the NO-induced stress is related to the absence of *Ph*-2/2HbO (Parrilli at al., 2010a).

1.3.2 Impact of the bacterial hemoglobins in biotechnology

Limited availability of O_2 is a diffused problem especially in the large-scale production processes, causing decreased growth, lowered product yield and increased formation of undesired fermentative products (Konz et al., 1998). These problems can be partially solved by improving process parameters and bioreactor configuration (Konz et al., 1998).

A first attempt to alleviate problems caused by O_2 limitations was taken by Khosla and Bailey (1988), when they expressed the active VHb in *E. coli*. The heterologous expression of VHb improved both the microaerobic growth of the bacterium in the bioreactor and the production of primary and secondary metabolites and recombinant proteins (Khosla and Bailey, 1988; Khosla et al., 1990). Successively, this protein was successfully expressed in several microorganisms of biotechnological significance (Magnolo et al., 1991; DeModena et al., 1993; Chen et al., 1994; Kallio and Bailey, 1996; Brünker et al., 1998; Minas et al., 1998) and in tobacco (Holmberg et al., 1997).

The heterologous expression of VHb seems to be also important to enhance antibiotic production and the results obtained using an industrial *Saccharopolyspora erythraea* strain producing erythromycin confirmed it (Bru¨ nker et al., 1998; Minas et al., 1998). Thus, this technology may generally be applied to improve the metabolism of industrially important antibiotic-producing strains.

This technology has also been applied in bioremediation processes, where the O_2 is required in several steps of the degradation pathway of some serious contaminants, such as the decomposition of benzoic acid by *Xanthomonas maltophilia* (Liu et al., 1996) or of dinitrotoluene by *Burkholderia* sp. (Patel et al., 2000).

The flavoHb from *Ralstonia eutropha* (FHP) has been shown to have a stronger effect on the increase of the growth and of the heterologous protein production than VHb, when expressed in *E. coli* in a microaerobic bioreactor (Frey et al., 2000). This enhancement was even greater in a strain engineered to express a fusion protein of VHb and the C-terminal reductase domain of FHP.

Therefore, the research of novel globin genes and their expression, in native, engineered, or mutated forms, seems a promising biotechnological strategy to improve the physiological properties of various hosts.

Moreover, the heterologous expression of globin genes might defend cells from nitrosative stress, for example by protecting several enzymes such as aconitase and cytochromes, and therefore favour the growth. In fact, the inactivation of cytochromes by NO would reduce O_2 consumption, resulting in a lower ATP generation and slow down of the growth.

In addition, the involvement of the microbial globins in the NO defence is of great interest for the clinical microbiology and pharmaceutical industries. In fact, pathogenic microorganisms make profit from this resistance mechanism during the

infection of the potential host. Because of the number of antibiotic-resistant pathogens is increasing, the globin genes could be novel drug targets for a therapy.

1.4 Objectives of the PhD project

The high- and sub-Antarctic notothenioid fish, belonging to species phylogenetically related but living in a large range of latitudes, represent an excellent opportunity to identify the biological and biochemical characters responsible for thermal adaptation. A thorough understanding of the structural and functional properties of their Hbs is of vital importance, as it will allow further investigations into their possible applications, such as the production of new potential HBOCs.

On this base, in the second chapter of my PhD thesis, the results obtained on the structural and functional characterisation of the O_2-transport system of sub-Antarctic notothenioids are described and compared with respect to high-Antarctic species, to provide a deeper understanding of the processes involved in cold adaptation. For this aim two different sub-Antarctic and one high-Antarctic species have been selected:

- *E. maclovinus* (family Eleginopidae), the sister group of high-Antarctic notothenioids, is crucial to understand notothenioid diversification representing the "starting point" for the notothenioid radiation.
- *D. eleginoides* (family Nototheniidae), found in sub-Antarctic waters, is interesting since it belongs to the most diversified family of the suborder Notothenioidei, in which most of the members have an Antarctic distribution.
- *Trematomus bernacchii* (family Nototheniidae), commonly found in the Antarctic continent, represents a good cold-Hb model, since its structure has been extensively studied and presents only one major Hb (accounting for over 95% of the total blood content), easy to purify and to obtain in large quantity.

In response to the adaptation to the extreme temperatures, Antarctic fish Hbs show unique structural and functional properties that make them potentially less sensitive to the undesirable effects of the PEGylation, one of the most promising strategies used in the production of possible HBOCs. Therefore, in collaboration with Prof. Mozzarelli and Dr. Bruno (University of Parma, Italy), further objective of the PhD project, treated in the third chapter, has been the study of the PEGylated Antarctic Hbs, in order to obtain a product showing similar O_2-binding properties to whole blood. For this aim *T.bernacchii* Hb has been selected.

In the Antarctic bacterium *Ph*TAC125, considered one of the best model organisms for structural and functional genome analyses, several genes encoding 2/2 Hbs and a flavoHb (Médigue et al., 2005), have been identified. While the flavoHb is widely recognised to be involved in the NO detoxification, the role of the 2/2 Hbs is still not clear. In collaboration with Prof. Poole (University of Sheffield, UK), the last objective of my PhD thesis, reported in fourth chapter, has been the identification of the physiological function of *Ph*-2/2HbO, by *in vivo* and *in vitro* experiments.

CHAPTER II

STRUCTURAL AND FUNCTIONAL CHARACTERISATION OF HIGH- AND SUB-ANTARCTIC NOTOTHENIOID HEMOGLOBINS

2.1 Introduction

Diversification of the major group of Antarctic fishes of the perciform suborder Notothenioidei, largely confined within Antarctic and sub-Antarctic waters, has occurred in parallel with the climatic changes. Fishes of this suborder provide an excellent opportunity for studying the functional importance of evolutionary adaptations to temperature. To understand the unique biochemical features of Hbs of high-Antarctic notothenioids, particularly interesting also for their potential biotechnology applications, it is important to acquire information on their sub-Antarctic relatives and their Hbs.

In my PhD thesis, the O_2-transport system of two sub-Antarctic notothenioids *E. maclovinus* and *D. eleginoides* was investigated and compared with respect to high-Antarctic species, and in particular to the Hb of *T. bernacchii*.

Sub-Antarctic species are ideal subjects for studying the Hb systems that did not undergo the historical exposure to the Antarctic climate. *E. maclovinus* and *D. eleginoides* are endemics to the temperate and sub-Antarctic waters of southern South America and distribute around the Falkland Islands (Falkland Islands Government, 2003; Brickle et al., 2005). *D. eleginoides* has also been found in waters of Southwest Pacific (Macquarie Island) and Southern Ocean (South Georgia).

In this chapter, the primary structure and the O_2-binding properties of the Hbs from these sub-Antarctic species are described.

Moreover, in collaboration with different groups, a detailed functional and structural characterisation of the major component Hb1 of *E. maclovinus* was obtained by ligand binding kinetics, Resonance Raman spectroscopy, and X-ray crystallography.

2.2 Results and discussion

2.2.1. Purification of Hbs and separation of globins. Purification of *E. maclovinus* and *D. eleginoides* Hbs was achieved by ion-exchange chromatography. Unlike most Antarctic notothenioids, the blood of *E. maclovinus* displays high Hb multiplicity, with the presence of one cathodal (HbC) and two anodal (Hb1 and Hb2) components (data not shown). Hb1 and Hb2 account for approximately 65-70% and 5% of the total, respectively; HbC, usually found in Antarctic notothenioid species in trace amounts (less than 1%), is 20-25% of the total, similar to the high-Antarctic notothenioid *T. newnesi* (D'Avino et al., 1994). On the contrary, the blood of *D. eleginoides* shows a major (Hb1) and a minor (Hb2) component (accounting for 95% and 5%, respectively) (data not shown), similar to many high-Antarctic notothenioids.

Hbs are highly sensitive to the environment, therefore, their structural and functional properties in part reflect the thermal conditions encountered by species during their evolutionary history. Fish commonly exhibit high Hb multiplicity with marked differences in the O_2-binding properties and in their sensitivity to allosteric

effectors (Weber, 1990; di Prisco and Tamburrini, 1992; Feuerlein and Weber, 1994; Weber et al., 2000; Fago et al., 2002). Hb multiplicity is often interpreted as a molecular adaptation resulting from gene-related heterogeneity and gene duplication events, probably in response to differences and fluctuations in the physicochemical features (essentially temperature and O_2 availability). Whereas high-Antarctic notothenioids lost globin variability and concentration/multiplicity, unnecessary in a thermostable environment, and the erythrocyte number was reduced to counterbalance the increase in blood viscosity produced by subzero seawater temperature (Wells et al., 1980), most temperate and sub-Antarctic notothenioid fishes display higher Hb multiplicity.

Similar to the sub-Antarctic notothenioids *C. gobio* and *B. diacanthus* (family Bovichtidae) (Giordano et al., 2006, 2009; Coppola et al., 2010), the three Hbs of *E. maclovinus* may have been maintained to cope with the temperature changes at the north of the Polar Front (di Prisco et al., 2007). Some aspects of life style of *E. maclovinus* differentiate it from the stenohaline and sedentary Antarctic notothenioids. Examples are the rapidity of its escape response similar to eurythermal temperate non-notothenioids (Fernández et al., 2002), its rate of O_2 consumption that place it in an active category (Vanella and Calvo, 2005), and the highest fecundity (Brickle et al., 2005). It is one of only two euryhaline notothenioid species (Eastman and Lannoo, 2008).

As occurs in the sub-Antarctic *P. urvillii* (Verde et al., 2004), the O_2-transport system of *D. eleginoides* has features very similar to those of most Antarctic notothenioids. The absence of Hb multiplicity can be considered a synapomorphy, connecting this species to the other notothenioids, above all remembering that *D. eleginoides* belongs to a family in which the most of the members have an Antarctic distribution.

2.2.2. Separation of globins and primary structure. The globins of the hemolysate of *E. maclovinus* and *D. eleginoides* were separated by reverse-phase High-Performance Liquid Chromatography (HPLC) (data not shown). In *E. maclovinus*, the elution profile indicates four globins, two α-chains (α^1 and α^2) and two β chains (β^1 and β^2) (data not shown), as established by amino-acid sequencing and mass spectrometry. The chains composition of the major component Hb1 is $(\alpha^1\beta^1)_2$. HbC and Hb2 have the α chain and β chain, respectively, in common with Hb1. Differently, the hemolysate of *D. eleginoides* contains three globins (data not shown), one α chain (α^1) and two β chains (β^1 and β^2). The chain compositions of Hb1 and Hb2 are $(\alpha^1\beta^1)_2$ and $(\alpha^1\beta^2)_2$, respectively.

The complete amino acid sequences of the α and β chains (142 and 146 residues, respectively) of *E. maclovinus* Hb1 and HbC and of *D. eleginoides* Hb1 and Hb2 are reported in **Figure 2.1**.

Although sub-Antarctic notothenioids have never developed cold adaptation, the amino-acid sequence reveals high identity with the globins of Antarctic notothenioids, indicating a common phylogenetic origin within notothenioids and suggesting that the primary structure of the cold Hbs have been subjected to modifications only to a limited extent. The *E. maclovinus* and *D. eleginoides* Hbs show the classical two Root-effect motifs (the Asp48α/His55α and His69β/Asp72β pairs) found in *T. bernacchii* Hb and the terminal His146β whose role in the Root effect is still disputed (Mazzarella et al., 2006a).

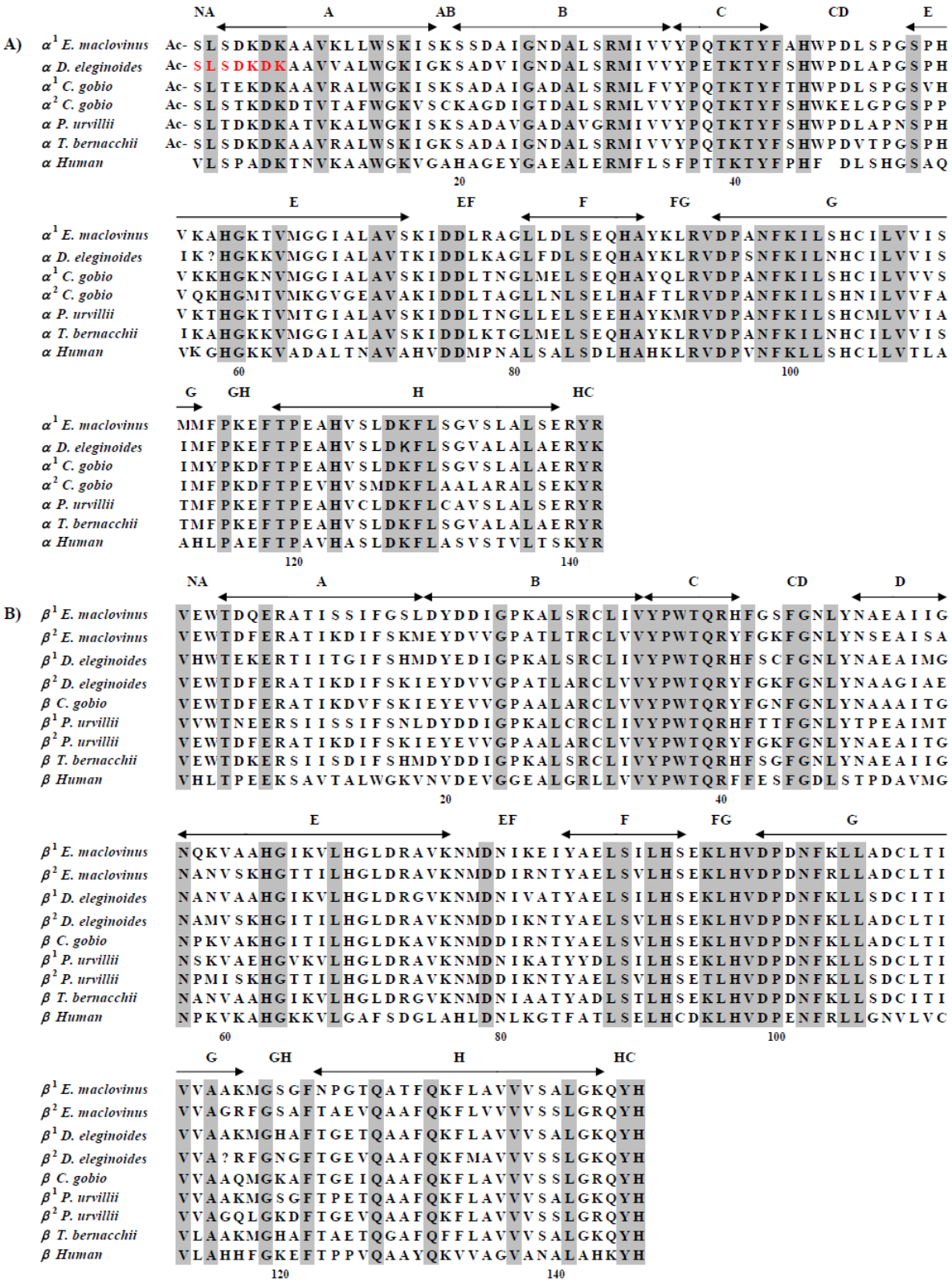

Figure 2.1. Sequence alignment of the α- **(A)** and β- **(B)** chains of *E. maclovinus* and *D. eleginoides* Hbs with *C. gobio*, *P. urvillii*, *T. bernacchii* and human α- and β-chains. Identical residues are in gray. In red the residues identified by homology.

These important residues, pH dependent, are the major candidates for proton uptake upon deoxygenation (Root protons).

As most of notothenioid α globins, the α-chains of *E. maclovinus* HbC and Hb1 and *D. eleginoides* Hb1 and Hb2 show some non-conservative substitutions, e.g. Gln replaces Leu in F7; moreover, in the α^1 chain of *E. maclovinus* Thr replaces Lys in E10, similar to most of sub-Antarctic species, while it is conserved in *D. eleginoides*, as well as in *T. bernacchii*. The primary structure of *E. maclovinus* and *D. eleginoides* Hbs also contains two replacements in the side chains that form the $\alpha^1\beta^2$ "dovetailed" switch region (ProαCD2, ThrαC3, ThrαC6, HisβFG4), important for cooperativity, quaternary transition T→R, and generally highly conserved in vertebrate Hbs. ProαCD2 is replaced by Ala and Ser, and ThrαC3 is replaced by Gln and Glu, in *E. maclovinus* and *D. eleginoides*, respectively; in both species ThrαC6 and HisβFG4 are conserved.

As in the sub-Antarctic *C. gobio*, in the β chains of *E. maclovinus* Hbs and in β^2 of *D. eleginoides*, Met D6, invariant residue in vertebrates (including most teleosts), is not conserved; it is replaced by Ile and Ser in *E. maclovinus* Hb1 and HbC, respectively and by Ala in *D. eleginoides* Hb2. On the contrary this residue is conserved in the major component Hb1 of *D. eleginoides*, as well as in *P. urvillii* Hb1 (Verde et al., 2004). Similar to all notothenioids and Arctic Gadidae (Verde et al., 2002, 2006a), the β chains contain two cysteyl residues occurring at B13 and G11, usually absent in other teleost.

2.2.3. Oxygen-binding properties. A decrease in the O_2 affinity occurs along the lineage of high-Antarctic notothenioids (di Prisco et al., 2007), probably correlated to the high concentration of O_2 in the cold-Antarctic waters. In contrast, the affinity in Hbs of the sub-Antarctic notothenioids is higher.

The O_2-binding experiments of the major Hb1 components of *E. maclovinus* and *D. eleginoides* were performed at 5°C, in the absence and presence of allosteric effectors, such as chloride ions (NaCl) and organophosphates (ATP), and compared with those of *T. bernacchii* Hb, carried out in the same conditions.

In **Figure 2.2**, the results obtained with *E. maclovinus* Hb1 **(A)**, *D. eleginoides* Hb1 **(B)** and *T. bernacchii* Hb **(C)**, are illustrated in terms of variation of O_2 affinity (log p_{50}) as a function of pH. As expected, the major Hbs of *E. maclovinus* and *D. eleginoides* show higher O_2 affinity and Bohr effect than the high-Antarctic notothenioid Hbs (di Prisco et al., 2007). The values of O_2 affinity, in the two former species are similar to Hbs of sub-Antarctic notothenioids *C. gobio* (Giordano et al., 2009), *B. diacanthus* (Coppola et al., 2010) and *P. urvillii* (Verde et al., 2004). In fact, the p_{50} values, at pH 7.46 and in presence of 3 mM ATP, for Hb1 of *E. maclovinus* and *D. eleginoides* and for *T. bernacchii* Hb are 29.9, 23.9 and 46.26 mmHg (**Figures 2.2A, B and C**), respectively. The difference between these values is remarkable, considering that *T. bernacchii* Hb shows higher O_2 affinity compared to the other high-Antarctic notothenioids (di Prisco et al., 2007). These Hbs show a strong Bohr effect, under all experimental conditions (**Figures 2.2A, B and C**), strongly enhanced by the physiological ligand ATP.

The cooperativity of O_2 binding is maximal at alkaline pH, in all Hbs investigated. In *E. maclovinus* Hb1 and *D. eleginoides* Hb1 it is significantly higher than that observed in *T. bernacchii* Hb (e.g., at pH 8.49 and in presence of ATP, the values of nHill are 2.5, 2.43 and 1.7, respectively) (**Figures 2.2D, E and F**).

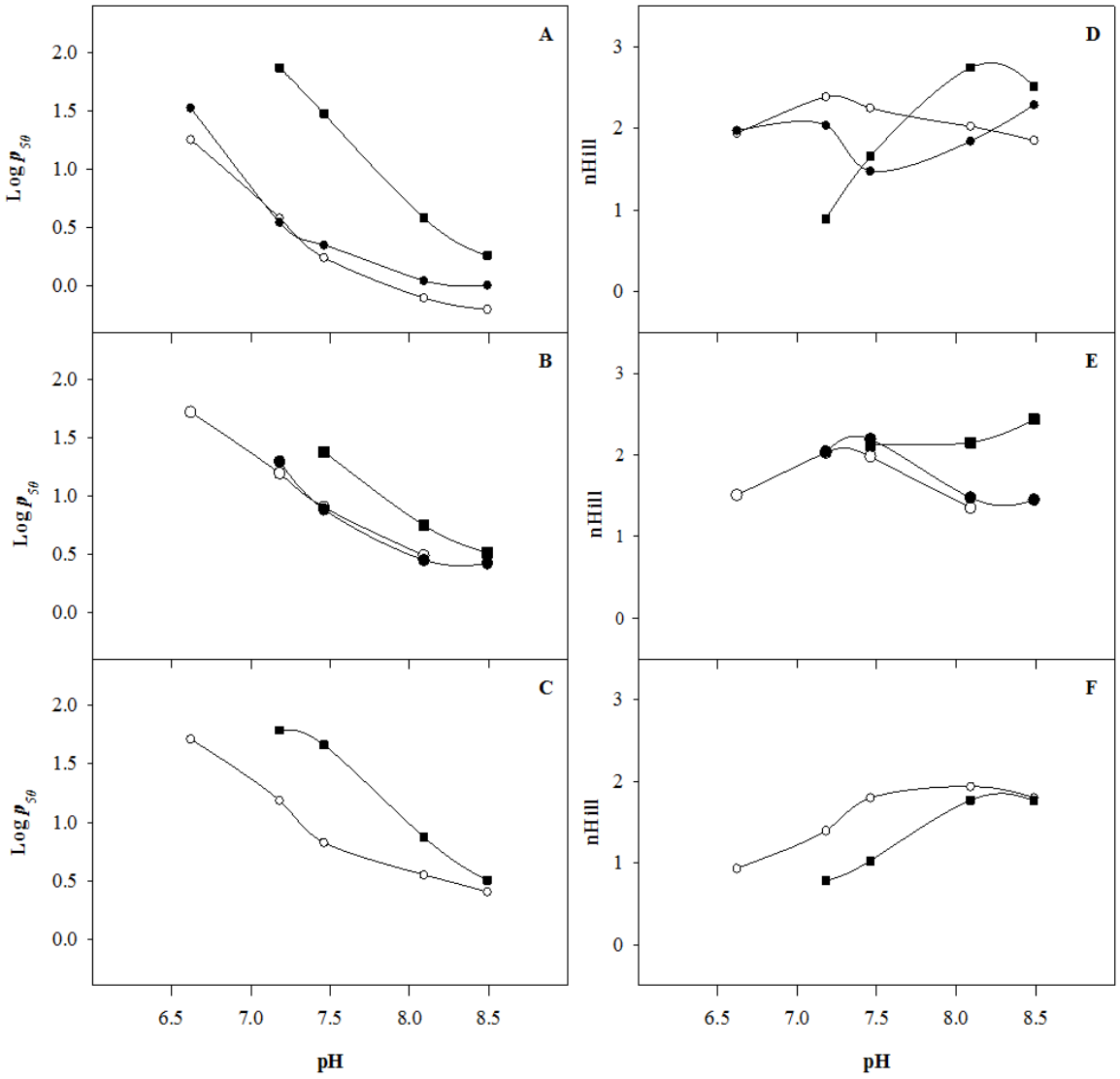

Figure 2.2. O_2-equilibrium isotherms (Bohr effect) and subunit cooperativity (nHill) as a function of pH of *E. maclovinus* Hb1 **(A, D)**, *D. eleginoides* Hb1 **(B, E)** and *T. bernacchii* Hb **(C, F)**. 100 mM HEPES, absence of effectors (empty circles); 100 mM NaCl (filled circles); 100 mM NaCl, 3 mM ATP (filled squares). The experiments were performed at 5°C.

Figures 2.3A and B show the O_2 saturation at atmospheric pressure (Root effect) as a function of pH of *E. maclovinus* Hb1 and *D. eleginoides* Hb1. All Hbs exhibit a marked Root effect, in keeping with a choroid *rete* very well developed in sub-Antarctic notothenioids. The percentage of saturation decreases at low pH and the O_2 release induced by protons is enhanced by ATP. At pH 6.25 the magnitude of the Root effect, expressed as percentage of the decrease in O_2 saturation, in Hb1 *E. maclovinus* is about 60% in the presence of ATP while about 35% in stripped conditions (**Figure 2.3A**). In *D. eleginoides* Hb1 it is about 60-70% in absence and in presence of ATP, respectively (**Figure 2.3B**).

In the case of *E. maclovinus* Hb1 also detailed O_2-binding isotherms were carried out at 10°C and pH 7.0, in the absence and in the presence of ATP (**Figure 2.4**). It is immediately evident that the addition of ATP causes a substantial loss of cooperativity, such that the Hb is still partially saturated even at atmospheric-O_2 pressure, as predicted by the observed Root effect (**Figure 2.3A**). The parameters employed for the non-linear least-squares fitting of experimental data are reported in **Table 2.1**. These results show clearly that the allosteric constant L_0 is the parameter that mainly undergoes the effect of ATP, decreasing drastically and implying an ATP-driven destabilization of the R state in the unliganded Hb.

Moreover, the ATP does not behave as a simple allosteric effector, since its addition is also accompanied by a 7-8 fold decrease of the O_2-equilibrium constant for the T state (K_T); on the contrary, no ATP-induced effect is observed for the O_2 affinity of the R structure (K_R).

	K_T (M^{-1})	K_R (M^{-1})	$L_0 = [R_0]/[T_0]$
no ATP	$5.4(\pm0.7)\times10^{4}$	$7.6(\pm0.9)\times10^{5}$	$8.6(\pm1.3)\times10^{-4}$
with ATP	$8.7(\pm1.1)\times10^{3}$	$7.6(\pm0.9)\times10^{5}$	$1.0(\pm0.3)\times10^{-8}$

Table 2.1. Parameters employed for the non-linear least-squares fitting of O_2-binding isotherms.

2.2.4. Kinetics of oxygen dissociation. The kinetics of O_2 dissociation were obtained by stopped flow in collaboration with Prof. Coletta (University of Rome).

The temperature dependence of the O_2-dissociation kinetics by CO displacement was carried out in order to obtain the rate constant from the fully liganded species. These observations were limited to the protein in the absence of ATP since only in this case the observed rate constant(s) (k_r, the dissociation rate constant of the R state) correspond(s) to the fully oxygenated form.

Experiments performed at 10°C, in the absence of ATP, indicate the presence of a single exponential both for *E. maclovinus* and *T. bernacchii*, excluding the possibility of chain heterogeneity for the R state; the value of k_r for *E. maclovinus* Hb1 ($9.7(\pm1.2)\ s^{-1}$) is similar to that observed for Hb from *T. bernacchii* ($10.7(\pm2.3)\ s^{-1}$) and, on the basis of the known K_R ($7.6(\pm0.9)\times10^{5}\ M^{-1}$) (**Table 2.1**), allows to predict the O_2 binding rate constant for Hb1 from *E. maclovinus* k'_r ($7.4(\pm0.9)\times10^{6}\ M^{-1}s^{-1}$).

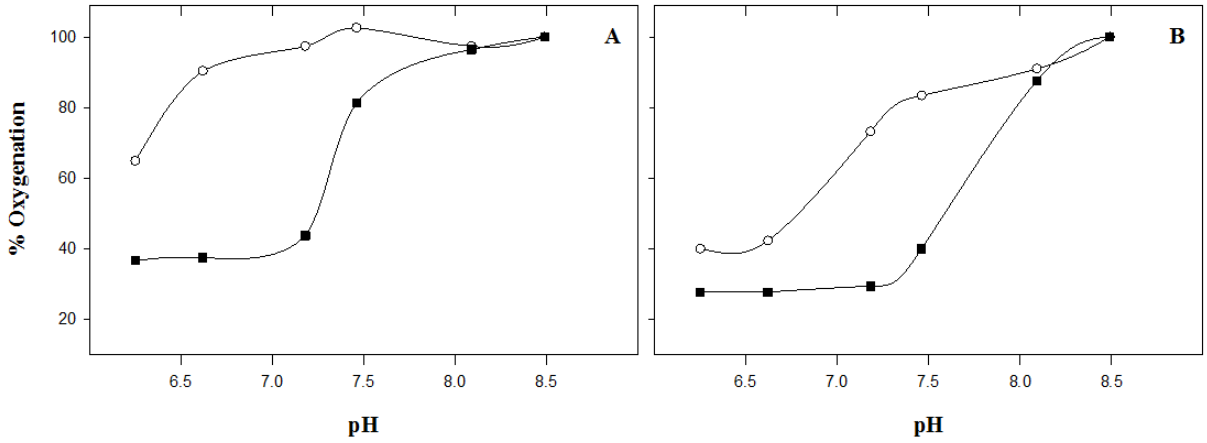

Figure 2.3. O_2-saturation at atmospheric pressure (Root effect) as a function of pH of *E. maclovinus* Hb1 **(A)** and *D. eleginoides* Hb1 **(B)**. 100 mM HEPES, absence of effectors (empty circles); 3 mM ATP (filled squares). The experiments were performed at room temperature.

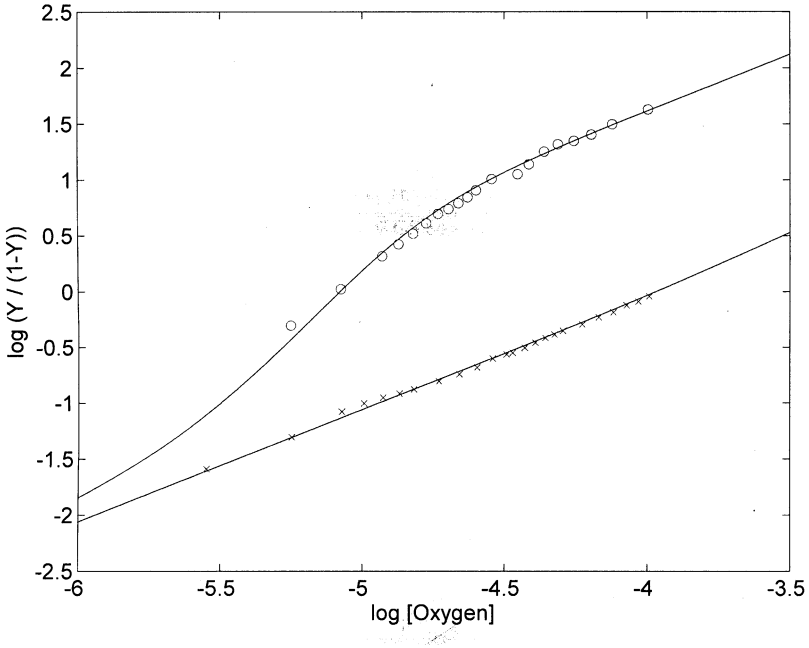

Figure 2.4. Detailed O_2-binding isotherms of *E. maclovinus* Hb1, at pH 7.0 and 10°C in 100 mM HEPES, absence of effectors (o) and in presence of 3 mM ATP (x). Continuous lines represent non-linear least-squares fitting of experimental data.

The resulting activation enthalpy ($\Delta H^{\ddagger}$) for O_2 dissociation from fully oxygenated *E. maclovinus* Hb1 (+46.4(±6.3) kJ/mol), is a value dramatically lower than that obtained from *T. bernacchii* (+80.8(±9.7) kJ/mol), suggesting a markedly different structural change associated to the Fe-O_2 rupture and the exit of the ligand from the protein moiety. Furthermore, the fairly high value of the activation free energy for both Hbs ($\Delta G^{\ddagger}$ = +64.8(±7.2) kJ/mol for Hb1 from *E. maclovinus* and $\Delta G^{\ddagger}$ = +64.6(±7.1) kJ/mol for Hb from *T. bernacchii*) indicates that while for *E. maclovinus* Hb1 the O_2 dissociation is associated to a negative activation entropy ($T\Delta S^{\ddagger}$ = -18.4(±3.1) kJ/mol), in the case of Hb from *T. bernacchii* the activation entropy is positive ($T\Delta S^{\ddagger}$ = +16.1(±2.1) kJ/mol).

All these data, together with kinetics of full O_2 dissociation by sodium dithionite, allowed the identification of the O_2-dissociation rate constant of the T-state (k_t). In absence of ATP, the value k_t of *E. maclovinus* Hb1 is 80(±10) s^{-1}. This value, by virtue of the knowledge of K_T (5.4(±0.7)×10^4 M^{-1}) (**Table 2.1**), allows the determination of the kinetic O_2 binding constant for the T state (k'_t = 4.3(±0.6)×10^6 $M^{-1}s^{-1}$).

Interestingly, the full O_2 dissociation kinetics in the presence of ATP can be satisfactorily described by employing the same k_r (9.7(±1.2) s^{-1}) used for the O_2 dissociation kinetics in the absence of ATP. The resulting value of k_t (31(±4) s^{-1}) is slower compared to that in the absence of ATP, suggesting that the major effect of ATP on the reduction of O_2 affinity to the T-state is exerted through an almost 20-fold decrease of the O_2 binding rate constant k'_t (2.7(±0.4)×10^5 $M^{-1}s^{-1}$).

2.2.5. CO-rebinding kinetics. The rebinding of ligands to heme proteins in solution, following nanosecond-laser photolysis, reflects the ligand migration inside the protein matrix and the reactivity towards freely diffusing molecules. It is essential to understand the molecular mechanisms underlying protein-ligand interactions. Although the O_2 is the physiological ligand of Hb, the carbon monoxide (CO) is often preferred to avoid the irreversible oxidation and side reactions in *in vitro* experiments.

CO-rebinding kinetics of *E. maclovinus* Hb1 and *T. bernacchii* Hb were carried out in collaboration with Prof. Viappiani, during my training period at the University of Parma.

Laser photolysis of the CO complex of these Hbs in solution results in multiphasic-rebinding kinetics, very similar to each other.

Figure 2.5 shows CO-rebinding kinetics, at 10°C and pH 7.0 for solutions of *E. maclovinus* Hb1 equilibrated with 1 atm and 0.1 atm CO. A simple visual inspection of these kinetics allows a qualitative distinction between a nano-to-microsecond first order phase, and a second order phase occurring on longer time scales. Analysis of the kinetics with multiple exponential relaxations reveals a clear CO-concentration dependence for the time constants of the two longer lived transients. On the other hand, the lifetimes of the first three kinetic phases show little CO-concentration dependence and thus appear to be related to unimolecular reactions. These may arise either from geminate rebinding or from structural relaxations affecting the shape of the absorbance spectrum (Hofrichter et al., 1983; Jones et al., 1992).

CO-rebinding kinetics of *T. bernacchii* Hb, performed at 10°C and pH 7.0 and equilibrated with 1 atm CO (**Figure 2.6**), show several kinetic phases, which closely resemble those observed for *E. maclovinus* Hb1.

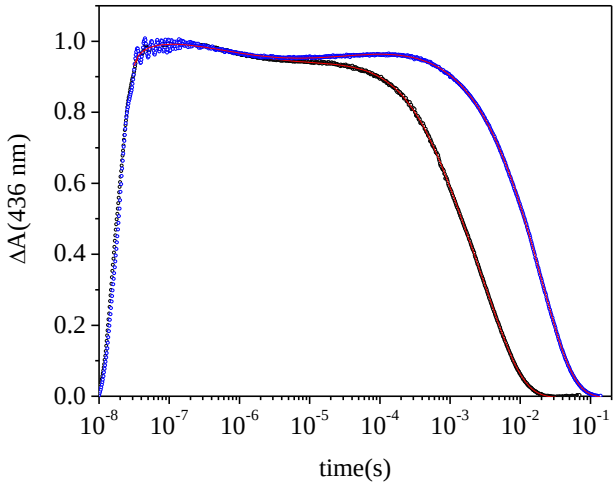

Figure 2.5. Transient absorbance after nanosecond photolysis of *E. maclovinus* Hb1-CO solutions equilibrated at pH 7.0, T = 10°C, with 1 atm CO (black) and 0.1 atm CO (blue). Red curves were the result of a fit with a sum of three stretched exponential and two exponential relaxations.

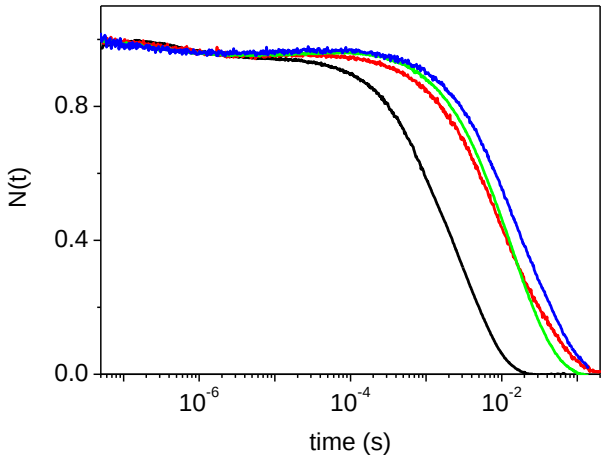

Figure 2.6. Normalised absorbance changes at 436 nm following photolysis at 532 nm of *E. maclovinus* Hb1-CO in the absence (black line) and presence (red line) of 3 mM ATP and of *T. bernacchii* Hb-CO, in the absence (green line) and presence (blue line) of 3 mM ATP. Experiments were conducted on solutions equilibrated with 1 atm CO, at T = 10°C and pH 7.0.

In addition, CO rebinding to *E. maclovinus* Hb1 and *T. bernacchii* Hb were carried out in presence and in absence of 3 mM ATP (**Figure 2.6**), showing a second order phase affected by allosteric effectors with the enhance of the formation of the slow rebinding species, although with a much smaller extent in *T. bernacchii*.

In both Hbs a peculiar finding is the presence of two kinetic phases with a negative pre-exponential factor, which cannot be explained in terms of geminate rebinding and probably arises from structural relaxations. This characteristic is not unexpected; in the human HbA, tertiary and quaternary relaxations are well know to occur following photolysis, leading to very similar spectral changes (Henry et al., 1997).

Because of the complexity of the observed rebinding kinetics, time resolved absorbance spectra were measured for *E. maclovinus* Hb1 and *T. bernacchii* Hb, to identify the contributions arising from rebinding of the photo-dissociated ligands, and separate them from those generated by conformational changes. The time resolution in these experiments does not allow resolution of the fastest (lifetime $\tau_1 \approx$ 5-10 ns) transient state.

In **Figure 2.7** the amplitudes of the spectral components obtained from the rebinding kinetics of *E. maclovinus* Hb1, measured at pH 6.0, pH 7.0 and pH 8.0, are compared. The first spectral component matches the carboxy minus deoxy absorption spectrum, thus the time course of the amplitude of the first component (V_1) represents the rebinding reaction progress. The spectral shape of the second component is indicative of a spectral change occurring after photodissociation (Henry et al., 1997). Following the interpretation for human HbA, this spectral component is associated with tertiary and quaternary relaxations, characterized by the same spectral shape. The time course of V_2 demonstrates that a time extended tertiary relaxation occurs in the nano-to-microsecond time scale, while a quaternary switch occurs in the microseconds time scale. It is evident that the structural relaxation proceeds much further at low pH than at alkaline pH, and the rebinding kinetics slows down accordingly due to the larger extent of T state formation.

The effect of ATP on the time evolution of the spectral components is shown in **Figure 2.7**, at pH 6.0 **(C)** and pH 8.0 **(D)**. At pH 8.0, in presence of ATP, a large shift towards the slowly rebinding T population is observed; on the contrary, at pH 6.0 only a little effect of ATP is viewed, either on the structural relaxation (V_2) or on the rebinding kinetics (V_1).

Figure 2.8 compares the spectral components and their time evolution, determined from the singular value decomposition (SVD) analysis of the time resolved differential absorption spectra following photolysis, of *E. maclovinus* Hb1-CO solutions, at pH 8.0, in the absence and in the presence of 3 mM ATP. These data are also compared to the single wavelength kinetics monitored at 436 nm, showing the major deviations from the rebinding curve (V_1), due to the substantial contributions to the absorbance changes coming from V_2. No major spectral change is induced by the presence of ATP in both components. However, as already observed above, it is clearly evident that the reaction slows down to the milliseconds time scale when ATP is added. In particular, V_2 appears to proceed further and for longer times, suggesting the formation of the low affinity quaternary T state.

Assuming that the allosteric effector results in a change in the populations of the species formed upon photolysis, but not in their CO rebinding rates, the kinetics were analyzed with a global approach using a sum of two stretched exponentials and two exponential relaxations. The resulting curve-fittings (**Figure 2.8**) reproduce accurately

the whole time course of each component, with the exception of the fastest portion of the kinetics, which is not resolved in the time resolved spectra experiments. The time constants and the stretching exponents were kept as shared parameters in the analysis, while amplitudes were left free to vary independently for each curve. Fitting of kinetics at pH 6.0 and pH 8.0 resulted in comparably satisfactory results.The data obtained show that two relaxations, described by stretched exponential relaxations, occur on the nanosecond and the microsecond time scales. These are most likely associated with tertiary (ns) and quaternary (μs) relaxations. The next two phases are associated with rebinding to the R and T states of *E. maclovinus* Hb1.

The **Table 2.2** reports the relative amplitude of the two kinetic phases determined from the rebinding curves (components V_1) as a function of pH, in the presence and in the absence of ATP. In the absence of ATP, the amplitude of the rebinding phase to R molecules drops from 100% at pH 8.0 to 18 % at pH 6.0. On the other hand, the fraction of T like molecules contributing to second order rebinding increases in the presence of ATP (from 0% to 62 % at pH 8.0), but this effect is almost negligible at pH 6.0.

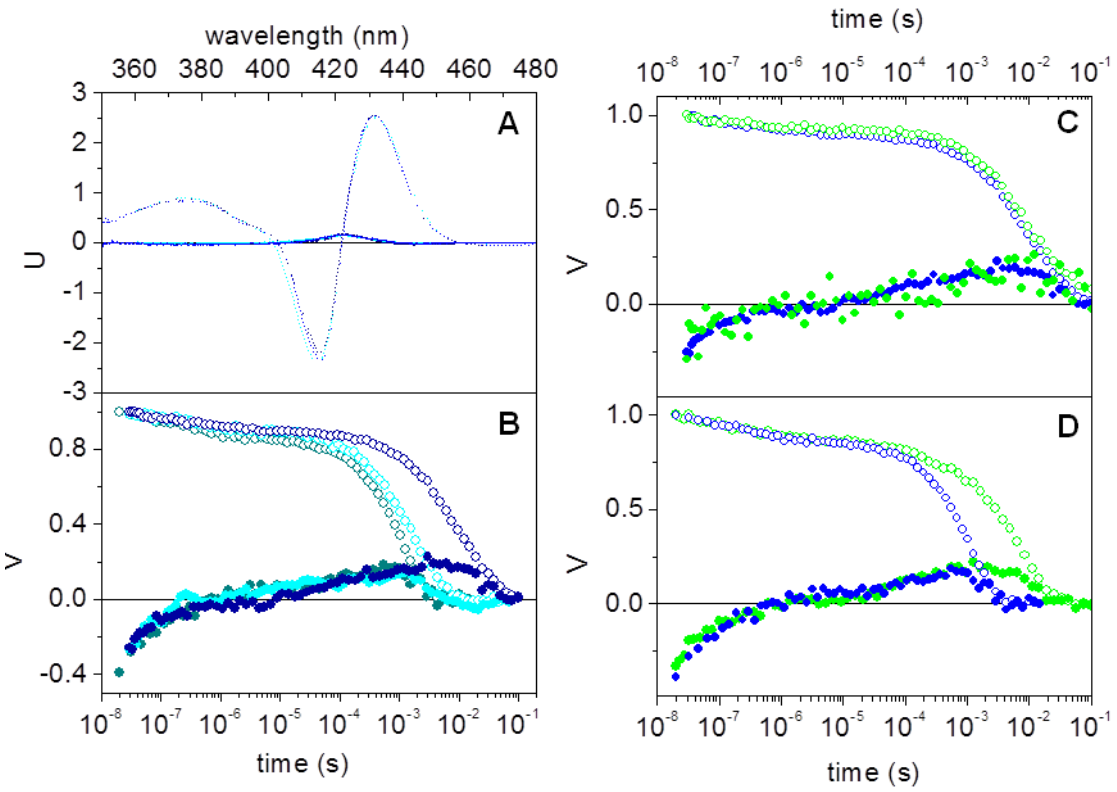

Figure 2.7. Spectral components (U×s) **(A)** and amplitudes **(B)** (open circles, component V_1; filled circles, component V_2) determined from the SVD analysis of time resolved differential absorption spectra of *E. maclovinus* Hb1-CO solutions equilibrated with 1 atm CO, at T = 10°C and at pH 6.0 (Blue), pH 7.0 (cyan), pH 8.0 (navy). The effect of ATP on the rebinding kinetics (open circles, component V_1) and the structural relaxation (filled circles, component V_2), at pH 6.0 **(C)** and pH 8.0 **(D)**, at T = 10°C and 1 atm CO. Experiments were conducted in the presence (green) and in the absence (blue) of 3 mM ATP.

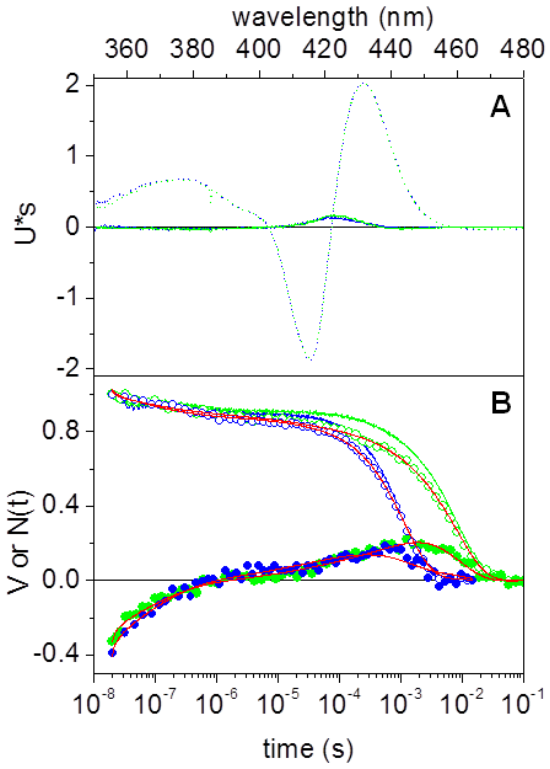

Figure 2.8. Spectral components (U×s) **(A)** and amplitudes **(B)** (open circles, component V_1; filled circles, component V_2) determined from the SVD analysis of time resolved differential absorption spectra of *E. maclovinus* Hb1-CO solutions equilibrated with 1 atm CO in the absence (blue) and in the presence (green) of 3 mM ATP, at T = 10°C and pH 8.0. For comparison, absorbance changes at 436 nm, under the same experimental conditions, are also shown as solid lines. Red curves are the best fit of the curves obtained after a global analysis with the equation:

$$A = A_1 e^{-\left(\frac{t}{\tau_1}\right)^{\beta_1}} + A_2 e^{-\left(\frac{t}{\tau_2}\right)^{\beta_2}} + A_3 e^{-\left(\frac{t}{\tau_3}\right)} + A_4 e^{-\left(\frac{t}{\tau_4}\right)}$$

		τ_1(ns)	β_1	τ_2 (µs)	β_2	R (%)	τ_3 (ms)	T (%)	τ_4 (ms)
pH = 6.0		49±8	0.7	11±9	0.2	18	1.5±0.1	82	16.2±0.1
	+ATP					14		86	
pH = 7.0						92		8	
	+ATP					46		54	
pH = 8.0						100		0	
	+ATP					38		62	

Table 2.2. Lifetime and relative amplitude for CO-rebinding kinetics to R and T state following photolysis of *E. maclovinus* Hb1-CO solutions, at 10°C and 1 atm CO.

Under these conditions, more than 80% of the molecules switch to T after photolysis, with only a 4% increase when ATP is added, suggesting that the shift in population is saturated by the low pH alone. It is worthwhile noting that lower CO partial pressures may of course allow relaxation to the T state to a larger extent. The data in **Table 2.2** have allowed to estimate the on-rates of the R and T states of Hb1 as $k_{ON,R} = 6.5\times10^5$ $M^{-1}s^{-1}$ and $k_{ON,T} = 0.6\times10^5$ $M^{-1}s^{-1}$.

Similar results are also observed in *T. bernacchii* Hb. The effect of pH on the amplitudes of the spectral components determined from the SVD analysis of the time resolved adsorption spectra is shown in **Figure 2.9A**. When pH is increased from 6.0 to 8.0, the rebinding kinetics become much faster. Moreover, the effect of the ATP is largest on the rebinding kinetics measured at pH 8.0 (**Figure 2.9B**). However, it is evident that the reaction slows down when ATP is added, but much less compared to *E. maclovinus* Hb1.

As in the case of *E. maclovinus* Hb1, the components V_1, obtained at pH 7.0, are compared to the single wavelength kinetics monitored at 436 nm (**Figure 2.10**). Also here, the single wavelength kinetics show large deviations from the rebinding curve (V_1), due to the substantial contributions to the absorbance changes coming from V_2.

The kinetics obtained at pH 7.0, in the presence and in the absence of ATP, were analysed with a global analysis approach. The resulting curve-fittings (data not shown) reproduce accurately the whole time course of each component and the fitted parameters were used to estimate the on-rates of the R and T states of *T. bernacchii* Hb, as $k_{ON,R} = 2.1\times10^5$ $M^{-1}s^{-1}$ and $k_{ON,T} = 0.26\times10^5$ $M^{-1}s^{-1}$.

2.2.6. Kinetics of CO binding and dissociation. In collaboration with Prof. Coletta (University of Rome), kinetics of CO binding and dissociation were obtained by stopped flow. The CO dissociation by NO replacement shows a marked biphasicity, both in *E. maclovinus* Hb1 and in *T. bernacchii* Hb, clearly indicating the presence of two populations in the fully liganded Hb-CO with drastically different CO dissociation rate constants. This behaviour might be explained by the presence of a significant fraction of T-liganded form, probably corresponding to the fast CO dissociating species.

These results are strengthened considering that kinetic curves of CO binding show that *E. maclovinus* Hb1 is not fully saturated for concentration of CO ≤ 100 μM, suggesting a low affinity for CO. Similar to the Antarctic fishes belonging to the family Artedidraconidae (Tamburrini et al., 1998), the CO binding to both Hb1 from *E. maclovinus* and to Hb from *T. bernacchii*, does not show any marked subunit heterogeneity, as observed instead for other temperate fish Hbs (Noble et al., 1970; Giardina et al., 1978; Saffran et al., 1978; Coletta et al., 1996). The observed low affinity is consistent with a very high thermodynamic stabilisation of the T-liganded form.

The values of the faster CO dissociation rate constants (at 10°C k_{off} is 4.5(±0.6) s^{-1} for *E. maclovinus* Hb1 and 4.8(±0.6) s^{-1} for *T. bernacchii* Hb) are very unusual and indicates a very weak Fe-CO bond in these species, possibly related also to a weak proximal Fe-His bond (Coletta et al., 1985).

2.2.7. Spectroscopic characterisation. In collaboration with Prof. Smulevich (University of Florence), the spectroscopic studies of *E. maclovinus* Hb1 were performed, by electronic absorption UV-Vis and Resonance Raman spectra.

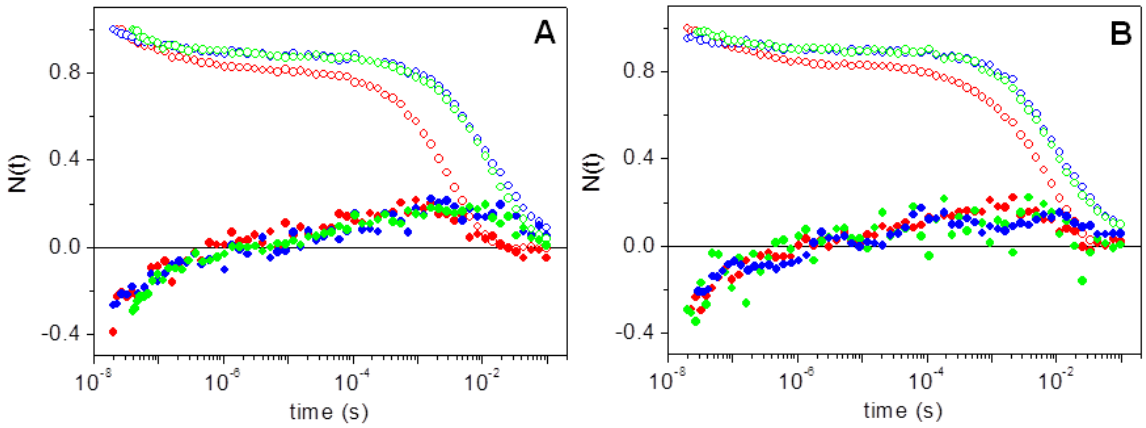

Figure 2.9. Comparison of the time courses of the amplitudes (open circles, component V_1; filled circles, component V_2) determined from the SVD analysis of time resolved differential absorption spectra, collected after photolysis of *T. bernacchii* Hb-CO solutions equilibrated with 1 atm CO, at T = 10°C and at pH 6.0 (Blue), pH 7.0 (green), and pH 8.0 (red), in the absence **(A)** and in the presence **(B)** of a 3 mM of ATP.

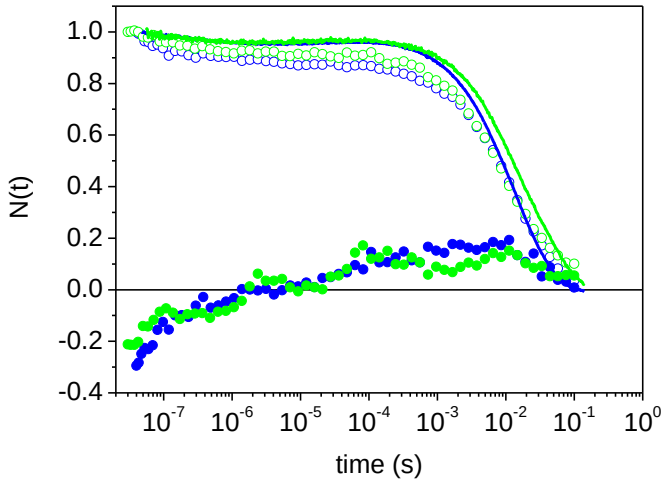

Figure 2.10. Amplitudes (open circles, component V_1; filled circles, component V_2) determined from the SVD analysis of time resolved differential absorption spectra of *T. bernacchii* Hb-CO solutions equilibrated with 1 atm CO at T = 10°C, pH 7.0 in the absence (blue) and in the presence of 3 mM ATP (green). Absorbance changes at 436 nm under the same experimental conditions are also shown as solid lines.

To gain insight about the heme pocket properties, the *E. maclovinus* Hb1 was studied in the ferrous oxidation state (Fe^{2+}) of the deoxy Hb and in CO and O_2 complex. These analyses show that the conformation and electrostatic field surrounding the heme pocket of ferrous Hb1 of *E. maclovinus* are essentially unmodified, with respect to Hb1 of the high-Antarctic *T. bernacchii* (Ito et al., 1995; Mazzarella et al., 2006a), the Hb1 of the sub-Antarctic *P. urvillii* (Verde et al., 2004) and the human HbA.

2.2.8. X-ray crystallography. In collaboration with Prof. Mazzarella's group (University of Naples), the carbomonoxy form of *E. maclovinus* Hb1 was crystallized in two different crystal forms, orthorhombic (Ortho) and hexagonal (Hexa), and high-resolution diffraction data were collected for both forms (1.45 and 1.49 A° resolution, respectively). The first of these two forms was analysed. The low temperature structure of *E. maclovinus* Hb1-CO was refined to an R-factor of 0.214 and includes 368 water molecules and two glycerol molecules.

The structure of *E. maclovinus* Hb1-CO displays all the features typically associated to Hbs in the R state. As generally found in tetrameric Hbs, the structure significantly differs from the deoxygenated state. Indeed, the superimposition, based on the BGH core (Baldwin and Chothia, 1979), of $\alpha_2\beta_2$ dimers between *E. maclovinus* Hb1-CO and the deoxy form of *T. bernacchii* Hb (Mazzarella et al., 2006a), after superimposition of their $\alpha_1\beta_1$ dimers, requires a rotation χ=14.7°. Interestingly, this value and the position of the rotation axis used for the superimposition are different from those obtained by comparing the liganded (Camardella et al., 1992) and unliganded (Mazzarella et al., 2006a) forms of *T. bernacchii* Hb (in this case χ=11.3°), suggesting that *T. bernacchii* Hb and *E. maclovinus* Hb1-CO have a slightly different quaternary structure in their carbomonoxy form.

The analysis of the tertiary structure of the *E. maclovinus* Hb1 α and β chains highlights the presence of an overall structure and coordination of the heme iron atom that are in line with those generally observed in tetrameric Hbs in their carbomonoxy state. The major differences are localised in the proximity of the β_2 C-terminal region, because of the interactions of one of the two glycerol molecules that causes apparent changes in this region. In particular, the side chain of Tyr145β_2 adopts a conformation typical of the T state, interposing between residues His92β_2 and Val98β_2 (tyrosine pocket). This tertiary variability in the tyrosine pocket is important for the allosteric transition (Baldwin and Chothia, 1979).

The peculiarities observed at low temperature essentially disappear at room temperature, and they must be attributed to the change in the solvation medium. This is a clear indication of an accessible cavity into the protein matrix, that can be modulated by effectors (in this case glycerol) to assume typical T-tertiary structure motifs. It is worth mentioning that despite the high resolution of the crystal structures of *T. bernacchii* Hb (in the deoxy, carbomonoxy or ferric forms), no glycerol molecule was observed. The inspection of the charge density maps clearly show that glycerol site corresponds to a region with a relatively high positive charge, and possibly the target of phosphate groups of ATP.

2.3 Conclusions

Fishes thriving in polar habitats offer many opportunities for comparative approaches to understanding protein adaptations to temperature. For the study of

temperature adaptations, Antarctica more than any other habitat on earth is indeed a unique natural laboratory. The variety of adaptations underlying the ability of modern Antarctic fish to survive at the freezing temperatures, represents the extreme of low temperature adaptations found among vertebrates. Fish Hbs function at the interface between organism and environment; consequently this molecule is suitable for identification of the key links between molecular and eco-physiological adaptations.

The aquatic habitats are such that fish may experience temporal and spatial variations in O_2 availability, salinity, ionic composition, pH and temperature. Notothenioidei, the dominant suborder in the Antarctic Ocean, have evolved reduction of hemoglobin concentration and multiplicity, perhaps as a consequence of temperature stability and other environmental parameters. The comparison of the biochemical and physiological adaptations of cold-adapted Antarctic notothenioids with sub-Antarctic and temperate notothenioids has been a powerful tool to understand whether (and to what extent) an extreme environment required specific molecular adaptations.

In contrast to high-Antarctic notothernioid Hbs, the Hbs of *E. maclovinus* and *D. eleginoides* are characterised by high O_2 affinity and cooperativity, and marked Root effect, which become evident at moderately acid pH values. In addition, in *E. maclovinus* Hb1 the Root effect is strongly enhanced by the presence of the allosteric effector ATP; in *T. bernacchii* Hb, the Root effect seems to correspond to an intrinsic property of the molecule, and therefore exhibited also in the absence of ATP.

Moreover, the ligand binding kinetics of *E. maclovinus* Hb1 indicate a strong stabilization of the low affinity T quaternary state, which is particularly pronounced in the presence of the physiological allosteric effector ATP. The crystal structure of the carbomonoxy form of Hb1 of *E. maclovinus*, the first sub-Antarctic Hb to be crystallized, hosts in an overall canonical structure, some peculiar features both at the level of the tertiary and quaternary structure, and provides a putative binding site for ATP.

These results indicate that over evolutionary time, the notothenioid Hb phenotype is undergoing dynamic changes in response to cold adaptation. Whereas the Hbs of the sub-Antarctic fish are proteins strongly regulated by the physiological effectors and with high cooperativity in O_2 binding, the high-Antarctic notothenioid Hbs have evolved a low-affinity O_2 transport system facilitating O_2 unloading. The survival with no obvious ill effects of *T. bernacchii*, in spite of the functional incapacitation of Hb by CO and also of gradual reduction of the hematocrit in specimens in which a cannula had been inserted in the caudal vein, leaves little doubt that, in the cold, stable environment of the Antarctic sea, routine O_2 transport is still possible even in the absence of functional Hb (di Prisco, unpublished results).

CAPTER III

PEGYLATED HEMOGLOBIN FROM *TREMATOMUS BERNACCHII*, A MODEL FOR HEMOGLOBIN-BASED BLOOD SUBSTITUTES

3.1 Introduction

Decoration of human and animal Hbs with PEG chains has been widely exploited since represents one the most promising strategies for the development of blood substitutes. In the case of human HbA, the destabilization of the tetramer brought by PEGylation led to products with different O_2-binding properties with respect to unmodified Hb, deemed responsible for some of their side effects observed in clinical trials, preventing their application as a replacement of red blood cells. In particular, one of the limits is the higher O_2 affinity of PEGylated products compared to Hb in red blood cells, impairing the adequate tissue oxygenation.

In this contest, the second part of the PhD project was focussed on the study of the action of PEGylation on the structural and functional properties of the O_2-transport system of Antarctic fish, and in particular of *T. bernacchii* Hb, a well characterized Hb endowed with a remarkably low O_2 affinity and high tetramer stability, in order to obtain a molecule with potential properties to be used for possible HBOCs.

For this aim, in the study here described, the O_2-binding properties, CO-rebinding kinetics and NO-dioxygenase reactivity of *T. bernacchii* Hb and its PEGylated products were investigate and compared with those of PEGylated human HbA.

These experiments were carried out in collaboration with Prof. Mozzarelli, Dr. Bruno and Prof. Viappiani, during my training period at the University of Parma.

3.2 Results and discussion

3.2.1. Cysteine reactivity and PEGylation. Preliminarily to PEGylation experiments, it was necessary estimate the reactivity of the cysteyl residues of *T. bernacchii* Hb, since they can be sites of potential PEG attachment.

The sulfhydryl reactivity towards 4,4'dithiodipyridine (4-PDS) of carboxy- and deoxy-Hb was very slow (data not shown) with the fastest-reacting cysteine completing the reaction in more than 24 hours. The low accessibility of cysteyl residues is in agreement with the structural data (Mazzarella et al., 2006a), indicating the absence of exposed cysteyl residues. 2-iminothiolane (IMT)-generated SH groups are therefore predicted to be the only reactive sites towards maleimido polyethylene glycol (MAL)-PEG

PEGylated *T. bernacchii* Hb and human HbA (hereafter named PEG-*Tb*Hb and PEG-Hb^{oxy}, respectively) have been obtained under aerobic conditions by a two-step procedure in which, the protein is first treated with IMT, which adds a spacer arm ending with a thiol group to lysine residues, then the thiols are made to react with MAL-PEG 5000 (**Figure 5.1**, see Material and Methods).

The PEGylation reaction was monitored for one hour. Samples collected at different times were then compared in a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE gel) (**Figure 3.1A**) to prove the efficiency in separating

non-conjugated and conjugated Hb chains (Caccia et al., 2009). The stained protein bands were analysed by densitometry (**Figure 3.1B**), to calculate the distribution of the number of conjugated PEG molecules per tetramer. The reaction seems to be completed in 30 min (Caccia et al., 2009). The densitometric analysis indicates that during the reaction about four PEG chains are added per tetramer, as compared to the 5-6 PEG chains/tetramer added to HbA under the same reaction conditions.

Nevertheless, in native electrophoresis, PEG-*Tb*Hb seems to have a slower migration compared to the human PEG-Hb^{oxy}, probably due to differences in electric charge (**Figure 3.2**). Interestingly, the PEG-*Tb*Hb derivative appears more homogeneous and does not show any traces of unmodified tetramer (**Figure 3.2**), widely known to be very toxic, as it can extravasate and filtered at glomerular level (see Introduction, paragraph 1.2.2), and which is consistently observed in all preparation of PEG-Hb^{oxy} (Ronda et al., 2011). The complete derivatization of *T. bernacchii* Hb, as indicated by these results, proves a valuable property of the protein as a blood substitute.

3.2.2. Oxygen-binding properties. O_2 affinity, cooperativity and the Bohr effect of PEGylated Hb derivatives and unmodified Hbs were measured, under different conditions (**Figure 3.3**).

The O_2-binding curves were determined at pH 7.0 and pH 8.0 and at 10°C. The analysis allows to calculate p_{50} and nHill values, reported in **Table 3.1**. At pH 7.0, HbA exhibits a p_{50} of 1.7 Torr, which decreases to approximately 0.4 Torr upon PEGylation (**Table 3.1**). The derivatization also results in loss of cooperativity, with nHill decreasing from around 2 to 1.2. *T. bernacchii* Hb, under the same conditions, shows a much higher p_{50} of 28.2(± 0.2) Torr. PEGylation results in an increase in O_2 affinity to 19.7(± 0.3) Torr, but remaining, however, 50-fold higher than PEG-Hb^{oxy}, under the same conditions. Cooperativity is significantly reduced, with the Hill coefficient decreasing from 2 to around 1.3.

Upon PEGylation, the changes in O_2-binding properties of *T. bernacchii* Hb, are therefore similar to those observed for PEG-Hb^{oxy}, in particular showing loss in cooperativity. Considering the stability of the *T. bernacchii* Hb tetramer, this effect is probably due to steric effects of the PEG moieties, which prevent the transition between the T and R states, rather than to dissociation of the tetramer, as seen in the human HbA.

Despite the loss in cooperativity, the p_{50} value of PEG-*Tb*Hb remains remarkably high. Moreover, ATP at saturating concentrations still acts as an allosteric effector (**Table 3.1**), raising p_{50} to 31.1 Torr. These data, combined with those relative to PEGylated Hbs of other species, particularly in bovine (Vandegriff et al., 1997) and canine Hbs (Acharya et al., 2007), show that, regardless of their different O_2 affinity, there is indeed a correlation between the increase in affinity and the PEGylation reaction. The non-specific effect of PEGylation in increasing the O_2 affinity is also demonstrated within the same preparation, through the electrophoretic separation of differently PEGylated HbA derivatives having different affinities (Ronda et al., 2011).

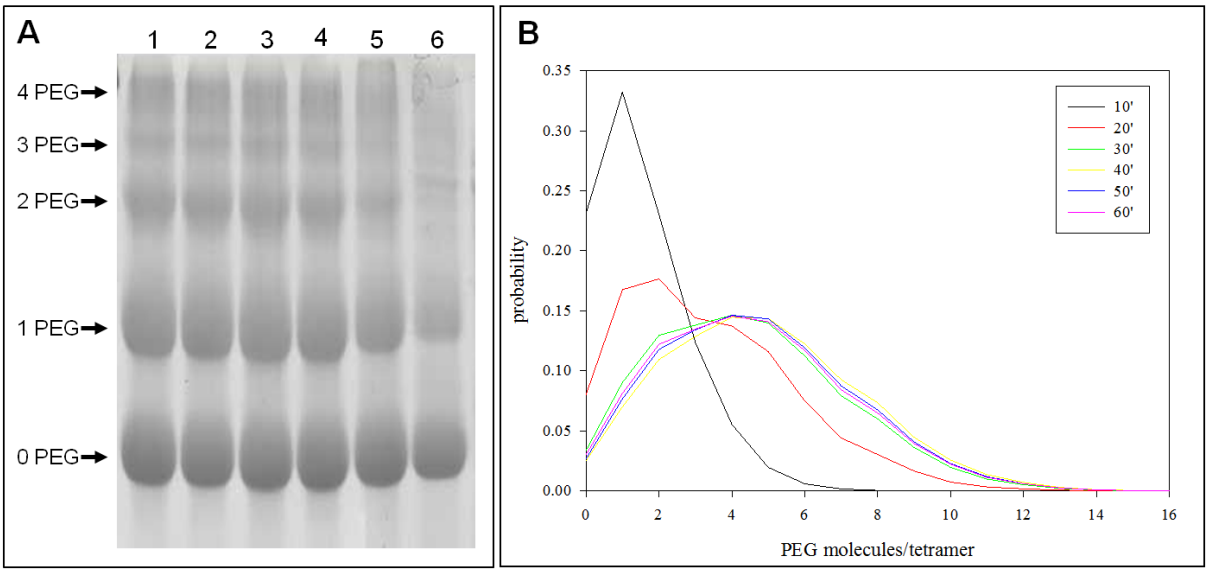

Figure 3.1. (A) SDS-PAGE analysis of PEG-*Tb*Hb. The reaction was stopped after 10' (lane 6), 20' (lane 5), 30' (lane 4), 40' (lane 3), 50' (lane 2) and 60' (lane 1). **(B)** Probabilistic analysis of the band intensities detected in the SDS-PAGE gel.

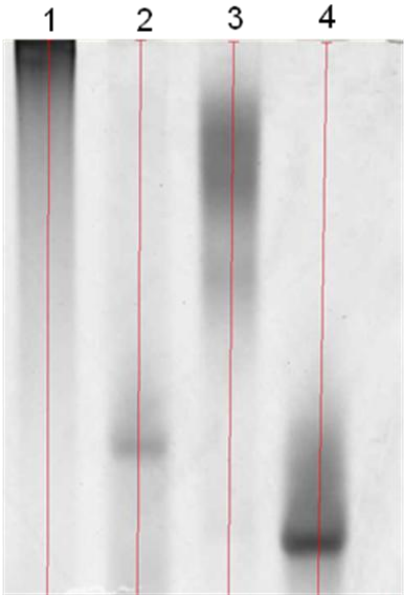

Figure 3.2. Gradient native PAGE of *T. bernacchii* Hb (lane 2), human HbA (lane 4) and their PEGylated derivatives (lanes 1 and 3, respectively).

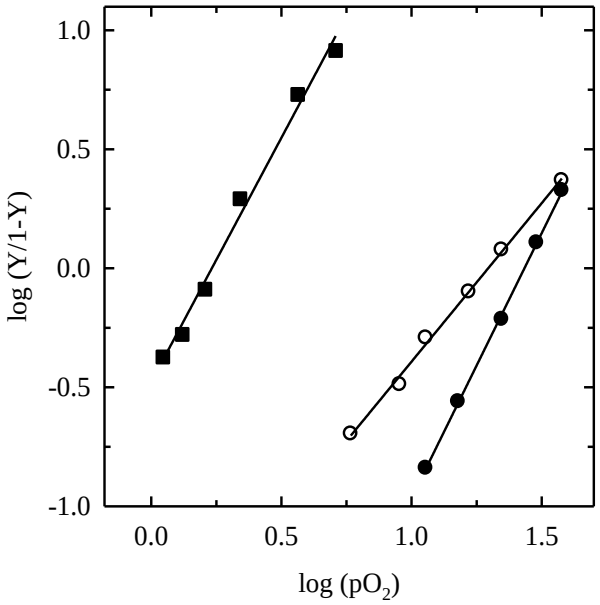

Figure 3.3. Hill plots of O_2-binding curves of HbA (closed squares), *T. bernacchii* Hb (closed circles) and PEG-*Tb*Hb (open circles), measured in 100 mM HEPES pH 7.0, 1 mM EDTA, 5 mM sodium ascorbate, 10^3 U/ml catalase, T = 10°C. Experimental points are fitted to the Hill equation, with calculated Hill's coefficients and p_{50}, reported in **Table 3.1**.

Protein	pH 7.0		pH 8.0	
	p_{50}	nHill	p_{50}	nHill
HbA	1.7 ± 0.1	2.05 ± 0.02	0.39 ± 0.3	1.72 ± 0.03
PEG-Hb^{oxy}	0.4a	1.21b		
*Tb*Hb	28.2 ± 0.2	2.06 ± 0.1	7.3 ± 0.1	1.75 ± 0.03
PEG-*Tb*Hb	19.7 ± 0.3	1.33 ± 0.02	5.3 ± 0.1	1.09 ± 0.02
PEG-*Tb*Hb + 3 mM ATP	31.1 ± 0.2	1.09 ± 0.01	4.4 ± 0.3	1.02 ± 0.03

Table 3.1. O_2-binding parameters of human HbA and *T. bernacchii* Hb (*Tb*Hb) and their PEGylated derivatives. 100 mM HEPES, 1 mM EDTA, 5 mM ascorbate, 10^3 U/ml catalase, T = 10°C.

3.2.3. Flash photolysis experiments. **Figure 3.4** compares the CO-rebinding kinetics measured after laser-flash photolysis, on the carboxy forms of HbA and *T. bernacchii* Hb solutions equilibrated with 1 atm CO, at 10°C and pH 7.0, with the corresponding signals observed in the PEGylated proteins.

Dependence of the kinetics on the CO concentration allows to distinguish between unimolecular and bimolecular processes (data not shown). As well established for HbA, CO rebinding comprises multiple phases: a nanosecond geminate process, due to unimolecular rebinding within the heme pocket or the protein matrix, and two second order processes, one in the microsecond time scale and the other in the millisecond time scale, attributed to bimolecular rebinding to quaternary R state and to proteins that have switched to quaternary T state, respectively (Henry et al., 1997). This means that tertiary and quaternary conformational changes are superimposed to ligand-rebinding kinetics because upon CO dissociation, proteins relax from the R state (liganded) to the T state (unliganded). As proposed by Kliger and coworkers (Goldbeck et al., 1996), a sum of six exponential decays functions is used in order to reproduce the experimental rebinding curves for HbA.

Figure 3.4 also shows the results of the global fitting on HbA, *T. bernacchii* Hb and on their PEGylated products, demonstrating a very good agreement between calculated and experimental curves. Besides the two exponential decays, necessary to describe the geminate phase (10 and 182 ns for HbA; 14 and 452 ns for *T. bernacchii* Hb), two processes ascribed to quaternary relaxation from R to T state are detected in the micro-millisecond time scale (1 and 140 µs for HbA; 110 µs and 1.5 ms for *T. bernacchii* Hb), one phase associated with the rebinding to R state (350 µs for HbA; 7 ms for *T. bernacchii* Hb) and one phase associated with the rebinding to T state (7 ms for HbA; 21 ms for *T. bernacchii* Hb).

Interestingly, as previously shown in HbA (Caccia et al., 2009), while PEGylation preserves the general features of the dynamics and reactivity of the protein, it partially prevents the R to T relaxation, so that upon PEGylation the fractional amplitude of the rebinding to R changes from about 27% to 80% in HbA and from 34% to 76% in *T. bernacchii* Hb.

3.2.4. NO dioxygenase activity. In order to develop a possible blood substitute, it is fundamental to consider not only the O_2-transport properties of these Antarctic Hbs and their PEGylated products, but also their influence on NO homeostasis. In fact, the scavenging of NO, associated with an extremely rapid deoxygenation reaction with oxy-Hb to form met-Hb and inert nitrate, is very important when Hb is present in blood vessels outside erythrocytes and represents one of the main determinant of the adverse effects of HBOCs (Eich et al., 1996; Doherty et al., 1998).

The reaction rates of the NO with unmodified *T. bernacchii* Hb and its PEGylated Hb derivatives were measured and compared with those of HbA and its PEGylation products. Analysis of the oxidation kinetics of *T. bernacchii* Hb in the oxygenated form, treated with NO (**Figure 3.5**), yields a k_{obs} of 7.2 $\mu M^{-1}s^{-1}$, indicating slower reactivity with respect to HbA, with a k_{obs} of 64 $\mu M^{-1}s^{-1}$, under the same experimental conditions and in agreement with literature data (Eich et al., 1996). PEGylation of *T. bernacchii* Hb seems to affect the NO dioxygenase reactivity only marginally, with a k_{obs} of 7.7 $\mu M^{-1}s^{-1}$.

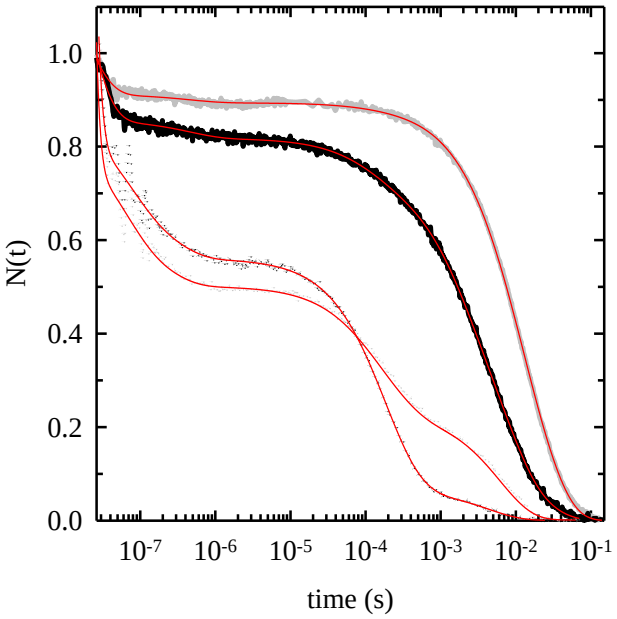

Figure 3.4. Effect of PEGylation on CO rebinding to HbA, *T. bernacchii* Hb and their PEGylated derivatives. The time courses of the deoxyheme fraction are shown for HbA (gray circles), PEGylated HbA (black circles), *T. bernacchii* Hb (gray solid line) and PEG-*Tb*Hb (black solid line), in 100 mM HEPES pH 7.0, 1 mM sodium EDTA, 1 atm CO, T = 10°C. The fitting curves are shown in red.

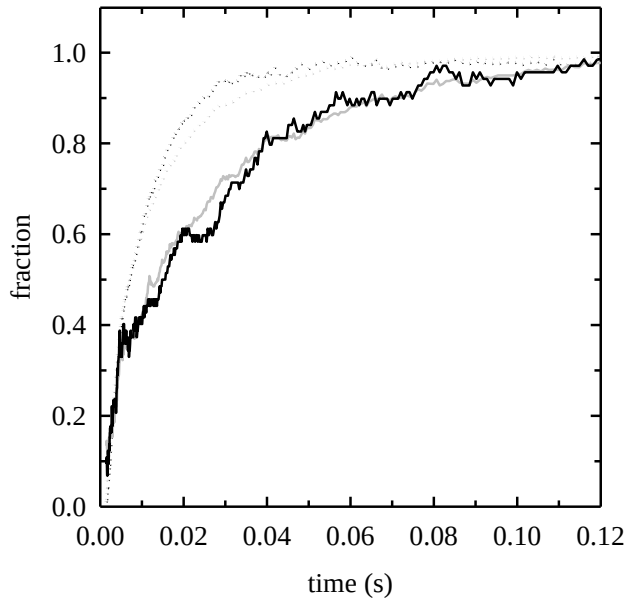

Figure 3.5. Determination of the effect of PEGylation on NO reductase activity of the oxygenated HbA (gray dotted line) and *T. bernacchii* Hb (gray solid line) and their PEGylated derivatives (black dotted line and black solid line, respectively). The reaction is carried out by rapid mixing under anaerobic conditions at 20°C and monitored at 405 nm. The absorbance differences were normalized.

A comparable decrease in the NO dioxygenase activity of HbA is achieved by mutating residues αE11, βE11, and βB10 (2-15 $\mu M^{-1}s^{-1}$) (Doherty et al., 1998). These mutants show reduced *in vivo* vasoactivity, directly correlated with the *in vitro* NO oxygenation rate.

The same pattern is observed in HbA, the PEGylation of which, under aerobic and anerobic conditions, yields k_{obs} of 83 $\mu M^{-1}s^{-1}$ and 86 $\mu M^{-1}s^{-1}$, respectively.

3.3 Conclusions

The Hbs from Antarctic fish are particularly interesting, because of their peculiar features (exceptionally low affinity for O_2, little or no dissociation of the tetramer into dimers, absence of cysteine β93) that make them potentially less sensitive to the undesirable effects of PEGylation.

The functional characterisation of the PEG-conjugated derivative of the highly stable Hb tetramer of *T. bernacchii* confirms some non-specific effects of PEGylation already observed in human, bovine and canine Hbs, including an increase in O_2 affinity, a decrease in cooperativity and a reduction of the R- to T-quaternary switching upon flash photolysis. However, these non-specific effects are accompanied by the partial retention of the remarkably low affinity for O_2, the sensitivity to allosteric effectors and the low NO dioxygenase reactivity.

These results indicate that PEGylated cold-adapted Hbs can cover a wide range of O_2-binding properties, potentially meeting the functional requirements of blood substitutes.

CAPTER IV

INVESTIGATION OF THE ROLE OF *Ph*-2/2HbO IN RESPONSE TO NITROSATIVE STRESS

4.1 Introduction

Although the presence of the O_2-binding Hbs had long been thought to be limited to vertebrates, recent discoveries have indicated an almost ubiquitous existence of these proteins in non-vertebrate, plants, and prokaryotic and eukaryotic microorganisms. On the basis of structural characteristics three types of globins have been identified: the monomeric single domain globins, chimeric globins and the 2/2 Hbs (as previously explained in the Chapter I).

In the Antarctic bacterium *Ph*TAC125 genome, multiple genes encoding 2/2 Hbs (annotated as *PSHAa0030*, *PSHAa0458*, *PSHAa2217*) and a flavoHb gene (*PSHAa2880*) have been identified. This multiplicity suggests that specific and different functions may be associated to these two classes of proteins (Médigue et al., 2005; Giordano et al., 2007; Verde et al., 2009; Parrilli et al., 2010a; Parrilli et al., 2010d; Howes et al., 2011; Giordano et al., 2011).

The structural characterisation of the globin encoded by the *PSHAa0030* gene (*Ph*-2/2HbO), belonging to group II, was performed by spectroscopy studies, kinetics measurements and computer simulation approaches (Giordano et al., 2007; Howes et al., 2011; Giordano et al., 2011). The results indicate high structural flexibility, probably linked to the peculiarity of the cold environment.

In addition, recent *in vivo* results demonstrated that inactivation of the gene encoding *Ph*-2/2HbO makes the mutant bacterial strain sensitive to high O_2 levels, hydrogen peroxide, and nitrosating agents (Parrilli et al., 2010a). Moreover, the transcription of the gene encoding the flavoHb occurs only in the mutant grown in microaerobiosis at 4°C, suggesting that the occurrence of the NO-induced stress is related to the absence of *Ph*-2/2HbO (Parrilli et al., 2010a).

On this basis, in the last part of my PhD thesis, the physiological role of the globin *Ph*-2/2HbO was investigated by *in vivo* and *in vitro* experiments in order to confirm, as previously suggested, its involvement in the NO detoxification. The *PSHAa0030* gene was cloned and then over-expressed in a mutant of *E. coli* defective in the flavoHb (*E. coli hmp*), hypersensitive to nitrosative stress. The mutant of *E. coli* including the *PSHAa0030* gene was studied analysing its growth properties and the O_2 uptake in presence of NO.

These experiments were carried out in collaboration with Prof. Poole, during my training period at the University of Sheffield.

4.2 Results and discussion

4.2.1. Cloning and expression of the PSHAa0030 gene in E. coli hmp. The *PSHAa0030* gene was cloned into the commercial vector pBAD/HisA (Invitrogen), under control of an L-Arabinose inducible promoter. The construction was verified by sequencing and named pBAD-2/2HbO.

To check the expression of *PSHAa0030* gene, the absolute absorption spectrum of whole *E. coli* cells, grown at 25°C, under aerobic conditions and in

presence of induction supplements, was performed using an SDB-4 dual-wavelength scanning spectrophotometer (**Figure 4.1A**) and compared with that obtained using whole *E. coli hmp* cells, grown in the same conditions, but transformed with the empty vector pBAD/HisA (**Figure 4.1B**). The absolute spectrum in **Figure 4.1A** shows characteristic peaks of the *Ph*-2/2HbO, indicating a correct expression of the protein in the cells. The presence of a Soret peak at 420 nm along with α and β peaks at 558 and 527 nm, very similar to the absorption spectra of the deoxy form of the purified *Ph*-2/2HbO, in the hexacoordinated conformation (Giordano et al., 2011), suggesting that the predominant form of this globin remains *in vivo*, in the ferrous form stabilised by an endogenous ligand.

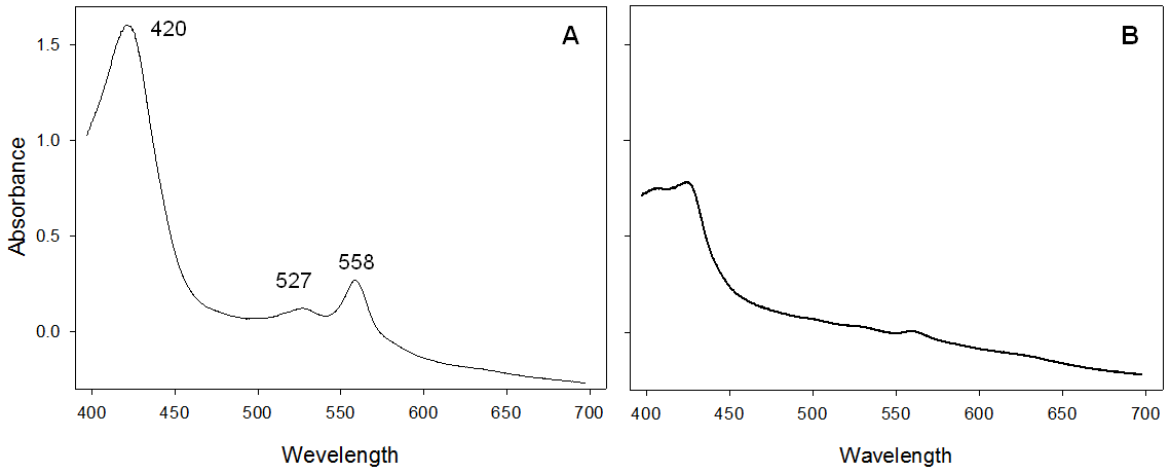

Figure 4.1. Absolute absorption spectra of *E. coli hmp* cells carrying pBAD-2/2HbO **(A)** and pBAD/HisA **(B)**.

4.2.2. Effect of nitrosative stress on bacterial growth of the NO-sensitive E. coli strain, expressing the PSHAa0030 gene. To elucidate whether the expression of *PSHAa0030* gene had any effect on the protection from NO toxicity in *E. coli hmp* cells, overnight cultures were grown at 25°C, under aerobic conditions, in presence of increasing concentrations of GSNO (a nitrosylating agent) or DETA-NONOate (a NO-releaser), with and without induction supplements. The ability of these cells to survive in presence of nitrosative stress was compared with that of cultures of *E. coli hmp* obtained in the same conditions but transformed with the plasmid carrying *hmp* gene (pPL341) or with the empty vector pBAD/HisA (**Figure 4.2**). In presence of GSNO, different concentrations of L-Arabinose were also tested to find the best condition for expression (**Figure 4.2A**).

In the medium containing a final concentration of L-Arabinose higher than 0.02%, the cultures of the *E. coli* mutant expressing *Ph*-2/2HbO show similar ability to cope with stress compared to the complemented strain, in presence both of GSNO and of DETA-NONOate (**Figure 4.2**). On the contrary, *E. coli hmp* strain, where the globin gene is not expressed, is unable to survive, suggesting a possible involvement of the globin in protecting the bacterium from nitrosative stress.

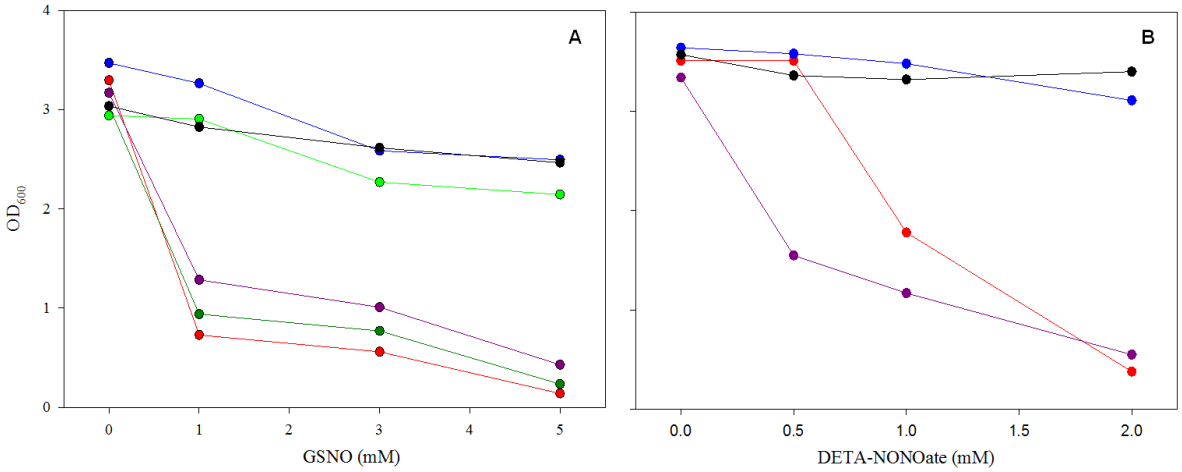

Figure 4.2. Susceptibility test of *E. coli hmp* strains transformed with different plasmids, in presence of GSNO **(A)** and DETA-NONOate **(B)**. Cultures of *E. coli hmp* carrying the *hmp* gene (black), the empty vector in presence of L-Arabinose 0.2% (dark pink), the *PSHAa0030* gene in absence (red) or in presence of 0.00002% (dark green), 0.02% (light green), and 0.2% (blue) of L-Arabinose. Cultures were grown in LB with the indicated concentration of GSNO or DETA-NONOate for 18 h at 25°C, 180 r.p.m., under aerobic conditions.

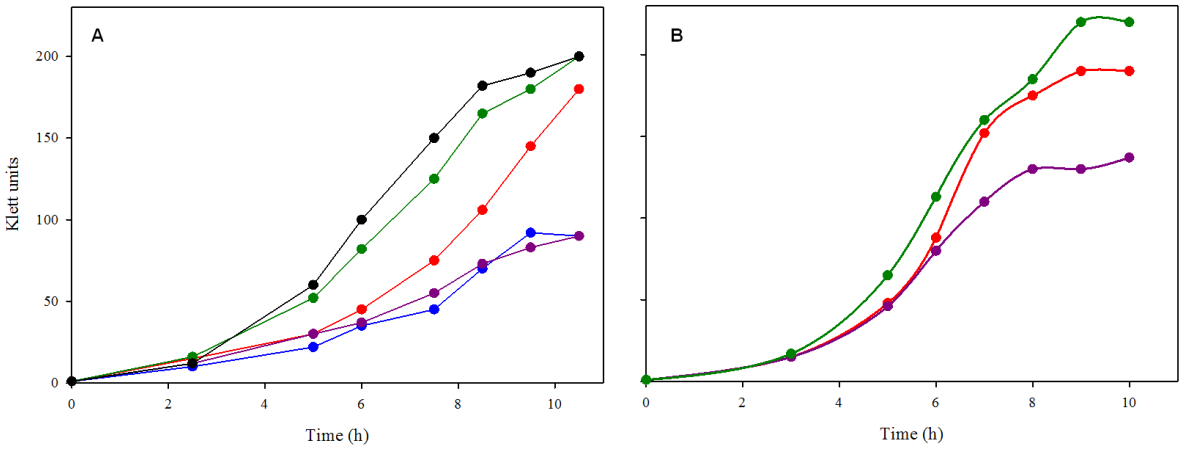

Figure 4.3. Growth profiles of *E. coli* strains during the exposure to 3 mM GSNO **(A)** and 1 mM DETA-NONOate **(B)**. Cultures of *E. coli* wild-type (black) and *E. coli hmp* carrying the *hmp* gene (dark green), the empty vector in presence of L-Arabinose 0.2% (dark pink), the *PSHAa0030* gene in presence (red) and in absence (blue) of L-Arabinose 0.2%. Cultures were grown in LB at 25°C, 180 r.p.m., under aerobic conditions.

To corroborate these results, cultures of *E. coli* wild-type and *E. coli hmp* carrying different plasmids were grown at 25°C, under aerobic conditions and treated with 3 mM GSNO (**Figure 4.3A**) and 1 mM DETA-NONOate (**Figure 4.3B**); changes in the growth behaviour were recorded.

The growth profiles, shown in **Figure 4.3A**, clearly demonstrate that respect to *E. coli* wild-type or complemented strains, the exposure to 3 mM GSNO, causes only a little decreasing of the growth of *E. coli hmp* carrying *Ph*-2/2HbO, obtained in presence of 0.2% final concentration of L-Arabinose. On the contrary, the growth profiles of control cells bearing either the vector empty pBAD/HisA obtained in the same conditions, or of cells carrying the plasmid pBAD-2/2HbO but grown in absence of L-Arabinose, are drastically reduced.

These results clearly demonstrated that the presence of *Ph*-2/2HbO provides substantial protection to the cells from NO toxicity.

4.2.3. NO consumption activity and respiration rate of E. coli hmp carrying Ph-2/2HbO. A further step to establish if *Ph*-2/2HbO provides protection to cells from nitrosative stress was to study the cellular respiration of different *E. coli* mutant strains, in presence and in absence of NO. *E. coli hmp*, carrying the *PSHAa0030* globin gene or the *hmp* gene or the empty vector, grown at 25°C, under aerobic conditions and in presence of needed induction supplements, were all tested for their ability to withstand NO inhibition of respiration.

Known amounts of whole cells were added in a closed O_2 electrode system, as described in the Chapter V, and the O_2 uptake measured before, during, and after the addition of Proli-NONOate, a NO-releaser. Because the toxicity of NO is dependent on dissolved O_2 concentration (Stevanin et al., 2000), additions of NO were made at three different O_2 tensions; the respiration was induced by the addition of glycerol (final concentration, 50 mM).

In **Figure 4.4** O_2 and NO consumption traces are showed for each of the three strains. All mutant strains respire O_2 at a similar rate without NO addition (data not shown). Following addition of NO, *E. coli hmp* carrying the empty vector shows prolonged inhibition of O_2 uptake (**Figure 4.4A**). At each NO addition, visualised by a rapid upward excursion of the NO electrode output, the O_2 uptake is immediately halted until the NO levels fall steadily. When NO levels become negligible, respiration continues until the chamber became anaerobic.

In the case of strain *E. coli hmp* complemented by pPL341 vector, the addition of NO give no inhibition of O_2 uptake and the NO is consumed so quickly that it is undetectable by the electrode (**Figure 4.4B**), confirming that the FlavoHb is able to detoxify NO, as reported previously (Hernández-Urzúa et al., 2003).

On additions of NO to whole cells of *E. coli hmp* carrying the *Ph*-2/2HbO only very little periods of inhibition of respiration are observed and the disappearance of NO is very fast (**Figure 4.4C**). When the NO concentration falls, reaching negligible amounts, O_2 uptake resumed at rate similar to that before of NO addition, in contrast to the cells bearing the vector empty. Interestingly, in absence of O_2, the disappearance of NO is very slow, indicating O_2-dependent NO consumption.

The slight upwards deflection in the O_2 electrode traces shown in **Figure 4.4** after inhibition of respiration presumably reflects polarographic drift or the back-diffusion of O_2 into the chamber through the capillary used for NO addition.

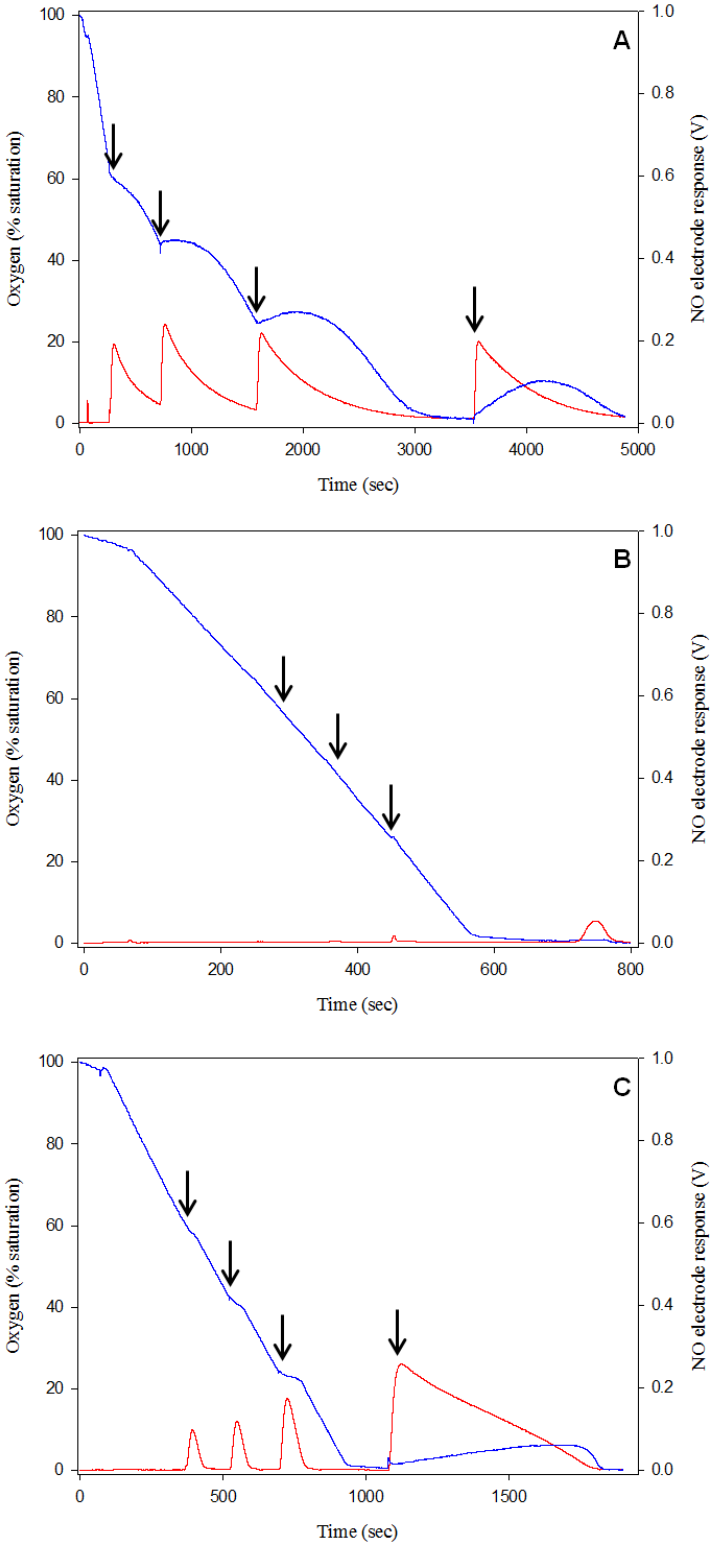

Figure 4.4. NO uptake and cellular respiration of *E. coli hmp* mutant cells, carrying pBAD/HisA **(A)**, the pPL341 **(B)** and pBAD-2/2HbO **(C)** vectors. Respiration was measured in a Clark-type O_2 electrode and NO uptake with an NO electrode. O_2 (blue) and NO (red) traces were followed simultaneously. Black arrows indicate addition of 1 μM PROLI-NONOate final concentration. Cultures were grown overnight at 25°C and 180 r.p.m.

The results here presented indicate that the respiration of *E. coli hmp* strain, after the expression the *Ph*-2/2HbO, is not more inhibited by NO, confirming that the globin participates in bacterial defences against nitrosative stress, under aerobic conditions.

4.3 Conclusions

A possible approach to establish the participation of *Ph*-2/2HbO in the protection from nitrosative stress was to test *in vivo* the influence of the heterologous expression of the gene *PSHAa0030* on protection from NO toxicity, in a mutant of *E. coli* defective in the flavoHb. In fact, the deletion of *hmp* gene alone abolishes in *E. coli* the NO-consuming activity (Liu et al., 2000) and is sufficient to make bacteria hypersensitive to NO and other nitrosative stresses (Membrillo-Hernández et al., 1999).

In the presence of O_2, the over-expression of the globin gene encoding *Ph*-2/2HbO conferred to the NO-sensitive mutant *E. coli hmp* enhanced resistance to NO and nitrosating agents and distinct NO consumption ability. On the contrary, growth curves and cellular respiration were strongly inhibited both in *E. coli hmp* containing the empty vector and in *E. coli hmp* grown in absence of the induction supplements carrying the pBAD-2/2HbO vector.

Taken together, these results suggest that, under aerobic conditions, *Ph*-2/2HbO provides protection of cells against attack by NO and related reactive nitrogen species, in functionally compensating the defect in NO detoxification of *E. coli hmp* strain, which lack the major NO-scavenging protein.

Finally, the NO-consumption activity of this cold globin was sustained in *E. coli* with repetitive NO addition indicating the presence of an electron donating partner in *E. coli* compatible with the Antarctic bacterium *Ph*TAC125, to carry out reduction of the ferric form of *Ph*-2/2HbO.

CHAPTER V

MATERIAL & METHODS

5.1. STRUCTURAL AND FUNCTIONAL CHARACTERISATION OF HIGH- AND SUB-ANTARCTIC NOTOTHENIOID HEMOGLOBINS

5.1.1. Materials. Trypsin (EC 3.4.21.4) treated with L-1-tosylamide-2-phenylethylchloromethylketone from Cooper Biomedical; 4-vinylpyridine from Sigma; dithiothreitol (DTT) from Fluka; sequanal-grade reagents from Applied Biosystems; High-Performance Liquid Chromatography **(**HPLC)-grade acetonitrile from Lab-Scan Analytical; oligonucleotides from MWG. All other reagents were of the highest purity commercially available.

5.1.2. Collection of specimens. Adult specimens of *E. maclovinus* and *D. eleginoides* were collected during ICEFISH 2004, near the Falkland Islands. Specimens of *T. bernacchii* were collected by means of gill nets or hook-and-line in the vicinity of Terra Nova Bay "Mario Zucchelli" Station (74°42'S, 164°07'E), Ross Sea, Antarctica, and kept in aquaria supplied with running, aerated sea water.

Blood was taken by heparinised syringes from the caudal vein. 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 (D'Avino and di Prisco, 1988). Saline-washed erythrocytes were frozen at 80°C until use.

5.1.3. Purification of hemoglobins. *E. maclovinus:* Separation of Hbs was achieved by Fast Protein Liquid Chromatography (FPLC) anion-exchange chromatography on a Mono Q-Tricorn column (1.0 x 10 cm), equilibrated with 20 mM Tris-HCl pH 7.6 (Buffer A). Elution was performed with a gradient from Buffer A to 20 mM Tris-HCl pH 7.6, 100 mM NaCl (Buffer B). In *E. maclovinus*, a mixture of HbC and Hb1 was eluted at 50% Buffer B, Hb1 at 57% Buffer B and a mixture of Hb1 and Hb2 at 60% Buffer B. HbC was purified to homogeneity from the first peak by a second anion-exchange chromatography under the same conditions. Moreover, to perform the X-ray crystallography experiments, Hb1 was further purified by ion-exchange chromatography on a DE52 column equilibrated with 10 mM Tris-HCl pH 7.6 and eluted stepwise with the same buffer. The Hb-containing pooled fractions were dialysed against 10 mM HEPES pH 7.6. All steps were carried out at 0-5°C. CO-Hb solutions were stored in small aliquots at -80°C until use.

D. eleginoides: Separation of Hbs was achieved by FPLC anion-exchange chromatography on a Mono Q-Tricorn column (1.0 x 10 cm), equilibrated with 20 mM Tris-HCl pH 8.5 (Buffer A). Elution was performed with a gradient from Buffer A to 20 mM Tris-HCl pH 8.5, 500 mM NaCl (Buffer B). In *D. eleginoides*, a mixture of Hb1 and Hb2 were eluted at 20 and 25% Buffer B, respectively. The Hb-containing pooled fractions were dialysed against 10 mM HEPES pH 7.6. All steps were carried out at 0-5°C. CO-Hb solutions were stored in small aliquots at -80°C until use.

T. bernacchii: Purification of Hb at 98% was achieved by FPLC ion-exchange chromatography on a DEAE Sepharose Fast Flow column (0.5 x 5 cm) equilibrated with 10 mM Tris-HCl pH 7.6 (Buffer A). Elution was performed with a gradient from Buffer A to 500 mM Tris-HCl pH 7.6. Hb1 was eluted at 25% Buffer B. The Hb-containing pooled fractions were dialysed against 10 mM HEPES pH 7.6. All steps were carried out at 0-5°C. CO-Hb solutions were stored in small aliquots at −80°C until use.

5.1.4. Purification of globins. Globins of *E. maclovinus* and *D. eleginoides* were purified by reverse-phase HPLC, Beckman *Gold Nouveau,* on C_4 Vydac (4.6 x 250mm) and on micro-Bondapak-C_{18} (0.39 cm x 30 cm; Waters) columns, respectively, 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 (Tamburrini et al., 1996). Before loading, samples were incubated in a denaturating solution of 5% β-mercaptoethanol and 1% TFA at room temperature.

5.1.5. Amino-acid sequencing. Fractionation of tryptic peptides and subsequent amino-acid sequencing were carried out for *E. maclovinus* and *D. eleginoides* globins. Alkylation of thiol groups with 4-vinylpyridine was carried out as described in (D'Avino and di Prisco G, 1989; Tamburrini et al., 1992; Tamburrini et al., 1996). Sulfydryl groups were treated with 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, Beckman *Gold Nouveau,* on a C_4 Vydac (4.6 x 250mm), 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 at 100°C for a few minutes. Tryptic peptides were purified by reverse-phase HPLC, on a micro-Bondapak-C18 column (0.39 cm×30 cm; Waters), equilibrated with 0.1% TFA in water (Solvent A) and 0.08% TFA in 99.92% acetonitrile (Solvent B); absorbance at 220 nm and 280 nm was monitored. Sequencing was performed using an Applied Biosystems Procise 494 automatic sequencer, equipped with on-line detection of phenylthiohydantoin amino acids.

Nucleotide sequences of *E. maclovinus* and *D. eleginoides* globins were established by cloning and sequence analysis of globin cDNAs: total RNA was isolated from the spleen using TRI Reagent (Sigma). First-strand cDNA synthesis was performed according to the manufacturer's instructions (Promega) using an oligo (dT)-adaptor primer. The β-globin cDNA was amplified by PCR using oligonucleotides designed on the N-terminal regions as direct primers and the adaptor primer as the reverse primer. Amplifications were performed with 2.5 units EuroTaq (EuroClone Genomics), 1X Reaction Buffer, 3 mM MgCl2, 200 mM of each dNTP, Reverse primer 0.6 mM, Forward primer 1.2 mM. The PCR programme consisted of 30 cycles of 1 min at 94°C, 1 min at 38°C (*E. maclovinus*)/40°C (*D.*

eleginoides), 1 min at 72°C, ending with a single cycle of 10 min at 72°C. The N-terminal regions of globins were obtained by amino-acid sequencing. Amplified cDNA was purified and ligated in the pDrive Cloning Vector (Qiagen). *E. coli* (strain TOP 10) was transformed with the ligation mixtures. Standard molecular-biology techniques (Sambrook et al., 1989) were used in the isolation, restriction and sequence analysis of plasmid DNA.

5.1.6. Mass Spectrometry. The molecular mass of S-pyridylethylated α and β chains and of peptides (less than 10 kDa) was measured by MALDI–TOF MS on a PerSeptive Biosystems Voyager-DE Biospectrometry Workstation. Analyses were performed on pre-mixed solutions prepared by diluting samples (final concentration, 5 nmol ml^{-1}) in 4 volumes of matrix, namely 10 mg ml^{-1} sinapinic acid in 30% acetonitrile containing 0.3% TFA (globins), and 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in 60% acetonitrile containing 0.3% TFA (peptides).

5.1.7. Absorption spectrum. To evaluate the oxidation state of Hbs of high- and sub-Antarctic fishes and to calculate their concentration, absorption spectra were registered in the visible region between 350 nm and 700 nm in a spectrophotometer VARIAN Cary 300 by homology with HbA. 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 Hb-CO, 541 (ε = 13.5) and 576 nm (ε = 14.6) for Hb-O_2, due to two $\pi \rightarrow \pi^*$ transitions (Di Iorio, 1981). In order to obtain the fully oxygenated forms, Hbs were previously reduced with a few crystals of sodium dithionite for 1 minute. The side-reaction products and excess reagent were removed on a Sephadex G-25 column (Pharmacia) equilibrated with 10 mM TRIS-HCl pH 7.6. CO binding was achieved by bubbling samples with gas. To obtain deoxygenated spectra, excess solid dithionite was added to the oxygenated Hbs.

5.1.8. Oxygen affinity and Root effect. For each experiment of O_2 binding, an aliquot of CO-Hb stored at 80°C (see paragraph 5.1.3) was thawed, converted to the oxy form by exposure to light and O_2, and immediately used; no oxidation was spectrophotometrically detectable, indicating that final Met-Hb formation was negligible (<2%).

O_2 equilibria were measured in 100 mM HEPES/MES in the pH range 6.25-8.4 at 5°C. O_2-binding isotherms were carried out in 100 mM HEPES pH 7.0 at 10°C. The final Hb concentration was 0.5-1.0 mM on a heme basis. Experiments were performed in duplicate and a standard deviation of ±3% for p_{50} values was calculated. In order to achieve 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). Sensitivity to chloride was assessed by adding NaCl to a final concentration of 100 mM. The effects of ATP were measured at a final ligand concentration of 3 mM, a large excess over the tetrameric Hb concentration.

O_2 affinity (measured as p_{50}) and cooperativity (nHill) were calculated from the linearised Hill plot of log S/(1-S) against log pO_2 at half saturation, where S is the fractional O_2 saturation. The Root effect was determined in 100 mM HEPES/MES in the pH range 6.25-8.4 at room temperature by calculating the mean absorbance difference at three wavelengths (540, 560 and 575 nm) between the spectra at pH 8.4 (fully oxygenated Hb) and pH 6.25, and the spectra after deoxygenation using sodium dithionite. O_2 saturation is given by $A_{540} + A_{575} - 2\ (A_{555})$.

5.1.9. Rebinding kinetics. The laser flash photolysis setup was described previously (Abbruzzetti et al., 2006; Banderini et al., 2004). The CO-rebinding curves were measured by monitoring changes in absorbance at 436 nm or by measuring transient spectra in the Soret band after nanosecond-laser photolysis at 532 nm (Abbruzzetti et al., 2006). The laser pulse repetition rate was kept at 3 Hz. Difference spectra at each time delay, calculated from the kinetic series by subtracting the spectrum of the CO-form at equilibrium, were analyzed by SVD (Henry and Hofrichter, 1992). The SVD analysis was performed using the software MATLAB (The Mathworks, Inc., Natick, MA). The data matrix, **D**, consists of difference absorbance values measured as a function of two variables: the wavelength of the probe beam and the time delay between the photolysis beam and the opening of the gate of the CCD image intensifier. The singular value decomposition of **D** can be written as: $\mathbf{D = USV^T}$, where the columns of **U** are a set of linearly independent, orthonormal basis spectra, the columns of **V** describe the time-dependent amplitudes of these basis spectra, and the matrix **S** is a diagonal matrix of non-negative singular values which describe the magnitudes of the contributions of each of the outer products of the i-th column vectors $U_iV_i^T$ to the data matrix D. On the basis of the singular values and the autocorrelation, only the first two components were retained for further analysis. The time course of the V's was then analyzed with a sum of decays, some of which required the use of a stretching exponent.

5.2. PEGYLATED HEMOGLOBIN FROM *TREMATOMUS BERNACCHII*, A MODEL FOR HEMOGLOBIN-BASED BLOOD SUBSTITUTES

5.2.1. Materials. IMT, HEPES, EDTA, PBS, sodium ascorbate, catalase and the reagents for the Hayashi enzymatic reducing system (Hayashi et al., 1973) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and MAL-PEG (5600 Da-MW) from Nektar Molecule Engineering (Nektar Therapeutics, San Carlos, CA, U.S.A.). All other reagents were of the best available commercial quality.

5.2.2. Purification of hemoglobins. Purification of *T. benacchii* Hb at 98% was achieved by FPLC ion-exchange chromatography as previously described in the paragraph 5.1.3. The human HbA was purified as described previously (Ronda et al., 2008).

5.2.3. Cysteine titration. Preliminarily to PEGylation experiments, the reactivity of the cysteyl residues of *T. bernacchii* Hb was evaluated in both the deoxy- (T) and carboxy- (R) states using 4-PDS (Ainsworth and Treffry, 1974). For the titration under anerobic conditions, the protein solution was incubated in a helium flux until the absorption spectrum shifted to the deoxy-Hb form. A separately deoxygenated stock solution of 4-PDS was anaerobically added.

5.2.4. Hemoglobins PEGylation. The PEGylation reaction was carried out following the protocol published for HbA in aerobic conditions (Caccia et al., 2009; Winslow, 2004). *T. bernacchii* Hb or HbA was treated in the presence of IMT (80 moles/tetramer moles) and then with MAL-PEG 5600 Da-MW (12 moles/tetramer moles), at 10°C to prevent any heme oxidation during the reaction (**Figure 5.1**). *T. bernacchii* Hb was treated in the presence of CO, subsequently removed under O_2 flow before measurements. IMT and MAL-PEG reactions were quenched using lysine

and cysteine in excess, respectively. Less than 5% met-Hb was formed during the reaction. To monitor the PEGylation reaction, small aliquots of the reaction mixture were taken every 10 minutes, and the reaction was quenched by addition of lysine and cysteine in excess. The samples were analyzed by SDS-PAGE. Gels were stained with Coomassie Bio-Safe (R) and scanned using a Bio-Rad GS-800 densitometer. Gel images were evaluated using the Quantity One software (Bio-Rad). Under denaturing conditions, SDS-PAGE applied to PEGylated Hbs was able to separate PEGylated Hb into unmodified globin chains and globin chains with different PEGylation degree (Caccia et al., 2009). To evaluate the homogeneity of the final products, electrophoresis under native conditions was carried out in 8-2% gradient gel and analyzed. Gradient native gels were prepared using a homemade setup (Ronda et al., 2011). The gradient concentration profile can be described by the equation: $\mathbf{D = A + (B-A)e^{-x/c}}$, where **D** is the acrylamide concentration of the mixture, **A** is the acrylamide concentration of the diluent solution, **B** is the initial concentration of acrylamide in the mixing chamber, and **c** is the volume (ml) of B in the mixing chamber. For the gradient gels, we used the following parameters: A = 2%, B = 8%, and c = 15 ml (half of the final gel volume).

5.2.5. Oxygen affinity. O_2-binding curves of human HbA, *T. bernacchii* Hb and their PEGylated derivatives, called PEG-Hb^{oxy} and PEG-*Tb*Hb, respectively, were measured with a modified tonometer (Ronda et al., 2008). Before titration, the stock solutions of the proteins were diluted in a solution containing 100 mM HEPES, 1 mM sodium EDTA, at either pH 7.0 or 8.0, to a final concentration of 30 µM. Sodium ascorbate and catalase were added to final concentrations of 5 mM and 10^3 U/ml, respectively, to prevent significant autoxidation during the measurement. For *T. bernacchii* Hb and PEG-*Tb*Hb, experiments were carried out in the presence and absence of the allosteric effector ATP at a final concentration of 3 mM. For the experiments on *T. bernacchii* Hb and PEG-*Tb*Hb stored in the carbomonoxy form, CO was removed by exposure to pure O_2 for 2 hours prior to titration. The samples where then exposed to O_2 partial pressures ranging from 0 to 760 Torr generated using an Environics 4000 gas mixer and pre-mixed helium/O_2 bottles, at 10°C. Absorption spectra (see paragraph 5.1.7) were collected in the 350-700 nm range using a Cary 4000 (Varian Inc.) spectrophotometer. The O_2 saturation at each partial O_2 pressure was determined by deconvoluting the spectra in the 450-700 nm range to a linear combination of the reference spectra of deoxy-, oxy- and met-Hb, plus a baseline. The deoxy reference spectra were obtained for HbA and *T. bernacchii* Hb in the presence of sodium dithionite, whereas reference spectra for the oxy forms were obtained in pure O_2 in the presence of a reducing system (Hayashi et al., 1973). The nHill and P_{50} were calculated by linear regression of the Hill's plots corresponding to the O_2-binding curves within the saturation range 20-80%.

5.2.6. Rebinding kinetics. The laser flash photolysis setup was described previously (Abbruzzetti et al., 2006; Bruno el al 2007). The measurements were performed using the circularly polarized second harmonic of a Q-switched Nd:YAG laser (Surelite II Continuum) and a cw Xe arc lamp as a monitoring beam. The transient absorbance signals were measured at 436 nm with a 5-stage photomultiplier.

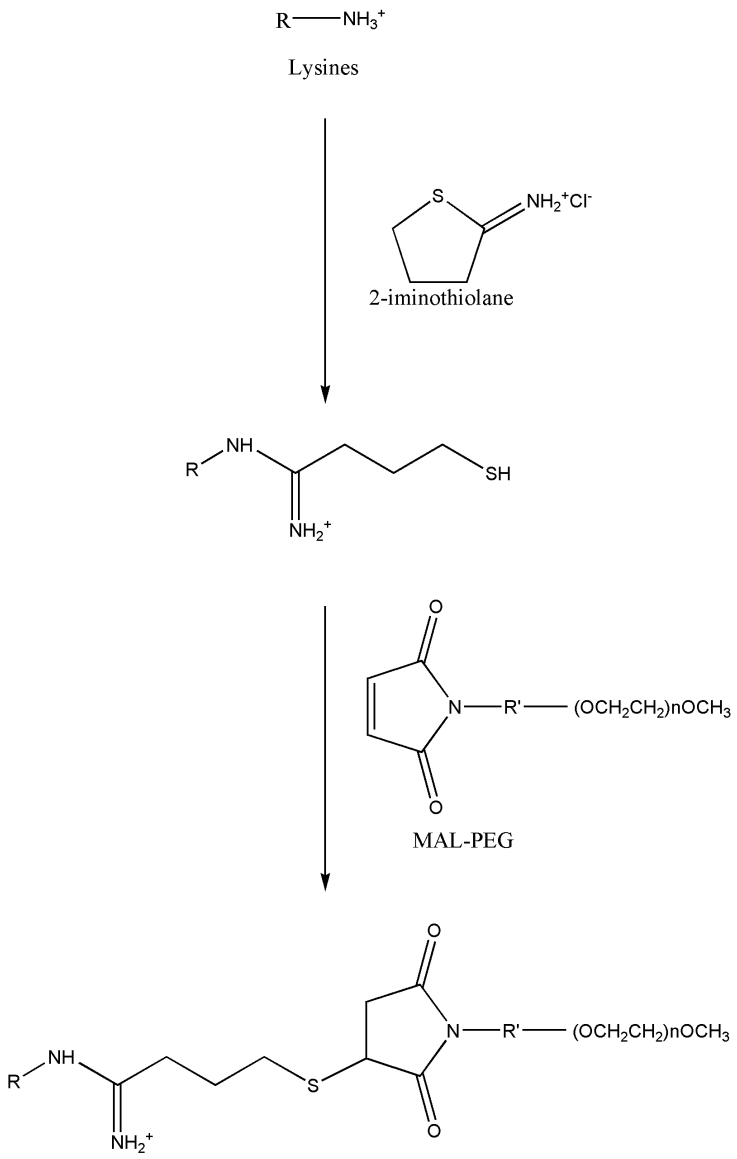

Figure 5.1. Scheme of PEGylation reaction; Hb is first treated with IMT, which adds a spacer arm ending with a thiol group to lysine residues, then the thiols react with MAL-PEG 5000.

5.2.7. NO dioxygenase activity. The rates of the NO dioxygenase reactivity at a single NO concentration were determined for HbA, *T. bernacchii* Hb, PEG-Hb^{oxy} and PEG-*Tb*Hb by rapid mixing using a stopped-flow apparatus (SX.18MV, Applied Photophysics). The NO solutions were generated by equilibrating a previously deoxygenated PBS solution at pH 7.4 with a gas mixture of 0.1% NO in nitrogen. The exact concentration of NO was measured by titration of the solution with deoxygenated HbA under anaerobic conditions. Time courses were collected at 12 μM concentration of NO and 3 μM concentration of protein by following absorbance changes at 405 nm. Between 5 and 10 traces were collected and averaged. All measurements were carried out under strict anaerobic conditions at 20°C.

5.3. INVESTIGATION OF THE ROLE OF *Ph*-2/2HbO IN RESPONSE TO NITROSATIVE STRESS

5.3.1. Strains and culture conditions. The *E. coli* RKP3036 (*hmp*) strain (Poole's Lab) was used for cloning and expressing *PSHAa0030* gene, to test cell survival in the presence of NO-donors and GSNO. The *E. coli* MG1655 wild-type strain and *E. coli* RKP3910 strain (*E. coli* RKP3036 transformed with the pPL341 vector carrying *hmp* gene) were used as positive control. *E. coli* RKP3919 (*E. coli* RKP3036 carrying the empty vector pBAD/HisA) was used as negative control.

Cells were grown in Luria-Bertani (LB) medium at 25°C, under aerobic conditions. When required, Amp 100 μg/ml and Kan 35 μg/ml was added.

5.3.2. Cloning and expression of *PSHAa0030* gene. The *PSHAa0030* gene encoding the protein *Ph*-2/2HbO was obtained from pET28a/trHbO vector (Giordano et al., 2007) and cloned into the L-Arabinose inducible, amp resistant and His-tagged pBAD/HisA vector (Invitrogen). The restriction enzyme cut-sites (NcoI and XhOI) were designed for insertion in pBAD/HisA without the His-Tagged region. The construction was verified by sequencing and named pBAD-2/2HbO.

For the overexpression of cold-adapted *Ph*-2/2HbO gene in *E. coli hmp*, the strain transformed with pBAD-2/2HbO was inoculated in LB medium supplemented with Amp 100 μg/ml and Kan 35 μg/ml. The culture was induced with 0.2 mM δ-aminolevulinic acid; 0.012 mM $FeCl_3$; 0.2% L-Arabinose.

5.3.3. Absorption spectra. Absorption spectra of whole cells of *E. coli hmp* carrying pBAD/HisA and pBAD-2/2HbO, were measured using an SDB-4 dual-wavelength scanning spectrophotometer at room temperature (Kalnenieks *et al.*, 1998) (University of Pennsylvania Biomedical Instrumentation Group, and Current Designs Inc., Philadelphia, PA). Samples were generally scanned at 10 samples per point and a 0.5 nm step size. Data were analysed using SoftSDB (Current Designs) and Sigma Plot 11.0.

Two 250 ml Klett flasks containing 50 ml LB medium, Amp 100 μg/ml, Kan 35 μg/ml and the induction supplements (0.2 mM δ-aminolevulinic acid; 0.012 mM $FeCl_3$; 0.2% L-Arabinose), were inoculated with 500 μl of overnight starter cultures and incubated overnight (around 18 h) at 25˚C, 180 r.p.m., under aerobic condition. Cells were harvested at 5.5 krpm for 15 min at 4˚C and the pellets were re-suspended in 6 ml of sodium phosphate Buffer 0.1 M, pH 7.0.

2.5 ml of whole cells were added to a plastic cuvette. The samples were analysed between 400 nm - 700 nm to have a native spectrum. All absolute spectra were read against a buffer baseline.

5.3.4. *S*-nitrosoglutatione and NO-donors. GSNO was prepared according to the following method (Hart, 1985). To 1.54 g of glutathione, 9 ml of ice-cold dH_2O, 415 µl of concentrated (12.1 M) HCl and 0.345 g of $NaNO_2$ were added and stirred in an ice-filled beaker for 40 min. Acetone (10 ml) was then added and the solution stirred for a further 10 min. The red precipitate was then filtered off and washed with ice cold 1 ml dH_2O 5 times, washed three times with 10 ml acetone and a further 3 times with 10 ml diethyl ether. The precipitate was then dried in a vacuum desiccator overnight. The dried product was stored in a desiccator at room temperature, in the dark. Working stock solutions of GSNO were typically 100 mM concentrations, made by dissolving 33.6 mg GSNO in 1 ml dH_2O.

DETA-NONOate (Enzo Life Science) and PROLI-NONOate (Bioquote Limited) were used as NO-donors. The half-life of DETA-NONOate is 20 h at 37°C and 56 h at 22-25°C in 0.1 M phosphate buffer, pH 7.4. The half-life of PROLI-NONOate is 1.8 sec at 37°C in 0.1 M phosphate buffer, pH 7.4.

5.3.5. GSNO and NO susceptibility test. Coltures of *E. coli hmp* strain, transformed with different plasmids were grown in 2 ml of LB medium, containing amp 100 µg/ml and Kan 35 µg/ml, in plastic universal tubes. Induction supplements: 0.2 mM δ-aminolevulinic acid; 0.012 mM $FeCl_3$; different concentrations of L-Arabinose (0-0.2-0.02-0.0002%) were added to *E. coli hmp* carrying *Ph*-2/2HbO or the empty vector. No addition to the positive control *E. coli hmp* transformed with pPL341 vector. GSNO and DETA-NONOate were added at different concentrations at t = 0. Cultures were incubated overnight (approximately 18 h) in the dark, at 25°C, 180 r.p.m. and under aerobic condition and the optical density at 600 nm was recorded.

5.3.6. Growth curves. 10 ml LB medium in 250 ml Klett flasks, containing Amp 100 µg/ml and Kan 35 µg/ml, were inoculated with 100 µl of overnight starter cultures. Induction supplements (0.2 mM δ-aminolevulinic acid; 0.012 mM $FeCl_3$; L-Arabinose 0.2%) were added to *E. coli hmp* carrying *Ph*-2/2HbO or the empty vector. No addition to the positive control *E. coli hmp* transformed with pPL341 vector. 3 mM GSNO/1 mM Noc-18 were added in each flask, at t = 0. Cells were incubated in the dark, at 25°C, 180 r.p.m. and under aerobic condition.

5.3.7 NO uptake and cellular respiration. 250 mL klett flasks containing 40 ml LB medium, supplemented with Amp 100 µg/ml and Kan 35 µg/ml, were inoculated with 0.4 ml of overnight starter cultures of *E. coli hmp* carrying pBAD-2/2HbO, pBAD/HisA and pPL341. In the first two flasks, the induction supplements (0.2 mM δ-aminolevulinic acid; 0.012 mM $FeCl_3$; 0.2% L-Arabinose) were added. Coltures were incubated overnight (around 18 h) at 25°C, 180 r.p.m., under aerobic conditions. Cells were harvested at 5.5 krpm for 15 min at 4°C and pellets obtained were washed with 10 ml of Tris-Buffer 50 mM, pH 7.5 twice and re-suspended in 5 or 10 ml of same buffer (the volume depends on the pellet size) to normalise the OD of the suspensions.

The respiration rates of whole cells was measured using a Clark-type polarographic O_2 electrode (Rank Bros), operating at a polarising voltage of 0.60 V, consisting of a perspex chamber kept at constant temperature of 25°C using a water jacket around the chamber and stirred magnetically with a membrane-covered electrode situated at the bottom of the chamber (Stevanin et al., 2000). The electrode was calibrated using air-saturated buffer which was then treated with a few grains of sodium dithionite to achieve anoxia.

A World Precision Instruments ISO NOP sensor (2 mm diameter) was used for direct measurement of NO. In all experiments, the NO electrode was used in conjunction with the O_2 electrode, inserted into the Perspex chamber through an adapted lid. The NO electrode was calibrated as described by the manufacturer. Briefly, sequential volumes of solution $NaNO_2$ 50 µM (e.g. 100 µl, 200 µl, 400 µl, 800 µl) were added to 20 ml of 0.1 M H_2SO_4/KI stirring solution, in which the NO electrode was suspended.

After the O_2 and NO electrodes calibration, 200 µl of whole cell suspension was diluted with buffer in the O_2 electrode chamber to a final volume of 2 ml and a tight-fitting lid was applied. The respiration was started using 50 mM glycerol. ProliNONOate 1 µM was added, through a hole in the lid using a Hamilton syringe, at successively lower O_2 concentrations and respiration followed until all the O_2 was depleted in the chamber.

5.3.8. Markwell protein assay. The protein content of whole cells was determined using the protocol of (Markwell et al., 1978). Reagent A (100 parts) (consisting of 20 g Na_2CO_3, 4 g NaOH, 1.6 g sodium tartrate and 10 g SDS per 1 L dH_2O) were mixed with 1 part of reagent B (4% copper sulphate); 3 ml of this was incubated with varying volumes of sample for 1 h at room temperature. Folin-Ciocalteau reagent (0.3 ml) (diluted 1:1 with dH2O) was added followed by further 45 min incubation. Absorbance was read at 660 nm and protein concentrations determined by comparison to a set of BSA standards.

5.3.9. Heme assay. 0.6 ml of sonicated samples (without clarification) + 0.6 ml of reagent (0.4 M NaOH + 4.2 M Pyridine) were added to a quartz cuvette (with stopper), and analysed between 500 nm - 700 nm using an SDB-4 dual-wavelength scanning spectrophotometer. The sample was reduced by adding few grains of sodium dithionite to the cuvette followed by gentle mixing to have a reduced spectrum. A new sample was used to have the oxidised spectrum, by the addition of potassium ferricyanide. A reduced minus oxidised difference spectra was obtained and the concentration of heme calculated from absorbance difference at 556 and 539 nm for the dithionite-reduced and ferricyanide-oxidised samples.

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1. Giordano, D., Russo, R., **Coppola, D.**, di Prisco, G., Verde, C., 2010. Molecular adaptations in hemoglobins of notothenioid fishes. Journal of Fish Biology 75, 301-318.
2. **Coppola, D.**, Giordano, D., Vergara, A., Mazzarella, L., di Prisco, G., Verde, C., Russo, R., 2010. The hemoglobins of sub-Antarctic fishes of the suborder Notothenioidei. Polar Science 4, 295-308.
3. Merlino, A., Vitagliano, L., Balsamo, A., Nicoletti, F.P., Howes, B.D., Giordano, D., **Coppola, D.**, di Prisco, G., Verde, C., Smulevich, G., Mozzarella, L., Vergara, A., 2010. Crystallization, preliminary X-ray diffraction studies and Raman microscopy of the major hemoglobin from the sub-Antarctic fish *Eleginops maclovinus* in the carbomonoxy form. Acta Crystallographica Section F F66, 1536-1540.
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Contributions to 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 extreme environments" Sant Feliu de Guixol, Spain.
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3. **Coppola, D.**, Vergara, A., Viappiani, C., Bruno, S., Russo, R., Merlino, A., Vitagliano, L., Mazzarella, L., di Prisco, G., Verde, C., 2010. Characterisation of hemoglobins of the non-Antarctic notothenioid fishes *Eleginops maclovinus* and Dissostichus eleginoides, International Polar Year Science Conference, Oslo, Norway.
4. **Coppola, D.**, Vergara, A., Nicoletti, F.P., Howes, B.D., Bruno, S., Russo, R., Giordano, D., Ciaccio, C., Merlino, A., Vitagliano, L., Viappiani, C., Coletta, M., Smulevich, G., Mazzarella, L., di Prisco, G., Verde, C., 2010. Structural and functional characterisation of hemoglobins of *Eleginops maclovinus*, a sub-Antarctic notothenioid fish, Proteine 2010, Parma, Italy.
5. **Coppola, D.**, Bruno, S., Ronda, L., Abbruzzetti, S., Viappiani, C., Giordano, D., Mazzarella, L., di Prisco, G., Mozzarelli, A., Verde, C., 2010. Low affinity PEGylated hemoglobins from Antarctic fishes, XVIth International Conference on Oxygen Binding and Sensing Proteins, Antwerp, Belgium.

6. Giordano, D., **Coppola, D.**, Riccio, A., di Prisco, G., Poole, R., Verde, C., 2011. The 2/2 Hemoglobin of Cold-Adapted Bacterium *Pseudoalteromonas haloplanktis* TAC125, CAREX Conference on Life in Extreme Environments, Dublin.

Experience in Italian and foreign laboratories

The work described in this thesis has been carried out at the Institute of Protein Biochemistry (IBP), National Research Council (CNR), Naples, under the supervision of Dr. Cinzia Verde, with the exception of some periods spent in other Institutions (see below):

March - May 2010: Visiting Scientist at University of Parma, under the supervision of Prof. Cristiano Viappiani (Department of Physics) and Prof. Andrea Mozzarelli (Biochemistry and Molecular Biology Department), to study (i) the kinetic properties of the hemoglobin from Antarctic fishes and (ii) their chemical modification (e.g. PEGylation).

September 2010 - July 2011: Visiting Scientist at University of Sheffield, under the supervision of Prof. Robert Poole (Molecular Biology and Biotechnology Department), to study globins from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 and their involvement in the nitrosative stress.

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REVIEW PAPER

Molecular adaptations in haemoglobins of notothenioid fishes

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Since haemoglobins of all animal species have the same haem group, differences in their properties, including oxygen affinity, electrophoretic mobility and pH sensitivity, must result from the interaction of the prosthetic group with specific amino-acid residues in the primary structure. For this reason, fish globins have been the subject of extensive studies in recent years, not only for their structural characteristics, but also because they offer the possibility to investigate the evolutionary history of these ancient molecules in marine and freshwater species living in a great variety of environmental conditions. This review summarizes the current knowledge on the structure, function and phylogeny of haemoglobins of notothenioid fishes. On the basis of crystallographic analysis, the evolution of the Root effect is analysed. Adaptation of the oxygen transport system in notothenioids seems to be based on evolutionary changes, involving levels of biological organization higher than the structure of haemoglobin. These include changes in the rate of haemoglobin synthesis or in regulation by allosteric effectors, which affect the amount of oxygen transported in blood. These factors are thought to be more important for short-term response to environmental challenges than previously believed.

Key words: Antarctic fish; biology; ecology; evolution; haemoglobin; molecular adaptation.

THE ENVIRONMENT AND NOTOTHENIOIDEI

The attention of physiologists and ecologists has long been attracted by environments that lie at the limits of the physical conditions capable of supporting life. In particular, the polar regions demand striking adaptations at the molecular, cellular or whole-organism level to allow organisms to survive, grow and reproduce (Clarke *et al.*, 2007*a*).

Antarctica, more than any other habitat on Earth, offers a unique natural laboratory for fundamental research on the evolutionary processes that shape biological diversity. The variety of adaptations underlying the ability of modern Antarctic fish to survive at the freezing temperatures of the environment represents the extreme of low temperature adaptations among vertebrates.

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Over the past million years, the Antarctic shelf has been subjected to tectonic and oceanographic events that began to alter the composition of the fish fauna and to initiate the process of faunal replacement (Clarke & Crame, 1992). Fragmentation of Gondwana into the modern southern continents and the displacement of the Antarctic continent to its current geographic location have been the most significant events responsible for these changes. The crucial opening of the Drake Passage between southern South America and the Antarctic Peninsula occurred 23·5–32·5 million (M) years before present (M B.P.) (Thomson, 2004) and possibly even as early as 41M B.P. (Scher & Martin, 2006). The Drake Passage lead to the development of the Antarctic Circumpolar Current (ACC) and this in turn was at least partially responsible for cooling of Antarctic waters from *c*. 20° C to the present extreme values near −1·8° C (Clarke, 1983).

The Antarctic Polar Front (APF), the northern boundary of the ACC, is a well-defined, roughly circular oceanic system, running between 50 and 60° S (Fig. 1). Along the APF, the surface layers of the north-moving Antarctic waters sink beneath the less cold and less dense sub-Antarctic waters, generating virtually permanent turbulence. Just north of the APF, the water temperature has an abrupt rise of *c*. 3° C, a critical factor for the isolation and adaptation of the ecosystem. The APF

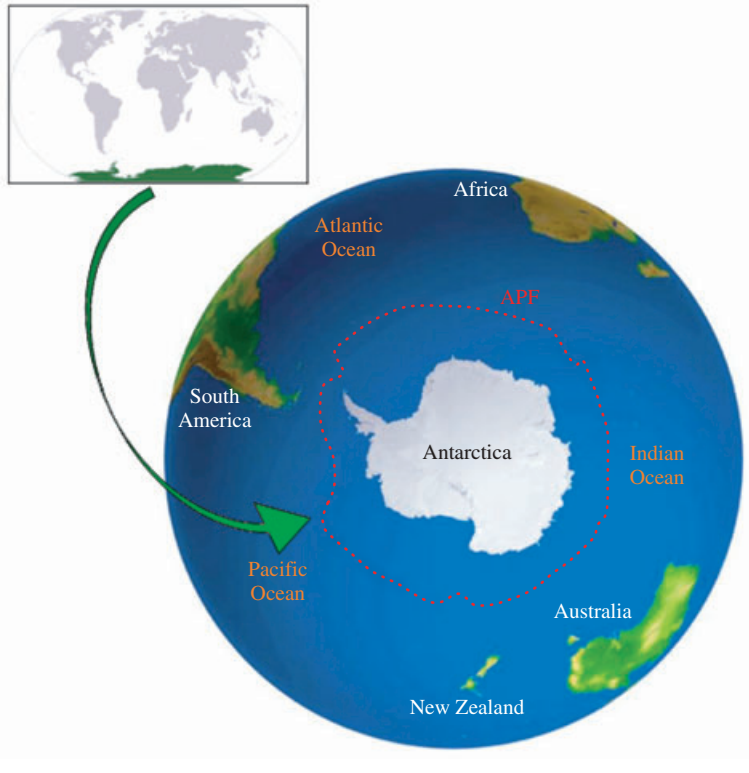


FIG. 1. Geographical location of Antarctica. The continent is surrounded by South America, Africa, Australia and New Zealand. The dashed lines denotes the Antarctic Polar Front, the northern boundary of the Antarctic Circumpolar Current, running between 50 and 60° S.

greatly limited the opportunities for migration, forcing the shallow-water fish fauna to either adapt to the changing climate or become extinct.

One group of teleost fish, the suborder Notothenioidei, became largely dominant as a consequence of success in adapting to the challenging environmental conditions, *e.g.* low temperature, sea ice, habitat reduction and seasonality of primary production (Clarke & Johnston, 1996). The ancestral notothenioid stock probably arose as a sluggish, bottom-dwelling teleost species that evolved some 40–60M B.P. in the shelf waters (temperate at that time) of the Antarctic continent and experienced extensive radiation, dating from the late Eocene, *c*. 24M B.P. (Near, 2004). Convincing arguments provided by Eastman & McCune (2000) have considered notothenioids as one of the very few examples of marine-species flock, due to the geographic, thermal and hydrologic isolation of the Antarctic shelf.

The perciform suborder Notothenioidei is the dominant component of the Southern Ocean fauna. High-Antarctic notothenioids are stenothermal (Eastman, 1993, 2005). They live at the freezing point of sea water (near $-1{\cdot}9^{\circ}$ C) and die at temperatures of $4–6^{\circ}$ C. High-Antarctic notothenioids are distributed south of the APF. Non-Antarctic notothenioids, which comprise sub-Antarctic as well as temperate species, are found north of the APF and compose 22% (28 of 129 species) of notothenioid biodiversity (Eastman, 2005). Notothenioids exhibit considerable morphological and ecological diversity and on the high-latitude shelves they account for 77% of the fish diversity, 92% of abundance and 91% of biomass (Eastman, 2005). This level of dominance by a single taxonomic group is unique among piscine shelf fauna of the world.

Bovichtidae, Pseudaphritidae, Eleginopidae, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae and Channichthyidae are the families of the suborder (Eastman, 2005). All Bovichtidae (except one species), monotypic Pseudaphritidae and Eleginopidae and some species of Nototheniidae inhabit waters north of the APF (Fig. 2). Molecular phylogeny has recently begun to provide indications about the time of radiation in the Antarctic. Initial divergence took place *c*. 40M B.P. during the Eocene (Near, 2004). In fact, Bovichtidae, Pseudaphritidae and Eleginopidae essentially never experienced near-freezing water temperatures because they presumably diverged and became established in waters around areas corresponding to New Zealand, Australia and South America before Antarctica became isolated.

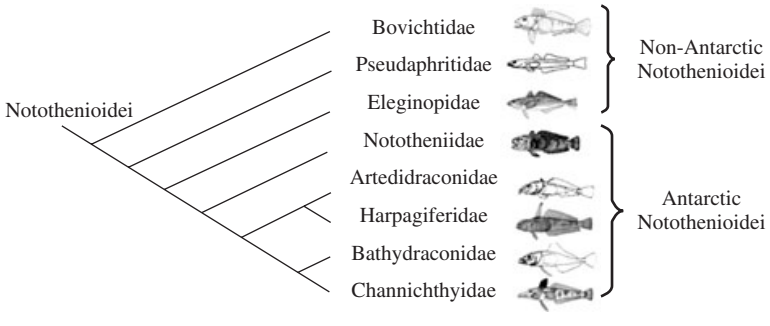


FIG. 2. Families of the suborder Notothenioidei; Bovichtidae (except one species), monotypic Pseudaphritidae and Eleginopidae are non-Antarctic.

The absence of any detectable antifreeze glycoprotein (AFGP) coding sequence, important for enabling survival as the ocean chilled to sub-zero temperatures, in some non-Antarctic species, *i.e. Bovichtus variegatus* Richardson, *Pseudaphritis urvillii* (Valenciennes) and *Eleginops maclovinus* (Cuvier), is consistent with this hypothesis (Cheng *et al*., 2003). The finding of the genes but lack of AFGPs in another temperate notothenioid, the more recent nototheniid *Notothenia angustata* Hutton (Cheng *et al*., 2003) supports the hypothesis that this species had developed cold adaptation before migration from the Antarctic continental shelf to temperate latitudes that occurred much later than other non-Antarctic species.

The availability of phylogenetically related notothenioid taxa living in a wide range of latitudes (in the Antarctic, sub-Antarctic and temperate regions) is a tool to potentially detect adaptive characters in response to climate change.

Climate change affects marine, terrestrial and limnetic polar systems. The great constancy of water temperature in the Southern Ocean has supported the evolution of stenothermal animal life, for the sake of an energy efficient mode of life in the cold (Pörtner, 2006). Stenothermal marine species appear particularly vulnerable to even small increases in temperature (Cook *et al*., 2005; Clarke *et al*., 2007*b*). Thus, the rate of current changes in relation to the capacity of extant species to acclimate or adapt is a crucial study area for the future management of polar ecosystems.

MOLECULAR ADAPTATIONS IN NOTOTHENIOIDS

In the process of cold adaptation, the evolutionary trend of Antarctic fish has led to unique specializations in many biological features in comparison with temperate and tropical species.

Examples include efficient microtubule assembly at temperatures as low as $-1{\cdot}9^{\circ}$ C (Detrich *et al*., 1989, 2000; Redeker *et al*., 2004), apparent loss of inducible heat-shock response (Hofmann *et al*., 2000; Place *et al*., 2004; Place & Hofmann, 2005), still possessed by some of non-Antarctic notothenioids (Hofmann *et al*., 2005), enzyme-structural constraints (Fields & Somero, 1998; Russell, 2000; Hochachka & Somero, 2002; Collins *et al*., 2003; D'Amico *et al*., 2003; Feller & Gerday, 2003; Fields & Houseman, 2004; Johns & Somero, 2004), decreased membrane fluidity (Römisch *et al*., 2003), constraints in aerobic energy supply, mitochondrial functioning and the capacity of anaerobic energy production (Johnston *et al*., 1998; Pörtner, 2006) and higher levels of ubiquitin-conjugated proteins in tissues as evidence for cold denaturation of proteins *in vivo* (Todgham *et al*., 2007). Recently, the complete loss of the nicotinamide adenine dinucleotide (NADH) 6 dehydrogenase mitochondrial gene was reported for Antarctic notothenioids (Papetti *et al*., 2007), particularly surprising in the light of the fact that its absence was never reported in any other animal mitochondrial genome.

The biosynthesis of AFGPs is one of the most intriguing evolutionary adaptations discovered in the Antarctic fish. AFGPs allow to avoid freezing by binding water, thus preventing growth of ice crystals in the blood and other body fluids (DeVries, 1988; Cheng & DeVries, 1991). Produced by pancreatic tissue and the anterior portion of the stomach (Cheng *et al*., 2006), AFGPs are a family of polymers composed of a glycotripeptide monomeric repeat, -Thr-Ala-Ala-, with each Thr linked to the disaccharide galactose-*N*-acetylgalactosamine (DeVries, 1988; Cheng & DeVries, 1991).

High-Antarctic notothenioids have ample gene families for the production of large amounts of AFGPs. In non-freezing environments, where the antifreeze function becomes nonessential, the AFGP function is reduced as observed in non-Antarctic notothenioid fishes. In *N. angustata* and *Notothenia microlepidota* Hutton, living in cool temperate waters, the AFGP system is reduced with very low blood AFGP concentration and only two to three genes showing some replacements in the functional repeat -(Thr-Ala-Ala)- (Cheng *et al*., 2003). *Dissostichus eleginoides* Smitt, a non-Antarctic notothenioid of the family Nototheniidae, appears to have no functional AFGP sequences, consistent with its non-Antarctic distribution. The apparent absence of AFGP genes in *D. eleginoides*, however, is intriguing because the AFGP gene was thought to have evolved once, before the Antarctic notothenioid radiation, at the base of the family Nototheniidae. The hypothesis is that the species had the primordial AFGP genotype, lost or mutated following its migration to non-Antarctic habitats (Cheng *et al*., 2003).

The study of freezing avoidance in Notothenioidei is now developing along new perspectives, linked to the recent discovery of AFGP-deficient, but freeze-resistant, notothenioids in early life stages (Cziko *et al*., 2006). The absence of AFGP production in larvae suggests that suitable freezing resistance may temporarily be afforded by alternative mechanisms.

Specialized haematological features are striking adaptations developed by the Antarctic ichthyofauna during evolution at low temperature. In the seven red-blooded notothenioid families, the erythrocyte number is an order of magnitude lower than in temperate fish and is reduced by over three orders of magnitude in the 16 icefish species of Channichthyidae (Eastman, 1993), the most phyletically derived family, whose blood lacks haemoglobin (Hb) (Ruud, 1954). Icefish retain genomic DNA sequences closely related to the adult α-globin genes of its red-blooded notothenioid ancestors and contemporaries, whereas its ancestral β-globin-gene sequences have been deleted (Cocca *et al*., 1995; Zhao *et al*., 1998; di Prisco *et al*., 2002). The discovery within the icefish family of two distinct genomic re-arrangements, both leading to functional inactivation of the locus, seems to point towards a multistep mutational process (Near *et al*., 2006).

In channichthyids, no carrier has replaced Hb and the oxygen-carrying capacity of the blood is only 10% that of red-blooded fish. They cope with the lack of an oxygen carrier with increased blood volume and higher cardiac output (Egginton *et al*., 2002); they have large gills and highly vascularized, scaleless skin, which favours cutaneous respiration. Recent studies highlight how the loss of Hb and their associated nitrogen monoxide–oxygenase activity may have favoured the evolution of these compensatory adjustments (Sidell & O'Brien, 2006). Although there is little doubt about the adaptive value of AFGPs, the evolutionary meaning of the deletion of the entire β-globin gene and parts of the α-globin gene observed in icefish is controversial (Sidell & O'Brien, 2006). It can be argued that such a unique feature is only possible in the oxygen-rich Antarctic waters, whereas it would be deleterious, if not lethal, in warmer waters. This view is in agreement with Near *et al*. (2006), who noted that Hb loss in icefish did not appear to be selectively neutral but is rather maladaptive, as indicated by the development of compensatory adaptations that enhance oxygen delivery, such as cutaneous uptake of oxygen and decreases in metabolic oxygen demand.

The loss of Hb in icefishes is paralleled by the loss of myoglobin (Mb) in six icefish species through at least four mutational events (Sidell *et al.,* 1997; Grove *et al.,* 2004; Sidell & O'Brien, 2006). Despite the costs associated with loss of these haemoproteins, the constantly cold and oxygen-saturated waters of the Southern Ocean provided an environment in which fish are able to survive even without oxygen-binding proteins.

Recent evidence suggests that at least three of the adaptations evolved by icefish, expansion of tissue capillarity density, enlargement of the heart and increases in mitochondrial densities in the heart, may be due to the homeostatic responses mediated by nitrogen monoxide. The loss of Hb, together with enhanced membrane-lipid densities (accompanied by high concentrations of mitochondria), becomes explicable by the exploitation of high oxygen solubility and low metabolic rates in the cold, where an enhanced fraction of oxygen supply occurs through diffusive flux.

Mb has also been lost in many notothenioids, at least in certain tissues. No notothenioid has Mb in its skeletal locomotory muscle, a loss of tissue-specific gene expression that must have occurred early in the notothenioid radiation some 7–15M B.P. (Sidell *et al*., 1997). Unlike Hb, where a gene has disappeared from the genome, the gene encoding Mb is present in all icefish, even though in some cases the reading frame is disrupted, whereas in other cases the gene is transcribed but the message is not translated into protein (Grove *et al*., 2004).

Recently, the genome-wide investigations of transcriptional and genomic changes associated with cold adaptation of Antarctic notothenioids have been reported (Chen *et al*., 2008). Through comparative analysis of same-tissue transcriptome profiles of *Dissostichus mawsoni* Norman and temperate–tropical teleost fishes, Chen *et al*. (2008) identified 177 notothenioid protein families involved in mitigating stresses at freezing temperatures that were expressed many fold over temperate fishes, indicating cold-related upregulation. Further examination of the genomic and evolutionary bases for this upregulation demonstrated that evolution under constant cold has resulted in augmenting gene expression and gene functions contributing to the physiological fitness of Antarctic notothenioids in freezing polar conditions (Chen *et al*., 2008).

THE HAEMATOLOGY IN NOTOTHENIOIDS

FISH HAEMOGLOBINS

Fish Hbs are particularly interesting because the respiratory function of fish differs from that of mammals. In fish, gills are in contact with a medium endowed with high oxygen tension and low carbon dioxide tension; in contrast, in the alveoli of mammalian lungs, the carbon dioxide tension is higher and the oxygen tension is lower than that in the atmosphere. The capacity of fish to colonize a large variety of habitats appears strictly related to the molecular and functional differences of their Hb system.

As in mammals, fish Hb is a hetero-tetramer having two identical pairs of α- and β-globins (Fig. 3).

The overall affinity of Hb for oxygen is expressed as the gas partial pressure required to achieve half-saturation (p_{50}). The cooperative ligand binding in the Hbs of higher vertebrates, expressed in the molecule because the binding of the

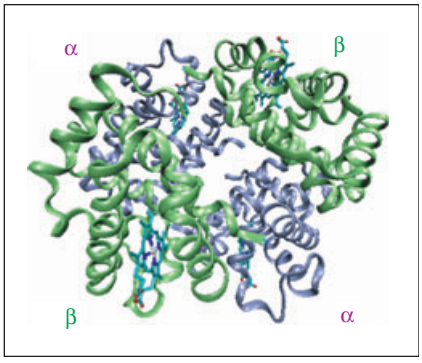


FIG. 3. Three-dimensional structure of emerald rockcod *Trematomus bernacchii* haemoglobin (pdb code 2h8D) (modified from Mazzarella *et al*., 2006*b*).

oxygen with one haem facilitates the binding of additional oxygen to the other haem sites, has important physiological consequences, because it allows oxygen to be efficiently released at relatively high oxygen partial pressure. During evolution, complex and sophisticated molecular mechanisms, such as effects of pH, carbon dioxide, organophosphates and temperature, have been developed to regulate oxygen transport by Hb in higher vertebrates.

Fish commonly exhibit pronounced Hb multiplicity with marked differences in the oxygen-binding properties and in their sensitivities to allosteric effectors, a differentiation that may serve to adapt oxygen transport to environmental variations and metabolic requirements (Weber, 1990; di Prisco & Tamburrini, 1992; Feuerlein & Weber, 1994; Weber *et al*., 2000; Fago *et al*., 2002). Hb multiplicity is usually interpreted as a sign of phylogenetic diversification and molecular adaptation, resulting from gene-related heterogeneity and gene-duplication events.

Red-blooded Antarctic notothenioids differ from temperate and tropical species in having fewer erythrocytes and reduced Hb concentration and multiplicity (none in channichthyids). The Hb content of erythrocytes is variable and in some species seems positively correlated with life style (Eastman, 1993). The vast majority of high-Antarctic notothenioid species have a single Hb (Hb 1), accompanied by minor Hbs (Hb C in trace amounts, and Hb 2, *c*. 5% of the total), having one of the globins in common with Hb 1 (di Prisco, 1998). High-Antarctic notothenioids have lost globin multiplicity, leading to the hypothesis that in the Antarctic thermostable environment the need for multiple Hbs may be reduced (Verde *et al*., 2006*a*, *b*). In comparison with high-Antarctic notothenioids, Hbs of many non-Antarctic notothenioids display higher multiplicity (di Prisco *et al*., 2007). It has been suggested that Hb multiplicity is more frequently found in fish that must cope with variable temperatures, whereas the presence of a single dominant Hb is usually associated with stable temperature conditions. This may explain why high-Antarctic notothenioids have a single major Hb, while sub-Antarctic and temperate notothenioids, such as *Cottoperca gobio* (Günther) and *Bovichtus diacanthus* (Carmichael) (family Bovichtidae) respectively, retained Hb multiplicity, presumably to cope with the small or large temperature changes in the respective habitats north of the APF (di Prisco *et al*., 2007).

Although a report (Sidell & O'Brien, 2006) challenges the ensuing hypothesis, the reduction in Hb content and multiplicity and erythrocyte number in the blood of high-Antarctic notothenioids is likely to counterbalance the potentially negative physiological effects (*i.e.* higher demand of energy needed for circulation) caused by the increase in blood viscosity produced by sub-zero seawater temperature.

The oxygen affinity of Hbs of many high-Antarctic species is quite low (di Prisco *et al.*, 2007), as indicated by the values of p_{50}. This feature is probably linked to the high oxygen concentration in the cold sea. In contrast, the affinity is higher in Hbs of the non-Antarctic notothenioids. The relationship between high affinity of non-Antarctic notothenioid Hbs and habitat features remains an open question as far as its structural basis is concerned. In fact, spectroscopic and modelling studies on Hb 1 of the temperate notothenioid *P. urvillii* have shown that all the non-conservative replacements in the primary structure of the α and β chains leave the conformation and electrostatic field surrounding the haem pocket essentially unmodified with respect to Hb 1 of the high-Antarctic *Trematomus bernacchii* Boulenger (Verde *et al.*, 2004*a*; Mazzarella *et al.*, 2006*b*).

THE ROOT EFFECT

The decreased oxygen affinity of Hb at lower pH values in the physiological range is known as alkaline Bohr effect (reviewed by Riggs, 1988), whose importance is to enable the animal to exchange oxygen and carbon dioxide at both lung or gill and tissue levels. In many Hbs of teleost fishes, the complete loss of cooperativity (indicated by a Hill coefficient equal to one), thus the inability to saturate the ligand sites at low pH even at high oxygen pressure, is a distinctive property with respect to the Bohr effect and is referred to as Root effect (reviewed by Brittain, 1987, 2005). The Root effect is responsible for a physiologically important response to lactic acidosis, which may induce complete oxygen unloading. The physiological significance of Root effect Hbs has been linked to the presence of at least one of two anatomical structures that require high oxygen pressure: the rete mirabile which supplies the gland that inflates the swimbladder with oxygen, and the choroid rete mirabile, a vascular structure that supplies oxygen to the poorly vascularized retina (Wittenberg &Wittenberg, 1974) (Fig. 4). Antarctic fish lack the swimbladder, and it is worth mentioning that only the few species possessing Hbs without a Root

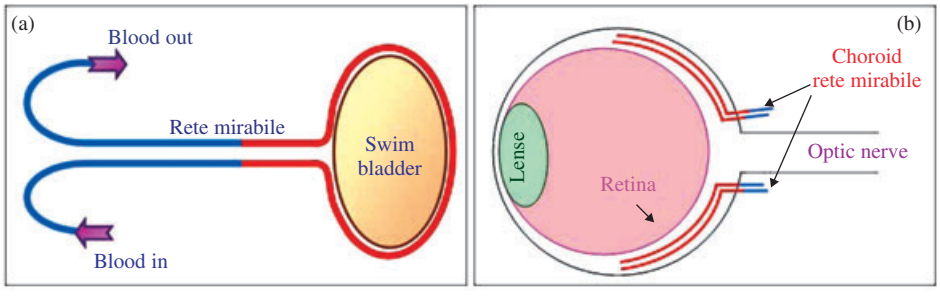


FIG. 4. Swimbladder (a) and choroid (b) retia mirabilia in fish. The retia are part of counter-current exchange systems. The acidic blood is in red, indicating oxygen release due to the Root effect.

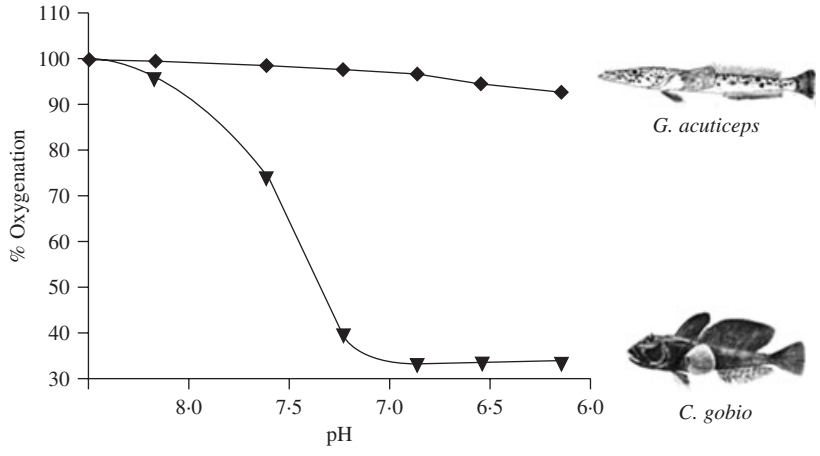
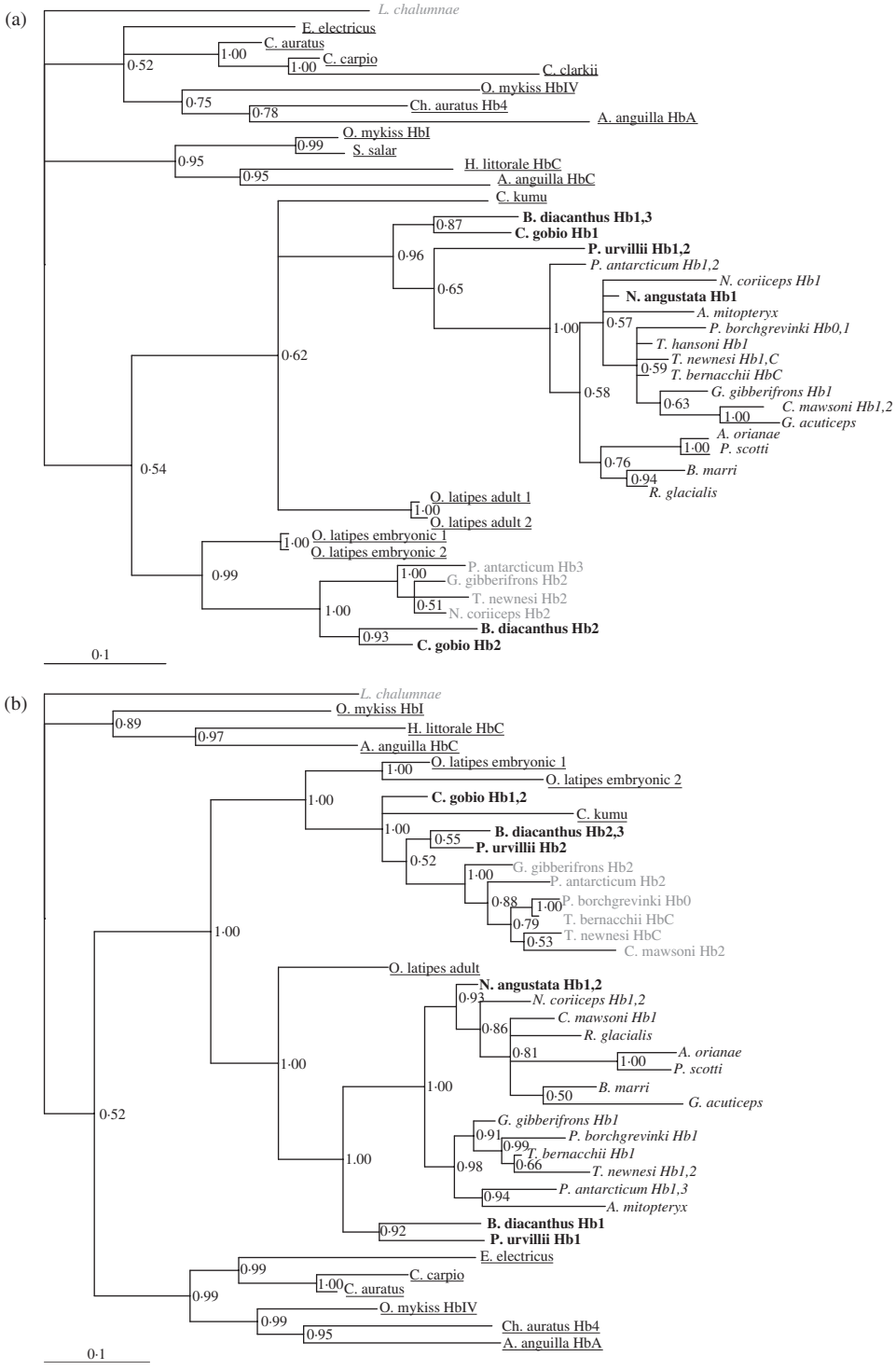


FIG. 5. Root effect of haemoglobins of the sub-Antarctic notothenioid *Cottoperca gobio* (Giordano *et al*., 2009), having the choroid rete, and the high-Antarctic notothenioid *Gymnodraco acuticeps* (Tamburrini *et al*., 1992), lacking the choroid rete.

effect, as well as those of the family Channichthyidae, are devoid of the choroid rete (Eastman, 1988). A general reduction in the Root effect is noticed during the evolution of the Antarctic notothenioids (di Prisco *et al*., 2007) (Fig. 5). Because high-Antarctic notothenioids still have Hbs endowed with Root effect also when the choroid rete is absent, this function may undergo neutral selection. Regarding neutrality, it has been argued that the possession of the Root effect may undergo neutral selection pressure in the simultaneous absence of retia mirabilia and presence of high Hb buffer capacity, as in some basal ray-finned fishes and in the ancestors of teleosts (Berenbrink *et al*., 2005; Berenbrink, 2007). This may generally also be the case in notothenioids with increased Hb surface–His content (Verde *et al*., 2008), because a role for some His residues as modulators of the Root effect has recently been postulated (Mazzarella *et al*., 2006*a*, see below). It remains to be shown to what extent each of these substitutions is the mechanistic cause of the reduced Root effect or the consequence of an altered selection pressure on Hb buffer properties once the Root effect was diminished (Verde *et al*., 2008).

Whatever the answer to the above questions may turn out to be, it seems that the multiple losses of the ocular oxygen-secretion mechanism in notothenioids are not necessarily associated with degenerate eyes or less visually oriented life styles, as an alternative oxygen supply route to the retina by a system of hyaloid capillaries is especially well developed in several notothenioid species that have lost the choroid *rete* (Eastman & Lannoo, 2004; Wujcik *et al*., 2007).

The study of the molecular bases of the Root effect has been tackled by many scientists over many years. Primary structures and analytical methods adopting an evolutionary perspective have provided useful indications on the physiology and evolution of the Root effect in fishes, but no unequivocal answer to the question of the structural implications. In recent years, X-ray crystallography succeeded in overtaking one of the classical views, which attempts to correlate all major changes in Hb function with a few residue substitutions, thus significantly contributing to



address the question in some instances. The extensive structural analysis of the Root effect by X-ray crystallography of Antarctic fish Hbs was stimulated by the good capacity to crystallize, together with the high sequence identity. These structural properties by themselves, however, are not sufficient to explain the presence of the Root effect. The current hypothesis is based on overstabilization of the T state, mainly induced by the inter-Asp hydrogen bond at the α_1/β_2 interface (Mazzarella *et al*., 2006*a*), possibly modulated by salt bridges involving histidyl residues (Mazzarella *et al*., 2006*b*).

Within the realm of tetrameric Hbs, there is one aspect in which Antarctic fish Hbs are exceptional. Although structurally and functionally analogous to mammalian Hbs, Antarctic fish Hbs follow a peculiar oxidation pathway when exposed to air or treated with chemical agents. The α and β chains of these proteins undergo distinct oxidation processes. Particularly unusual is the strong tendency of the β chains to form hexacoordinated bis-histidyl adducts in the ferric state (hemichrome) (Riccio *et al*., 2002; Vitagliano *et al*., 2004, 2008). Interestingly, the finding that Antarctic fish Hbs may assume states, such as hemichrome typically associated with Hbs with lower complexity, demonstrates that such states are also accessible to tetrameric Hbs. The bis-His co-ordination in the ferrous state, namely haemochrome (Rifkind *et al*., 1994), however, has never been observed. Thus, upon reduction, the hemichrome species is reversibly converted to the classical penta-co-ordinated deoxy form (Vitagliano *et al*., 2004). As yet, there is no clear understanding of the molecular constraints that prevent haemochrome formation in Antarctic fish Hbs. Current work by Mazzarella's team, based on a combined approach involving electron paramagnetic resonance (EPR) and crystallography, suggests a correlation between Root effect and hemichrome stability in Antarctic fish Hbs (Vergara *et al*., 2009).

MOLECULAR EVOLUTION AND PHYLOGENY

Four major α- and β-globin groups have been proposed in teleost fishes (Maruyama *et al*., 2004), *i.e.* Embryonic Hb Group (I), Notothenioid Major Adult Hb Group (II), Anodic Adult Hb Group (III) and Cathodic Adult Hb Group (IV) [Fig. 6(a), (b)] (Maruyama *et al*., 2004). Groups I and IV are still present in *Oryzias latipes* (Temminck & Schlegel) whereas Groups II and III were lost during the evolution of this species. Group I and IV are respectively located on chromosomes 8 and 19.

FIG. 6. Phylogenetic trees of fish α and β-globins (modified from di Prisco *et al*., 2007). The trees for α (a) and β-globins (b) were inferred by a Bayesian method, using the software Mr Bayes with mixed model. Antarctic notothenioid major globins are in italic, Antarctic notothenioid minor globins are in dark grey, non-Antarctic notothenioid globins in bold and temperate globins underlined. Full species names: *Latimeria chalumnae, Electrophorus electricus, Carassius auratus, Cyprinus carpio, Catostomus clarkii, Oncorhynchus mykiss, Chrysophrys auratus, Anguilla anguilla, Salmo salar, Hoplosternum littorale, Chelidonichthys kumu, Oryzias latipes, Notothenia coriiceps, Notothenia angustata, Pleuragramma antarcticum, Pagothenia borchgrevinki, Gobionotothen gibberifrons, Aethotaxis mitopteryx, Trematomus newnesi, Trematomus bernacchii, Cygnodraco mawsoni, Gymnodraco acuticeps, Racovitzia glacialis, Bathydraco marri, Pogonophryne scotti, Artedidraco orianae, Cottoperca gobio, Bovichtus diacanthus, Pseudaphritis urvillii, Eleginops maclovinus.*

According to previous results (Verde *et al*., 2004*a*, *b*), globin paralogues (*e.g.* gene copies originated by duplication in a given genome) currently found in Antarctic fish diverged *c*. 250M B.P.; hence, unlike AFGP, whose appearance coincided with cooling of the Antarctic continent (Chen *et al*., 1997), Hb diversification in major and minor groups appears less stringently correlated to changes in the environmental conditions. The time of the gene-duplication event that gave origin to the two paralogous groups of major and minor Hbs is similar, suggesting that they diverged long before the first stock of ancestral notothenioids.

The phylogenetic trees of the amino-acid sequences of α and β chains of Hbs of non-Antarctic notothenioids (temperate and sub-Antarctic), high-Antarctic notothenioids and temperate fish Hbs, obtained using a Bayesian method, are reported [Fig. 6(a), (b)] (di Prisco *et al*., 2007). The globins of major and minor Antarctic fish Hbs cluster in two separate, strongly supported groups, with the anodic and cathodic globins of temperate fish Hbs forming the first divergence lineage. The globin-gene trees are in agreement with the species trees obtained by sequence studies on mitochondrial RNA (Ritchie *et al*., 1996) and give strong support to the monophyly of Antarctic notothenioids, with non-Antarctic *C. gobio* as their sister taxon. The position of the *C. gobio* globins appears congruent with the phylogenetic evidence from nuclear and mitochondrial genes (Bargelloni *et al*., 2000), suggesting that *C. gobio* is the sister taxon of *P. urvillii*, *E. maclovinus* and also of the Antarctic notothenioids.

The obtained topology is in general agreement with the maximum-likelihood method (Giordano *et al*., 2006) and the hypothesis of four globin groups (Maruyama *et al*., 2004). According to this hypothesis, α-globins that belong to notothenioid minor Hbs are included in the 'Embryonic Hb Group' and those of the major notothenioid Hbs are grouped into the 'Notothenioid Major Adult Hb Group'. All β-globin sequences from the major Hb 1 components (shared by Hb 1 and Hb 2 in most Antarctic notothenioids) belong to the 'Notothenioid Major Adult Hb Group', β-globins that belong to notothenioid minor Hbs are included in the 'Embryonic Hb Group', with good bootstrap support.

In the phylogenetic trees, the basal position of *P. urvillii* Hbs appears congruent with the postulated divergence before the appearance of AFGPs. The α chain of *P. urvillii*, shared by Hb 1 and Hb 2, branches off the clade of the major Antarctic Hbs, and the same applies to the β chain of Hb 1. The β chain of *P. urvillii* Hb 2 is in a basal position with respect to the clade of the Antarctic minor Hbs (Verde *et al.,* 2004*a*). The α chain of *C. gobio* Hb 1 also branches off the clade of the major Antarctic Hbs, whereas the β chain shared by Hb 1 and Hb 2 is included in the clade of the minor Antarctic Hbs (Giordano *et al*., 2006).

In the majority of notothenioids, embryonic α and β-globins are expressed in trace or limited amounts in the adult stage, although in at least three species, namely *Trematomus newnesi* (D'Avino *et al*., 1994), *Pagothenia borchgrevinki* (Boulenger) (Riccio *et al*., 2000) and *Pleuragramma antarcticum* Boulenger (Tamburrini *et al*., 1996), embryonic globins are expressed at significant levels (*c*. 25% of the total). In these three species, however, the largest fraction of β chains is included in the 'Notothenioid Major Adult Hb Group'. A complete switch to exclusive expression of the embryonic β-globin gene seems to be occurred in adult *C. gobio* (Giordano *et al*., 2006).

CONCLUDING REMARKS

Gene expression patterns and, even more so, loss of genetic information, especially for Mb and Hb in notothenioid fishes, reflect the specialization of Antarctic organisms to a narrow range of low temperatures. These modifications become explicable by the exploitation of high oxygen solubility at low metabolic rates in the cold, where an enhanced fraction of oxygen supply occurs through diffusive oxygen flux (Pörtner *et al*., 2007). Conversely, limited oxygen supply to tissues upon warming is an early cause of functional limitation (Pörtner *et al*., 2007).

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 Hb loss include reduced costs for protein synthesis. As pointed out by Pörtner *et al*. (2007), however, the shift from Hb-mediated oxygen transport to mechanisms based on diffusion may account for higher vulnerability of icefishes and of notothenioids in general, to warmer temperatures.

The southern polar environment is experiencing significant climatic change, as shown by sea-ice reductions on the western side of the Antarctic Peninsula (Clarke *et al*., 2007*b*). Species such as Antarctic notothenioid fishes that have had a long evolutionary history at constant temperatures may be uniquely vulnerable to Global Warming (Somero, 2005).

Recently, Chen *et al*. (2008) have reported genome-wide studies of 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 upregulations of specific protein gene families. Many of these upregulated genes are involved in the function of antioxidants, suggesting that augmented capacities in antioxidative defence are likely to be important components in evolutionary adaptations in cold and oxygen-rich environment.

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The hemoglobins of sub-Antarctic fishes of the suborder Notothenioidei

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Abstract

Fishes of the perciform suborder Notothenioidei provide an excellent opportunity for studying the evolution and functional importance of evolutionary adaptations to temperature. To understand the unique biochemical features of high-Antarctic notothenioids, it is important to improve our knowledge of these highly cold-adapted stenotherms with new information on their sub-Antarctic relatives.

This paper focuses on the oxygen-transport system of two non-Antarctic species, *Eleginops maclovinus* and *Bovichtus diacanthus*.

Unlike most Antarctic notothenioids, the blood of *E. maclovinus* and *B. diacanthus* displays high hemoglobin (Hb) multiplicity. *E. maclovinus*, the sister group of Antarctic notothenioids, has one cathodal (Hb C) and two anodal components (Hb 1, Hb 2). *B. diacanthus*, one of the most northern notothenioids, has three major Hbs. The multiple Hbs may have been maintained as a response to temperature differences and fluctuations of temperate waters, much larger than in the Antarctic. Although non-Antarctic notothenioids have never developed cold adaptation, the amino-acid sequence reveals high identity with the globins of Antarctic notothenioids.

Hbs of sub-Antarctic notothenioids are characterised by high oxygen affinity and Root effect. Phylogenetic analyses are consistent with the hypothesis that Bovichtidae and Eleginopidae diverged after they became established in more temperate waters north of the Antarctic Polar Front.

Keywords: Hemoglobin; Fish; Hemoglobin multiplicity; Root effect

1. Introduction

The formation of the Southern Ocean, which surrounds Antarctica and includes the Weddell and Ross Seas, was marked by the creation of a large mass of water, uniquely cold and thermally stable. The opening of the Drake Passage, occurred approximately 41 million years ago, and the formation of the Tasmanian Gateway few millions years after, are the two key events (Scher and Martin, 2006). The separation of the southern landmasses led to the development of the Antarctic Circumpolar Current (ACC), and this in turn was at least partially responsible for cooling of

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Antarctic waters from ~20 °C to the present extreme values near −1.8 °C (Clarke, 1983). Ocean changes played a crucial role in establishing the thermal conditions that have driven evolution of the Antarctic biota (Eastman, 1993). The cold and oxygen-rich waters south of the Antarctic Polar Front (APF; the northern boundary of the ACC, a well-defined roughly circular oceanic system, running between 50°S and 60°S) serve as important effectors of evolution of the Antarctic marine biota. The APF acts as a cold "wall" that hinders mixing of the waters of the Southern Ocean with those of the Indian, Pacific and Atlantic Oceans and limits migration of the fauna of the temperate ocean to the south, and vice versa (Coppes and Somero, 2007). Because of this, the APF acts as a barrier for gene flow, causing evolutionary processes to occur in isolation.

Diversification of the major group of Antarctic fishes of the perciform suborder Notothenioidei, largely confined within Antarctic and sub-Antarctic waters, has occurred in parallel with the climatic changes. This suborder comprises eight families and 122 species. Five families and 96 species are Antarctic, whereas three families and 26 species are non-Antarctic (Eastman, 2005). Among the five notothenioid families, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae and Channichthyidae, 15 species occur along the cool-temperate southern coast of South America and New Zealand (Eastman and Eakin, 2000). These cold-temperate species encounter water temperatures of approximately 5−15 °C. Three small basal families, 10 of 11 species in the family Bovichtidae, monotypic Pseudaphritidae and Eleginopidae are non-Antarctic and presumably diverged during the Eocene and became established in waters around areas corresponding to New Zealand, Australia and high-latitude South America. They essentially never experienced near-freezing water temperatures, and absence of detectable antifreeze-glycoprotein (AFGP) coding sequence in Bovichtidae, Pseudaphritidae and Eleginopidae supports this scenario for the basal notothenioid lineages (Cheng et al., 2003).

To understand the unique biochemical features of high-Antarctic notothenioids, it is important to enrich our knowledge of these highly cold-adapted stenotherms with new information on their sub-Antarctic relatives.

The survival of polar fish depends on the presence of AFGPs because they avoid freezing by binding water to AFGPs, thus preventing growth of ice crystals in their blood and other body fluids (DeVries, 1988; Cheng and DeVries, 1991). The occurrence of AFGPs in non-Antarctic notothenioids from South America and New Zealand waters has been examined in several studies (Cheng et al., 2003; Cheng and Detrich, 2007). The copy numbers of AFGP genes and the numbers of AFGPs reflect the harshness of the freezing threat that Antarctic notothenioids have to face, in comparison with temperate notothenioids, which reached temperate waters before the production of the high levels of AFGPs found in current Antarctic notothenioids (Cheng et al., 2003).

In *Notothenia angustata* and *Notothenia microlepidota*, living in cool-temperate waters, the AFGP system is reduced, with very low blood AFGP concentration and only two to three genes showing some replacements in the functional repeat Thr−Ala−Ala (Cheng et al., 2003). *Dissostichus eleginoides*, a non-Antarctic notothenioid of the family Nototheniidae, appears to have no functional AFGP sequences, consistent with its non-Antarctic distribution. The hypothesis is that this species had the primordial AFGP genotype, lost or mutated following its migration to non-Antarctic habitats (Cheng et al., 2003).

Another remarkable example of adaptation to extreme cold found in Antarctic notothenioid fishes is the apparent loss of inducible heat-shock response (HSR) at constantly cold temperatures (Hofmann et al., 2000). Interesting results showed that *Bovichtus variegatus* (family Bovichtidae) expresses heat-shock proteins (hsp) in response to heat stress, whereas *N. angustata,* a non-Antarctic notothenioid living in New Zealand waters, does not display the stress-inducible hsp synthesis at the protein-level (Hofmann et al., 2005). These results suggest that HSR, the up-regulation of heat-stress sensitive genes, was lost after evolution in the subzero, stenothermic environment of Antarctic waters during the divergence of Bovichtidae from the other Antarctic notothenioid families.

Red-blooded high-Antarctic notothenioid families have evolved a suite of physiological and molecular adaptations, accompanied by a decrease in Hb oxygen affinity and concentration (Verde et al., 2006b). Hb-less Channichthyidae represent the extreme of this trend (Ruud, 1954).

This paper provides preliminary data on the oxygen-transport system of two non-Antarctic species, *Eleginops maclovinus* and *B. diacanthus*.

E. maclovinus, locally known as mullet or róbalo, is a notothenioid belonging to the family Eleginopidae and is the only species of its genus. It is an important component of the ichthyofauna in the coastal temperate and sub-Antarctic waters of South

America; it is also found in coastal waters around the Falkland Islands (Falkland Islands Government, 2003; Brickle et al., 2005). *E. maclovinus* is the sister group of Antarctic notothenioids that dominate the cold shelf waters of Antarctica and it is of interest to understand notothenioid diversification representing the "starting point" for the notothenioid radiation (Eastman and Lannoo, 2008). The status of *Eleginops* as sister group of Antarctic notothenioids is supported by phylogenetic analyses employing both morphological (Balushkin, 2000) and molecular data, including partial (Bargelloni et al., 2000) and complete 16S rRNA (Near et al., 2004) mtDNA gene sequences.

South Atlantic *B. diacanthus*, belonging to the phyletically basal family Bovichtidae, is the klipfish from Tristan da Cunha (37°S) and lives near the northern limit for notothenioids (Eastman, 1993). Here this species experiences mean monthly temperatures of 13–19 °C, and can tolerate summer tide-pool temperatures up to 27.4 °C (Andrew et al., 1995).

2. Materials and methods

2.1. Collection of specimens

Specimens of *E. maclovinus* and *B. diacanthus* were collected during ICEFISH 2004. Adult *E. maclovinus* was collected near the Falkland Islands and adult *B. diacanthus* in the temperate waters of Tristan da Cunha. Blood was taken by heparinised syringes from the caudal vein. Hemolysates were prepared as described previously (D'Avino and di Prisco, 1988). Saline-washed erythrocytes were frozen at −80 °C until use.

2.2. Purification of Hbs and oxygen binding

Separation of *E. maclovinus* Hbs was achieved by FPLC anion-exchange chromatography on a Mono Q-Tricorn column (1.0 × 10 cm), equilibrated with 20 mM Tris–HCl pH 7.6. Purification of *B. diacanthus* Hbs was achieved by chromatofocusing with Polybuffer Exchangers (PBE) 94 (Amersham Biosciences), equilibrated with 25 mM Tris-acetate pH 8.3. All steps were carried out at 0–5 °C. For oxygen binding, aliquots of CO-Hbs were stored at −80 °C. For each experiment, an aliquot was thawed, converted to the oxy form by exposure to light and oxygen, and immediately used; no oxidation was spectrophotometrically detectable, indicating that final Met-Hb formation was negligible (<2%).

2.3. Amino-acid sequencing

Globins of *E. maclovinus* and *B. diacanthus* were purified by reverse-phase HPLC on C_4 Vydac (4.6 × 250 mm) columns. Before loading, samples were incubated in a denaturating solution of 5% β-mercaptoethanol and 1% TFA at room temperature. Fractionation of tryptic peptides and subsequent amino-acid sequencing were carried out as previously described (Verde et al., 2002). Nucleotide sequences were established by cloning and sequence analysis of globin cDNAs:

a) *B. diacanthus*: total RNA was isolated from the spleen using RNA easy Extraction (Qiagen). First-strand cDNA synthesis was performed according to the manufacturer's instructions (BioLabs New England) using an oligo(dT)-adaptor primer. The β-globin cDNAs were amplified by PCR using oligonucleotides designed on the N-terminal regions as direct primers and the adaptor primer as the reverse primer. Primer sequences are available from the authors upon request. Amplifications were performed with 25 μl Hot StarTaq Master Mix Qiagen (2.5 units Taq Polymerase, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM of each dNTP), 5 pmol each of the above primers. The PCR programme consisted of 30 cycles of 1 min at 94 °C, 1 min at temperatures between 42 ÷ 54 °C, 1 min at 72 °C, ending with a single cycle of 10 min at 72 °C. The N-terminal regions of *B. diacanthus* Hbs were obtained by amino-acid sequencing. Amplified cDNA was purified and ligated in the pGEM-T Easy vector (Promega). *Escherichia coli* (strain JM109) was transformed with the ligation mixtures. Standard molecular-biology techniques (Sambrook et al., 1989) were used in the isolation, restriction and sequence analysis of plasmid DNA;
b) *E. maclovinus*: total RNA was isolated from the spleen using TRI Reagent (Sigma). First-strand cDNA synthesis was performed according to the manufacturer's instructions (Promega) using an oligo(dT)-adaptor primer. The β-globin cDNA was amplified by PCR using oligonucleotides designed on the N-terminal regions as direct primers and the adaptor primer as the reverse primer. Primer sequences are available from the authors upon request. Amplifications were performed with 2.5 units EuroTaq (EuroClone Genomics), 1X Reaction Buffer, 3 mM $MgCl_2$, 200 mM of each dNTP, Reverse primer 0.6 μM, Forward primer 1.2 μM. The PCR programme consisted of 40 cycles of

1 min at 94 °C, 1 min at 38 °C, 1 min at 72 °C, ending with a single cycle of 10 min at 72 °C. The N-terminal regions of *E. maclovinus* Hbs were obtained by amino-acid sequencing. Amplified cDNA was purified and ligated in the pDrive Cloning Vector (Qiagen). *E. coli* (strain TOP 10) was transformed with the ligation mixtures. Standard molecular-biology techniques (Sambrook et al., 1989) were used in the isolation, restriction and sequence analysis of plasmid DNA.

2.4. Oxygen affinity and Root effect

Hemolysate stripping was performed as described previously (Tamburrini et al., 1994). Oxygen equilibria were measured in 100 mM Hepes/Mes in the pH range 6.25–8.4 at 5 °C, at a final Hb concentration of 0.5–1.0 mM on a heme basis. Experiments were performed in duplicate, a standard deviation of ±3% for p_{50} values was calculated. In order to achieve stepwise oxygen saturation, a modified gas diffusion chamber was used, coupled to cascaded Wösthoff pumps for mixing pure nitrogen with air (Weber et al., 1987). Sensitivity to chloride was assessed by adding NaCl to a final concentration of 100 mM. The effects of ATP were measured at a final ligand concentration of 3 mM, a large excess over the tetrameric Hb concentration. Oxygen affinity (measured as p_{50}) and cooperativity (n_{Hill}) were calculated from the linearised Hill plot of $\log S/(1 - S)$ against $\log pO_2$ at half saturation, where S is the fractional oxygen saturation. The Root effect was determined in 100 mM Hepes/Mes in the pH range 6.25–8.4 at room temperature by calculating the mean absorbance difference at three wavelengths (540, 560 and 575 nm) between the spectra at pH 8.4 (fully oxygenated Hb) and pH 6.25, and the spectra after deoxygenation using sodium dithionite.

2.5. Phylogenetic analysis

Multiple alignments of α and β globin amino-acid sequences were performed with the program CLUSTAL X (Thompson et al., 1997). The sequence of *Latimeria chalumnae* was included in the analysis as out-group. Phylogenetic trees of globin sequences were inferred using the neighbour joining (NJ) method implemented in the program MEGA 3 (Kumar et al., 2004). The genetic distances were measured according to the *p*-distance model. Robustness of the NJ trees was assessed by bootstrap analysis with 10,000 replications.

3. Results and discussion

3.1. Purification of Hbs: the Hb multiplicity

Unlike most Antarctic notothenioids, the blood of *E. maclovinus* and *B. diacanthus* displays high Hb multiplicity.

The electrophoretic pattern of the hemolysate of *E. maclovinus* shows the presence of one cathodal (Hb C) and two anodal components (Hb 1 and Hb 2). Hb 1 and Hb 2 account for approximately 65–70% and 5% of the total, respectively (data not shown); Hb C, always found in Antarctic notothenioid species in trace amounts (i.e. less than 1%), was 20–25% of the total in *E. maclovinus*, similar to the high-Antarctic notothenioid *Trematomus newnesi* (D'Avino et al., 1994). Purification of *E. maclovinus* Hbs was achieved by ion-exchange chromatography, showing three components, Hb C, Hb 1, and Hb 2 (data not shown).

The globins of the hemolysate of *E. maclovinus* were separated by reverse-phase HPLC (data not shown). The elution profile indicates four globins, two α chains (α^1 and α^2) and two β chains (β^1 and β^2) as established by amino-acid sequencing and mass spectrometry. The chains composition of Hb 1 is $(\alpha^1\beta^1)_2$. Hb C and Hb 2 have the α chain and β chain, respectively, in common with Hb 1.

High multiplicity was also observed in *B. diacanthus*, one of the most northern notothenioids. The chromatofocusing of the hemolysate showed three major Hbs. The elution profile of the hemolysate of *B. diacanthus*, by reverse-phase HPLC, indicated four globins (data not shown), two α chains (indicated as α^1 and α^2, respectively) and two β chains (β^1 and β^2).

Fish commonly exhibit pronounced Hb multiplicity with marked differences in the oxygen binding properties and in their sensitivities to allosteric effectors, a feature that may serve to adapt oxygen transport to environmental variations and metabolic requirements (Weber, 1990; di Prisco and Tamburrini, 1992; Feuerlein and Weber, 1994; Fago et al., 2002; Weber et al., 2000). Hb multiplicity is usually interpreted as a sign of phylogenetic diversification and molecular adaptation resulting from gene-related heterogeneity and gene duplication events, probably maintained as a response to temperature differences and fluctuations of temperate waters, much larger than in the Antarctic. This may explain why high-Antarctic notothenioids have a single major Hb, whilst sub-Antarctic notothenioids, such as *E. maclovinus* and *B. diacanthus*, retained Hb multiplicity, presumably to cope with the small or large temperature changes in the respective

habitats north of the Polar Front (di Prisco et al., 2007). These properties may reflect the dynamic life history of fish and the different environmental conditions that species encounter. Similar to the other sub-Antarctic notothenioids, *Cottoperca gobio* (family Bovichtidae) displays Hb multiplicity, with two major Hbs in the hemolysate, probably required to cope with environmental variability (Giordano et al., 2006, 2009). In contrast, *Pseudaphritis urvillii*, similar to most Antarctic notothenioids, has a single major Hb and a minor component. The low amount of minor Hb can be considered a synapomorphy, connecting *P. urvillii* to the other notothenioids (Verde et al., 2004).

3.2. Primary structure and amino-acid-sequence identities

The amino-acid sequences of α^1, β^1 and β^2 of *E. maclovinus* Hb 1 and Hb C were deduced by sequencing peptides produced by trypsin digestion and the fragments generated after cleavage of the single Asp–Pro bond (D'Avino et al., 1994) (data not shown). The N termini of the α chains are not available to Edman degradation due to the presence of an acetyl group, similar to all teleost Hbs. The C terminus of β^2 was deduced from the sequence of cDNA. The amino-acid sequences of the α and β chains of *B. diacanthus* Hb 1, Hb 2 and Hb 3 were established by alignment of tryptic peptides for α^1 and α^2, and on the basis of nucleotide sequences, using primers designed on sequence stretches of β^1 and β^2 (data not shown).

Table 1 is an overview of sequence identities of α and β chains of notothenioids, Arctic and temperate fish Hbs. In notothenioids, the identity among the major components (Hb 1) is very high (right-hand). The minor components Hb 2 and Hb C usually have the β and α chain in common, respectively, with Hb 1. The chains which are not in common have low identity with Hbs of temperate non-notothenioids (as expected), but also with Hb 1 of all notothenioids. On the other hand, the identity with one another is high (left-hand). In many non-Antarctic notothenioids, the globin sequences have high similarity with major Hbs of Antarctic notothenioids. The sequence identity of the α chains of *E. maclovinus* Hb 1 and Hb C and of the α chains of *B. diacanthus* reaches values of 70–80% compared with major Hbs of Antarctic notothenioids.

Although non-Antarctic notothenioids have never developed cold adaptation, the amino-acid sequence reveals high identity with the globins of Antarctic notothenioids. This argues in favor of a common phylogenetic origin within notothenioids and suggests that the primary structure of the Antarctic Hbs have undergone modifications only to a limited extent. Unlike changes in amino-acid sequence that occurs at a much slower overall rate, other modifications in hematological features of Notothenioidei, such as Hb multiplicity, might be considered a short-term response to environmental changes. Short-term responses, such as regulatory processes or enhanced protein synthesis, may well be additional mechanisms of temperature compensation and represent phenotypic-plasticity response to environmental changes. When faced with new selection pressures, organisms can basically respond in several ways; one of these may be to adjust to the changed conditions (i) by means of phenotypic plasticity without altering their genetic constitution, or (ii) by genetic changes through the process of evolution occurring in the long run (Holt, 1990; Davis et al., 2005).

3.3. Oxygen affinity and Root effect

The oxygen affinity of Hbs of many high-Antarctic species is quite low (di Prisco et al., 2007), as indicated by the values of p_{50} (the oxygen partial pressure required to achieve half saturation). In contrast, the affinity in Hbs of the non-Antarctic notothenioids *C. gobio*, *B. diacanthus*, *P. urvillii* and *E. maclovinus* is higher. The relationship between oxygen affinity of notothenioid Hbs and habitat features is still not well understood. Spectroscopic and modelling studies on *P. urvillii* Hb 1 have shown that all the non-conservative replacements in the primary structure of α and β chains leave the conformation and electrostatic field surrounding the heme pocket essentially unmodified (Verde et al., 2004) with respect to Hb 1 of the high-Antarctic *Trematomus bernacchii* (Ito et al., 1995; Mazzarella et al., 2006b). Spectroscopic studies have demonstrated that the heme pocket of *E. maclovinus* Hb 1 is similar to that of *P. urvillii* Hb 1 (G. Smulevich, personal communication).

In many Hbs of teleost fishes, the complete loss of cooperativity (indicated by a Hill coefficient equal to one) and the inability to saturate the ligand sites at low pH, even at high oxygen pressure, is a distinctive property with respect to the Bohr effect. This feature is known as the Root effect (reviewed by Brittain, 1987, 2005).

A general reduction in the Root effect, is noticed during the evolution of the Antarctic notothenioids (di Prisco et al., 2007) corresponding to a variable scenario pertaining to the choroid *rete*. The physiological role of the Root effect is to secrete oxygen

Table 1

Sequence identity (%) of α (A) and β (B) chains of Hbs of notothenioids, Arctic and temperate fish. The abbreviations on top of the columns (*Ca*, *Ee*, etc) from left to right, correspond to the species from bottom to top. Adapted from Verde et al. (2008). Non-Antarctic notothenioids are highlighted in grey. The list of names of species are in Table 2, with the exception of *Gadus morhua* (accession numbers: α^2 O41425; β^2 P84611), *Thunnus thynnus* (accession numbers: α P11748; β P11749) and *Boreogadus saida* (accession number: β^1 P84607).

A. α chains

Identity (%)																																	
Species	*Ccl*	*Ca*	*Ee*	*Hl*	*Ss*	*Aa*	*Aa*	*Cc*	*Tt*	*Om*	*Om*	*Gm*	*Amin*	*Amin*	*Tn*	*Nc*	*Ck*	*Rg*	*Ps*	*Ga*	*Bm*	*Ao*	*Amyt*	*Cm*	*Pu*	*Cg*	*Cg*	*Na*	*Nc*	*Bd*	*Bd*	*Em*	*Tn*
Hemoglobin/Globin				C		A	C			IV	I	2	1	2, 3	2	2								1, 2	1, 2	2	1	1	1	a^2	a^1	1, C	1, C
T. bernacchii Hb 1, C	59	64	59	56	54	54	52	63	76	61	54	59	66	74	62	66	69	92	88	90	90	87	90	91	91	64	83	96	89	80	66	88	97
T. newnesi Hb 1, C	58	62	57	55	63	53	52	61	76	61	53	60	64	72	63	66	58	90	85	91	88	85	90	90	76	61	80	93	87	78	64	86	
E. maclovinus Hb 1, C	61	66	61	59	55	57	54	67	76	60	57	62	68	74	86	69	71	85	84	83	85	84	85	84	82	66	83	88	81	84	70		
B. diacanthus α^1	60	66	62	63	62	57	63	68	67	62	62	65	82	68	82	86	66	68	66	64	68	66	65	66	66	88	66	65	62	69			
B. diacanthus α^2	60	64	64	58	60	53	56	64	76	57	61	61	71	77	65	67	72	77	76	75	79	76	76	76	79	67	87	78	73				
N. coriiceps Hb 1	55	61	54	53	51	50	51	59	71	57	52	56	62	68	61	62	62	86	83	81	83	83	83	83	71	61	74	90					
N. angustata Hb 1	59	66	60	56	57	54	53	64	76	61	56	59	67	75	64	66	69	93	90	88	92	90	91	89	77	64	83						
C. gobio Hb 1	60	64	63	61	61	54	54	66	76	57	61	58	71	81	64	66	73	79	78	78	80	77	79	78	79	66							
C. gobio Hb 2	57	66	64	64	64	57	64	66	67	61	63	64	81	69	85	87	66	66	66	63	67	64	61	64	66								
P. urvillii Hb 1, 2	57	63	62	61	58	54	57	61	76	58	59	57	66	76	63	65	70	76	75	75	76	74	72	77									
C. mawsoni Hb 1, 2	58	63	57	56	54	53	52	63	76	61	55	61	64	72	64	69	69	88	85	92	88	84	84										
A. mitopteryx	58	61	56	53	53	53	50	61	73	58	53	61	64	70	64	63	67	88	84	85	85	84											
A. orianae	61	66	62	58	58	56	53	66	73	59	59	58	66	73	64	66	68	90	96	81	89												
B. marri	59	65	61	57	59	54	55	64	78	63	60	62	68	74	68	71	68	94	90	86													
G. acuticeps	57	61	55	54	54	52	52	61	74	61	54	59	64	71	65	66	66	86	82														
P. scotti	61	66	63	58	58	54	54	66	73	59	59	58	68	73	64	66	69	90															
R. glacialis	59	67	60	57	59	57	54	65	76	61	59	60	67	73	65	67	68																
C. kumu	64	70	63	58	59	57	55	71	69	57	59	61	67	71	63	66																	
N. coriiceps Hb 2	56	65	61	60	61	56	61	65	66	63	61	66	80	68	93																		
T. newnesi Hb 2	55	64	59	61	63	57	61	64	64	61	62	66	57	66																			
A. minor Hb 2, 3	61	69	64	63	62	57	56	67	73	59	61	58	72																				
A. minor Hb 1	59	66	64	67	69	57	64	66	68	61	67	65																					
G. morhua Hb 2	59	64	61	56	58	52	55	64	61	62	57																						
O. mykiss Hb I	64	67	67	71	91	56	68	66	61	60																							
O. mykiss Hb IV	63	68	65	58	63	60	54	66	58																								
T. thynnus	61	65	61	62	59	54	54	64																									
C. carpio	81	90	73	64	66	67	62																										
A. anguilla Hb C	54	64	59	68	69	54																											
A. anguilla Hb A	61	66	61	56	57																												
S. salar	62	67	69	73																													
H. littorale Hb C	58	63	63																														
E. electricus	66	76																															
C. auratus	76																																
C. clarkii																																	

B. β chains

Identity (%)

Species	*Ca*	*Ee*	*Hl*	*Aa*	*Aa*	*Cc*	*Tt*	*Om*	*Om*	*Bs*	*Gm*	*Amin*	*Amin*	*Tb*	*Tn*	*Pu*	*Cm*	*Ck*	*Rg*	*Ps*	*Ga*	*Bm*	*Ao*	*Amyt*	*Pu*	*Cm*	*Cg*	*Na*	*Nc*	*Bd*	*Bd*	*Em*	*Em*	*Tn*
Hemoglobin/Globin			C	A	C			IV	I	1, 2	2, 3	3	1, 2	C	C	2	2								1	1	1, 2	1, 2	1, 2	β^2	β^1	C	1	1, 2
T. bernacchii Hb 1	63	59	56	55	60	60	67	65	56	60	71	76	75	69	69	69	66	67	84	78	82	86	80	85	81	88	71	91	88	73	82	71	81	93
T. newnesi Hb 1, 2	59	56	54	55	57	56	65	60	53	57	70	72	75	67	67	67	64	63	82	77	80	84	78	82	77	84	69	84	84	71	80	69	76	
E. maclovinus Hb 1	65	63	54	56	64	63	68	64	61	63	71	75	81	72	71	71	67	66	77	73	76	77	76	78	82	78	73	82	80	72	83	70		
E. maclovinus Hb C	62	53	53	57	58	59	61	63	54	64	71	80	64	90	89	86	82	78	66	64	65	67	67	67	67	67	84	71	71	84	67			
B. diacanthus β^1	62	60	53	57	64	61	67	63	59	61	70	71	75	70	69	69	67	65	76	75	72	77	76	74	83	77	71	80	79	72				
B. diacanthus β^2	63	57	54	57	63	60	64	67	57	66	71	86	69	86	86	90	83	78	67	69	68	69	71	70	71	69	88	73	72					
N. coriiceps Hb 1, 2	58	56	56	54	60	57	66	63	53	58	69	75	78	70	70	69	65	68	86	82	80	89	82	81	78	87	72	93						
N. angustata Hb 1, 2	60	57	58	54	60	58	65	63	55	60	71	76	78	70	70	70	65	69	87	82	80	89	83	84	80	87	72							
C. gobio Hb 1, 2	62	57	54	59	63	59	65	66	60	65	71	84	71	86	85	89	82	77	68	66	68	70	68	71	69	68								
C. mawsoni Hb 1	58	56	53	54	59	55	66	61	52	58	70	73	75	68	68	67	67	63	89	83	84	87	82	80	76									
P. urvillii Hb 1	64	59	54	56	60	62	69	65	59	64	71	71	77	69	68	68	65	65	76	72	74	76	73	76										
A. mitopteryx	57	55	56	54	59	56	67	59	53	58	68	71	78	66	67	66	62	63	79	75	78	79	76											
A. orianae	58	56	55	53	58	57	65	61	52	60	69	71	74	67	67	67	63	65	80	90	77	81												
B. marri	58	54	55	54	59	56	65	60	53	59	67	73	76	67	67	67	65	67	87	78	84													
G. acuticeps	58	56	53	54	58	56	63	58	54	61	65	70	71	66	67	65	65	63	79	78														
P. scotti	57	57	54	52	57	56	63	60	50	60	67	69	73	65	65	65	62	61	80															
R. glacialis	56	54	54	53	56	54	65	60	50	57	68	71	75	66	65	66	61	63																
C. kumu	59	52	54	55	55	58	59	60	52	60	69	79	66	76	75	80	73																	
C. mawsoni Hb 2	57	54	48	56	60	54	60	59	54	58	67	78	71	89	89	84																		
P. urvillii Hb 2	60	53	51	56	60	57	63	63	52	64	71	84	68	88	86																			
T. newnesi Hb C	60	54	52	56	60	58	60	61	56	61	68	81	66	95																				
T. bernacchii Hb C	61	54	52	56	58	58	62	63	54	62	70	82	75																					
A. minor Hb 1, 2	63	58	57	56	59	60	71	63	58	60	71	73																						
A. minor Hb 3	63	56	54	57	58	61	65	64	58	65	72																							
G. morhua Hb 2, 3	63	56	52	58	58	60	69	63	54	62																								
B. saida Hb 1, 2	64	56	58	59	61	64	58	61	59																									
O. mykiss Hb I	63	50	55	53	66	63	58	58																										
O. mykiss Hb IV	78	70	58	72	60	72	62																											
T. thynnus	61	54	54	54	60	60																												
C. carpio	91	68	60	66	59																													
A. anguilla Hb C	64	63	69	56																														
A. anguilla Hb A	71	59	50																															
H. littorale Hb C	62	59																																
E. electricus	72																																	
C. auratus																																		

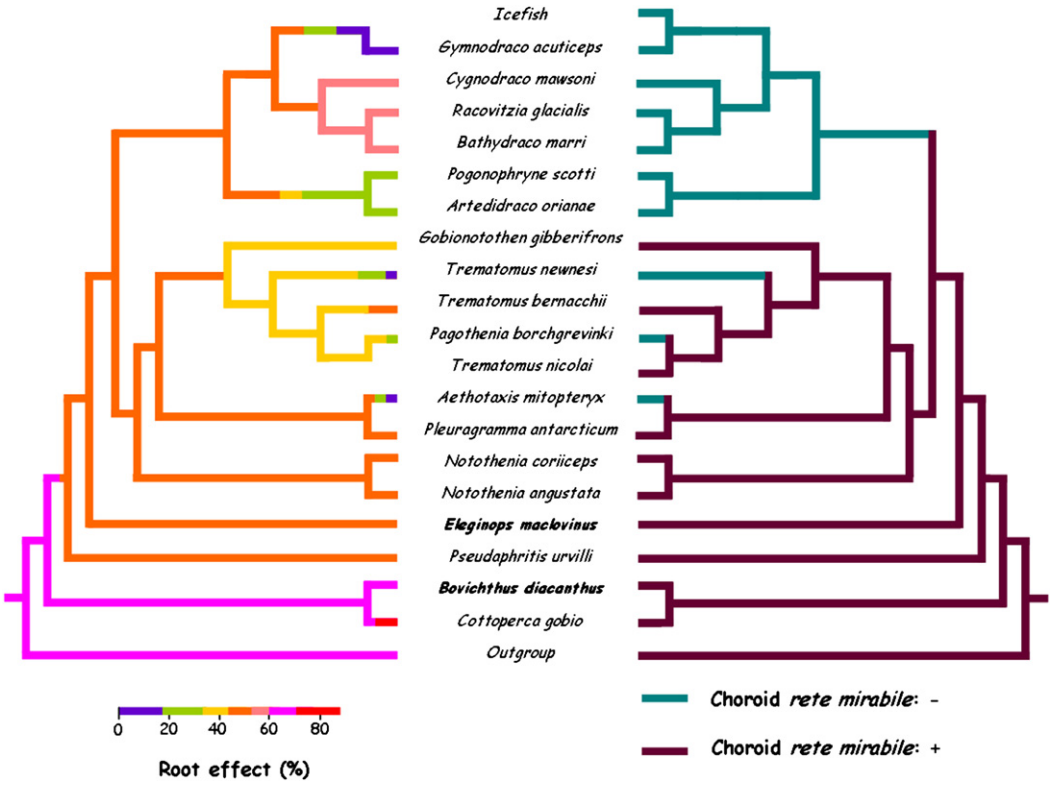

Fig. 1. Reconstruction of the evolutionary history of the Root effect. Adapted from Verde et al. (2008).

against high oxygen pressures into the swimbladder (when present) and the choroid *rete* (Wittenberg and Wittenberg, 1961; Wittenberg et al., 1964). It is likely that the eye choroid *rete* represents the most ancient anatomical structure associated with the presence of Root-effect Hbs (Wittenberg and Haedrich, 1974).

Antarctic fish lack the swimbladder. Among high-Antarctic notothenioids, many species have lost the choroid *rete*, although several retain portions of the *rete* and/or small vestigia of the choriocapillaris (Eastman, 1988, 1993, 2006). Fig. 1 shows the presence of the choroid *rete* and Root effect in Nototenioidei. The comparison of two phylogenetically related groups of non-Antarctic and high-Antarctic species shows that the choroid *rete* is very well developed in sub-Antarctic notothenioids *E. maclovinus*, *P. urvillii*, *C. gobio* and *B. diacanthus*, which are the most basal of the suborder. The Root effect drops to low values in the Artedidraconidae lineage, as well as in one Bathydraconidae (*Gymnodraco acuticeps*), as expected, but it is found at unexpectedly high levels in two species of the latter family (di Prisco et al., 2007). Hbs of sub-Antarctic notothenioids of the most basal notothenioid family are characterised by high Root effect (Fig. 1).

Since the identity among the major components (Hb 1) is very high in notothenioids (Table 1), some general structural explanations of the heterotropic proton regulation of oxygen affinity observed in *B. diachantus* and *E. maclovinus* major Hbs can be proposed. Indeed, the high sequence similarity suggests that some structural properties of these sub-Antarctic Hbs can be predicted according to the crystals structures available for high-Antarctic Hbs. A partial list of the available X-ray crystal structures of Antarctic fish Hbs is reported in a recent review (Verde et al., 2008). These structural studies strongly suggest that the key region responsible for pH modulation of oxygen affinity and cooperativity includes the aspartic triad at the $\alpha_1\beta_2$ interface (Mazzarella et al., 2006a, 2006b) and the CD corner of the α chain (Mazzarella et al., 2006b; Vergara et al., 2009). At the $\alpha_1\beta_2$ interface, two protons per tetramer are released upon oxygenation due to the breakage of an inter-Asp hydrogen bond between Asp95α and Asp101β (Ito et al., 1995; Mazzarella et al., 2006a, 2006b). This same structural evidence is also common to tuna fish (Yokoyama et al., 2004). At the CDα, the typical switch region in tetrameric Hbs (Baldwin and Chothia, 1979), an important order–disorder transition takes place upon pH increase due to deprotonation of His55α.

Table 2
Species and globin sequences investigated. Adapted from Giordano et al. (2006).

Species	Family	Subunit	Accession number/reference
Eleginops maclovinus[b]	Eleginopidae	Major α, β (Hb 1)	Unpublished
		Minor α (Hb C), β (Hb 2)	Unpublished
Bovichtus diacanthus[b]	Bovichtidae	α, β	Unpublished
Latimeria chalumnae[d]	Coelacanthidae	α, β	P23740, P23741
Chelidonichthys kumu[d]	Trigilidae	α, β	P80270, P80271
Anarhichas minor[c]	Anarhichadidae	α (Hb 1), α (Hb 2, Hb 3)	P83270, P83271
		β (Hb 1, Hb 2), β (Hb 3)	P83272, P83273
Chrysophrys auratus[d]	Sparidae	α, β (Hb 4)	Stam et al., 1997
Cottoperca gobio[b]	Bovichtidae	α (Hb 1)	P84653
		β (Hb 1)	P84652
Pseudaphritis urvillii[b]	Pseudaphritidae	α (Hb 1, Hb 2)	P83623
		β (Hb 1)	P83624
		β (Hb 2)	P83625
Notothenia coriiceps[a]	Nototheniidae	major α (Hb 1)	P10777
		minor α (Hb 2)	P16308
		β (Hb 1, Hb 2)	P16309
Notothenia angustata[b]	Nototheniidae	major α (Hb 1)	P29624
		minor α (Hb 2)	P16308
		β (Hb 1, Hb 2)	P29628
Pleuragramma antarcticum[a]	Nototheniidae	α (Hb 1, Hb 2)	Stam et al., 1997
		β (Hb 1, Hb 3)	Stam et al., 1997
		minor α (Hb 3), β (Hb 2)	Stam et al., 1997
Pagothenia borchgrevinki[a]	Nototheniidae	α (Hb 1, Hb 0)	P82344
		major β (Hb 1)	P82346
		minor β (Hb 0)	P83245
Gobionotothen gibberifrons[a]	Nototheniidae	major α, β (Hb 1)	P83611, P83612
		minor α, β (Hb 2)	P83613, P83614
Aethotaxis mitopteryx[a]	Nototheniidae	α, β	Stam et al., 1997
Trematomus newnesi[a]	Nototheniidae	major α, β (Hb 1)	P45718, P45720
		minor α (Hb 2), β (Hb C)	P45719, P45721
Trematomus bernacchii[a]	Nototheniidae	major α, β (Hb 1)	P80043, P80044
		minor β (Hb C)	P45722
Cygnodraco mawsoni[a]	Bathydraconidae	α (Hb 1, Hb 2)	P23016
		major β (Hb 1)	P23017
		minor β (Hb 2)	P23018
Gymnodraco acuticeps[a]	Bathydraconidae	α, β	P29623, P29625
Racovitzia glacialis[a]	Bathydraconidae	α, β	Tamburrini et al., unpublished
Bathydraco marri[a]	Bathydraconidae	α, β	Stam et al., 1997
Pogonophryne scotti[a]	Artedidraconidae	α, β	Stam et al., 1997
Artedidraco orianae[a]	Artedidraconidae	α, β	Stam et al., 1997
Salmo salar[d]	Salmonidae	α	P11251
Oncorhynchus mykiss[d]	Salmonidae	α, β (Hb I)	P02019, P02142
		α, β (Hb IV)	P14527, P02141
Anguilla anguilla[d]	Anguillidae	α, β (Hb C)	P80726 P80727
		α, β (Hb A)	P80945, P80946
Electrophorus electricus[d]	Electrophoridae	α, β	P14520, P14521
Hoplosternum littorale[d]	Callichthyidae	α, β (Hb C)	P82315, P82316
Cyprinus carpio[d]	Cyprinidae	α, β	P02016, P02139
Carassius auratus[d]	Cyprinidae	α, β	P02018, P02140
Catostomus clarkii[d]	Catostomidae	α	P02017
Oryzias latipes[d]	Adrianichthyidae		Maruyama et al., 2004

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

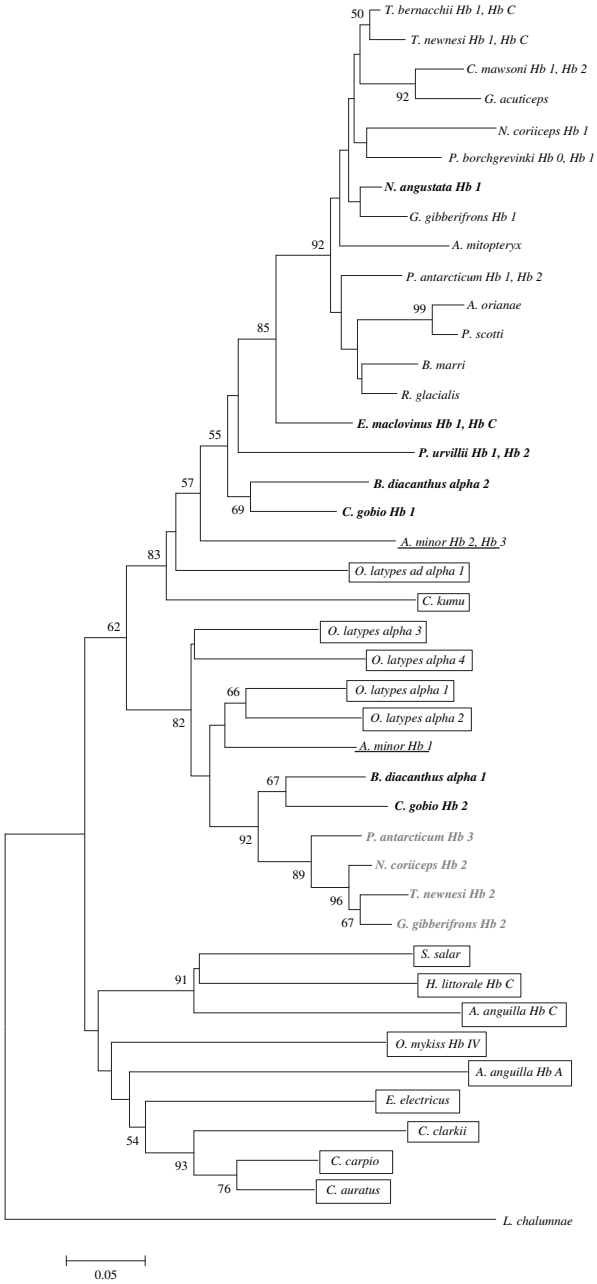

Fig. 2. Phylogenetic tree of amino-acid sequences of α chains of Arctic, Antarctic and temperate fish Hbs. Bootstrap values (percentage of 10,000 replicates) are given at the nodes. Notothenioid major globins are in black, notothenioid minor globins in grey, non-Antarctic notothenioid globins in bold, Arctic globins underlined, and temperate globins in box.

A punctual structural explanation of the higher affinity herein reported in sub-Antarctic Hbs than in high-Antarctic Hbs requires the establishment of the crystal structure of the former in its ligated and un-ligated forms. This study is in progress in *E. maclovicnus* Hb 1.

Interestingly, high-Antarctic Hbs exhibit at least three stable quaternary structures. Indeed, the classical relaxed state (R) (Camardella et al., 1992; Mazzarella et al., 1999), the tense (T) (Mazzarella et al., 2006a, 2006b) and an R/T intermediate quaternary structure [typical at least of the ferric (Riccio et al., 2002; Vergara et al., 2007) and partially oxidised states (Merlino et al., 2009; Vitagliano et al., 2008)] have been observed. Currently, the R/T quaternary structure is considered associated to ferric or partially oxidised states, though it cannot be excluded that the ferrous

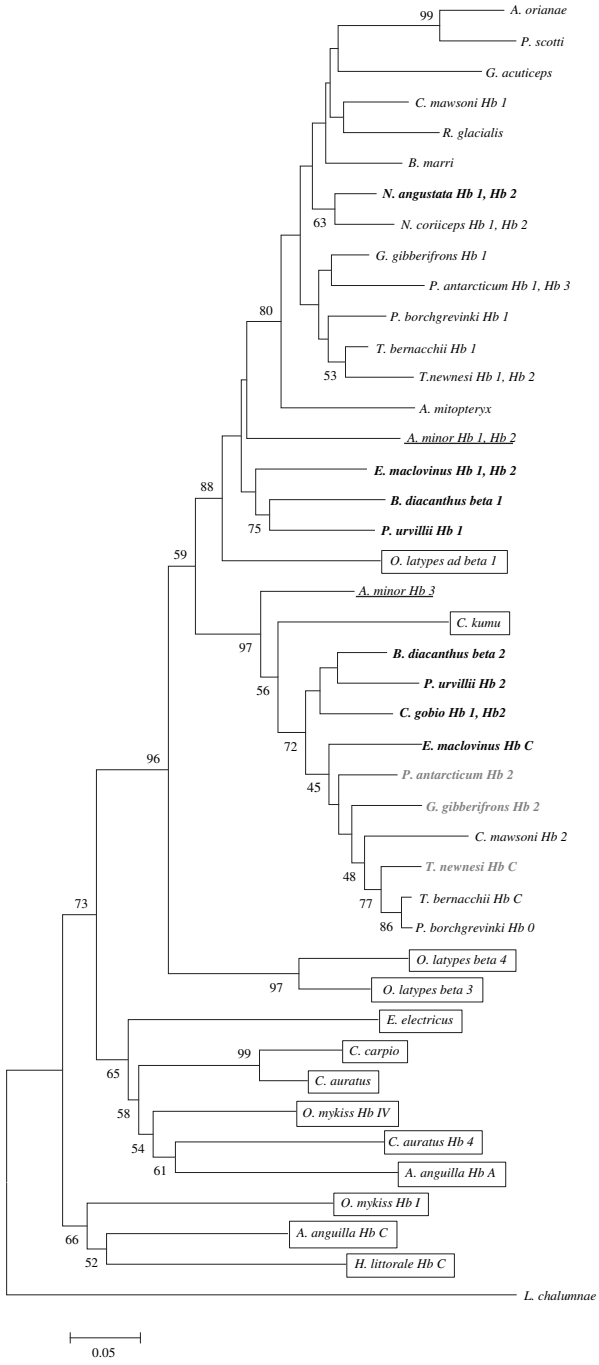

Fig. 3. Phylogenetic tree of amino-acid sequences of β chains of Arctic, Antarctic and temperate fish Hbs. Bootstrap values (percentage of 10,000 replicates) are given at the nodes. Notothenioid major globins are in black, notothenioid minor globins in grey, non-Antarctic notothenioid globins in bold, Arctic globins underlined, and temperate globins in box.

state may adopt this structure. Antarctic fish Hbs have an heterogeneous ferric state with α-heme in the aquomet state, and with β-heme in a bis-histidyl (hemichrome) state (Riccio et al., 2002; Vergara et al., 2007). It is an open question whether the accessibility to these multiple structural states are only a peculiarity of Antarctic fish Hbs or it extends to sub-Antarctic fish Hbs. Sequence similarity between the Hbs of *P. urvillii* and *C. gobio*, where hemichrome forms (Verde et al., 2004; Giordano et al., 2009), suggests that the

formation of the bis-histidyl adduct should be expected also in sub-Antarctic fish Hbs.

3.4. Molecular phylogeny

Table 2 reports the list of the species examined in this study and the accession numbers of α-globin and β-globin sequences used in the phylogenetic analysis. The sequences not available in data banks are indicated by references.

Two phylogenetic trees, one for α globins (Fig. 2) and one for β globins (Fig. 3) were obtained. Phylogenetic analysis was performed on the multiple alignments constructed with the program CLUSTAL X (Thompson et al., 1997; Verde et al., 2006a). The genetic distances were measured according to the *p*-distance model to evaluate to what extent the resulting tree differs from the expected interrelationships among species in a given cluster of orthologs (i.e. gene copies diversified by speciation). In both trees, anodal and cathodal globins of temperate fish Hbs form the first divergence lineage; the globins of major and minor Antarctic fish Hbs cluster in two separate, strongly supported groups. As a result of the isolation of Antarctica, the genotype of Notothenioidei diverged with respect to other fish groups in a way interpreted as typical of a species flock (Eastman and McCune, 2000).

The obtained topology is in general agreement with the maximum-likelihood method (Giordano et al., 2006) and the hypothesis of four globin groups (Maruyama et al., 2004), which include α and β globins, belonging to notothenioid minor Hbs, in the "Embryonic Hb Group", and α and β globins (shared by Hb 1 and Hb 2 in most Antarctic notothenioids) of the major notothenioid Hbs in the "Notothenioid Major Adult Hb Group".

The position of the *C. gobio* globins appears congruent with the phylogenetic evidence from nuclear and mitochondrial genes (Bargelloni et al., 2000), suggesting that *C. gobio* is the sister taxon of *P. urvillii*, *E. maclovinus*, and also of Antarctic notothenioids. The basal position of *E. maclovinus* and *B. diacanthus* Hbs is in agreement with the postulated divergence before the appearance of AFGPs. Their basal position in notothenioids is consistent with the hypothesis that Bovichtidae and Eleginopidae diverged after they became established in more temperate waters north of the APF. The α chain of *E. maclovinus*, shared by Hb 1 and Hb C, branches off the clade of major Antarctic Hbs, and the same applies to the β chain of Hb 1. The β chain of *E. maclovinus* Hb C is in a basal position with respect to the clade of Antarctic minor Hbs.

Anarhichas minor, an Arctic fish, is close to the notothenioid clades, according to the teleostean phylogeny (Verde et al., 2002, 2006a).

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Crystallization, preliminary X-ray diffraction studies and Raman microscopy of the major haemoglobin from the sub-Antarctic fish *Eleginops maclovinus* in the carbomonoxy form

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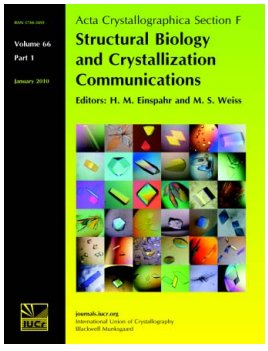

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Crystallization, preliminary X-ray diffraction studies and Raman microscopy of the major haemoglobin from the sub-Antarctic fish *Eleginops maclovinus* in the carbomonoxy form

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The blood of the sub-Antarctic fish *Eleginops maclovinus* (Em) contains three haemoglobins. The major haemoglobin (Hb1Em) displays the Root effect, a drastic decrease in the oxygen affinity and a loss of cooperativity at acidic pH. The carbomonoxy form of HbEm1 has been crystallized in two different crystal forms, orthorhombic (Ortho) and hexagonal (Hexa), and high-resolution diffraction data have been collected for both forms (1.45 and 1.49 Å resolution, respectively). The high-frequency resonance Raman spectra collected from the two crystal forms using excitation at 514 nm were almost indistinguishable. Hb1Em is the first sub-Antarctic fish Hb to be crystallized and its structural characterization will shed light on the molecular mechanisms of cold adaptation and the role of the Root effect in fish haemoglobins.

1. Introduction

Fish haemoglobins (Hbs) have been extensively studied not only to determine their structural and functional properties (Perutz, 1990), but also because they offer the possibility of investigating functional differentiation and molecular adaptation in species living in a large variety of environmental conditions. As in mammals, fish Hb is a heterotetramer. Much of our knowledge of the effect of the environment on vertebrate physiology and evolution comes from the study of fishes, which share most physiological mechanisms with mammals (Bonaventura *et al.*, 2004). Their bodies are submerged in water and their close physical and physiological relationship with the aquatic environment makes them sensitive sentinels of environmental challenge and offers important advantages in defining the organism–environment interface and responses to temperature adaptation (Verde *et al.*, 2007). The Antarctic notothenioids, including high-Antarctic, sub-Antarctic and temperate species, provide many opportunities for comparative approaches aimed at understanding protein thermal adaptations and their ability to counteract ongoing climate change (Verde *et al.*, 2008).

In addition to being highly sensitive to pH, several fish Hbs also become non-cooperative at acidic pH. The complete loss of cooperativity (indicated by a Hill coefficient of 1) and the inability to saturate the ligand sites at low pH values, even at high oxygen pressure, that are common to many fish Hbs are distinctive properties with respect to the Bohr effect and are known as the Root effect (Brittain, 2005).

Structural studies of Hbs from several temperate fish [tuna (Yokoyama *et al.*, 2004), spot (Mylvaganam *et al.*, 1996) and trout (Tame *et al.*, 1996)] displaying the Root effect have been conducted in their ferrous form. Furthermore, several high-Antarctic fish Hbs have been crystallized in both ferrous and ferric forms and their crystal structures have been determined (Camardella *et al.*, 1992; Ito *et al.*, 1995; Mazzarella, Bonomi *et al.*, 2006; Mazzarella *et al.*, 1999; Mazzarella, Vergara *et al.*, 2006; Vergara *et al.*, 2007, 2009, 2010; Merlino *et al.*, 2009; Vitagliano *et al.*, 2004, 2008; Riccio *et al.*, 2002). In particular, Hbs from *Trematomus bernacchii* (HbTb; Ito *et al.*, 1995; Mazzarella, Bonomi *et al.*, 2006; Mazzarella, Vergara *et al.*, 2006) and

T. newnesi (the major, Hb1Tn, and the cathodic, HbCTn, component; Mazzarella, Bonomi *et al.*, 2006; Mazzarella *et al.*, 1999; Vergara *et al.*, 2010) have been studied in their ferrous form. These studies have provided several possible structural explanations of the Root effect. The autoxidation process of Antarctic fish Hbs was extensively investigated in HbTb (Merlino *et al.*, 2009; Vitagliano *et al.*, 2004) and Hb1Tn (Vitagliano *et al.*, 2008), revealing the accessibility of a quaternary structure different from the canonical R and T states. In particular, the ferric states of HbTb (Vergara *et al.*, 2007) and Hb1Tn (Riccio *et al.*, 2002) are able to form an intermediate R/T state. In addition, it is noted that the Root effect can also affect the quaternary structure of the ferric state at acidic pH (Vergara *et al.*, 2009).

E. maclovinus, locally known as mullet or róbalo, is a notothenioid belonging to the family Eleginopidae and is the only species of its genus. It is an important component of the ichthyofauna in the coastal temperate and sub-Antarctic waters of South America; it is also found in coastal waters around the Falkland Islands (Brickle *et al.*, 2005). *E. maclovinus* forms a sister group of Antarctic notothenioids that dominate the cold-shelf waters of Antarctica and represents the 'starting point' for notothenioid radiation (Eastman & Lannoo, 2008).

Ion-exchange chromatography of the haemolysate of *E. maclovinus* shows the presence of three components (HbCEm, Hb1Em and Hb2Em; Coppola *et al.*, 2010). We studied the major component Hb1Em, a tetrameric Hb that displays the Root effect. Sequence alignments show a significant similarity to high-Antarctic fish Hbs. Indeed, Hb1Em shares 85% sequence identity to HbTb (Camardella *et al.*, 1992), the carbomonoxy (Camardella *et al.*, 1992) and deoxy (Ito *et al.*, 1995; Mazzarella *et al.*, 2006) structures of which have previously been determined. *T. bernacchii* is an Antarctic notothenioid living at 271 K, whereas *E. maclovinus* is a sub-Antarctic notothenioid adapted to 278–288 K. *E. maclovinus* essentially never experiences near-freezing water temperatures and the absence of a nucleotide sequence coding for antifreeze glycoproteins (AFGPs) in this fish supports this scenario (Cheng *et al.*, 2003).

Therefore, with the aim of obtaining insight into the mechanism of Hb cold adaptation and to understand the structural basis of the Root effect at low temperatures, we have undertaken the structural characterization of Hbs isolated from sub-Antarctic fishes. Here, we report the crystallization, Raman microscopy and preliminary diffraction studies of Hb1Em in the carbomonoxy form (Hb1EmCO).

2. Methods

2.1. Collection of specimens and purification

Specimens of *E. maclovinus* were collected during the ICEFISH 2004 cruise. Adult *E. maclovinus* were collected near the Falkland Islands. Blood was taken from the caudal vein using heparinized syringes. Haemolysates were prepared as described previously (D'Avino & Prisco, 1988). Saline-washed erythrocytes were frozen at 193 K until use.

Separation of *E. maclovinus* Hbs was achieved by FPLC anion-exchange chromatography on a Mono Q-Tricorn column (1.0 × 10 cm) equilibrated with 20 m*M* Tris–HCl pH 7.6 (buffer *A*). Elution was performed with a gradient from buffer *A* to 20 m*M* Tris–HCl pH 7.6, 100 m*M* NaCl (buffer *B*). A mixture of HbC and Hb1 was eluted at 50% buffer *B*, Hb1 was eluted at 57% buffer *B* and a mixture of Hb1 and Hb2 was eluted at 60% buffer *B* (Supplementary Fig. S1[1]). Hb1Em was further purified by ion-exchange chromatography on a DE52 column equilibrated with 10 m*M* Tris–HCl pH 7.6 and eluted stepwise with the same buffer. All steps were carried out at 273–278 K (Brickle *et al.*, 2005; Coppola *et al.*, 2010).

2.2. Crystallization

Hb1Em stock solutions were kept in the carbomonoxy form. The formation of the carbomonoxy form was monitored by optical spectroscopy. Crystallization trials were performed at 277 K in a CO atmosphere provided by flushing the gas in the flask containing the crystallization reactors. Crystals were obtained using the dialysis technique with microdialysis buttons. In buttons in which crystallization had occurred, crystals with different crystal habits were observed.

Crystals of the ferric form were prepared for resonance Raman experiments starting from carbomonoxy crystals by soaking a stabilizing solution containing 10 m*M* potassium ferricyanide into the crystals and then washing the excess off with stabilizing solution. The deoxy Hb crystals were obtained by treating crystals of the ferric form with degassed stabilizing solutions containing 10 m*M* sodium dithionite.

2.3. Data collection and processing

X-ray diffraction data were collected both in-house using a Rigaku MicroMax-007 HF generator equipped with a Saturn944 CCD detector and at the ELETTRA synchrotron. Cryoconditions for diffraction at 100 K were provided by the addition of 20% glycerol to the harvesting solution. Independent resonance Raman control experiments indicated that it was necessary to add 10 m*M* sodium dithionite to the harvesting and cryoprotecting solutions in order to avoid oxidation.

Diffraction data from the two different crystal forms were collected. In both cases, the oscillation range per image was 0.5°. The exposure time per image was 50 s for the data collected in-house and 20 s for the synchrotron data. Reflections were indexed, integrated and scaled using the *HKL*-2000 package (Otwinowski & Minor, 1997). The precision-indicating merging *R* factors ($R_{\rm p.i.m.}$) were calculated using the program *RMERGE* (Weiss, 2001; Evans, 2006).

2.4. Structure determination

Determination of the crystal structure was performed by molecular replacement using the program *AMoRe* (Navaza & Saludjian, 1997). HbTb (PDB code 3gkv; Merlino *et al.*, 2009), with which Hb1Em shares 85% sequence identity, was used as a search model.

2.5. Raman microscopy

During most of the analysis, crystals were kept in a 1 µl drop of mother liquor. Crystals were transferred from plates to a single hanging-drop reactor and then analyzed on a Raman microscope apparatus consisting of a confocal Raman instrument (Jasco NRS-3100) equipped with an Olympus microscope. Resonance Raman (RR) spectra were obtained at room temperature with the 514.5 nm line of an air-cooled Ar^+ laser (Melles Griot) focused to a spot size of approximately 4 µm by a 20× objective. A holographic notch filter was used to reject the excitation laser line from the scattered light. Raman scattering was dispersed through a monochromator (1200 grooves mm^{-1} grating) and collected using a Peltier-cooled 1024 × 128 pixel CCD photon detector (Andor DU401BVI). Typically, several 2 min spectra (7 cm^{-1} resolution) were recorded and averaged using a standard software routine. The RR spectra were

[1] Supplementary material has been deposited in the IUCr electronic archive (Reference: RP5054).

calibrated using indene as a standard. The frequencies were accurate to within 1 cm^{-1} for the intense isolated bands and to about 2 cm^{-1} for overlapped bands or shoulders. The laser power at the crystal was 1–2 mW for the ferric and deoxy forms and 0.1 mW for the carbomonoxy form (in order to avoid photolysis).

3. Results and discussion

Initial screenings for Hb1EmCO crystallization were conducted in dialysis buttons using the ammonium sulfate solutions that yielded crystals of the major Hb component of *T. newnesi* (Mazzarella *et al.*, 1999). 10 µl protein solution at 20 mg ml^{-1} in 100 m*M* Tris–HCl buffer pH 8.0 and 2 m*M* sodium dithionite was dialyzed against a 25 ml reservoir containing 2 *M* ammonium sulfate, 100 m*M* Tris–HCl pH 8.0 and 2 m*M* sodium dithionite. The quality of the crystals was then improved by fine-tuning the sulfate concentration. The best crystals were obtained in a week using 20 mg ml^{-1} Hb1Em and 1.8 *M* ammonium sulfate. In the same flask, different dialysis buttons contained crystals displaying two different morphologies (bipyramidal and rod-like).

Diffraction data (Fig. 1) were collected for the two types of Hb1EmCO crystals: Ortho (rod-like crystals) and Hexa (bipyramidal crystals). The maximum dimensions of the Ortho crystals were 0.05 × 0.05 × 0.4 mm, whereas those of the Hexa crystals were 0.1 × 0.1 × 0.2 mm. For the rod-like crystals, in-house data collections were carried out at 298 and 100 K. Data at room temperature were collected by mounting the crystal in a capillary filled with CO, whereas the data at 100 K were collected by flash-cooling in supercooled N_2 produced by an Oxford Cryosystem after the addition of 20% glycerol to the harvesting solution.

Diffraction data for the crystals with bipyramidal morphology were collected at the ELETTRA synchrotron-radiation facility. Crystals were flash-cooled at 100 K after addition of 20% glycerol to the harvesting solution.

All data sets were indexed and processed with the *HKL*-2000 suite of programs. Statistics of data processing are reported in Table 1. The two crystal forms belonged to space groups $P6_122$ (Hexa) and $P2_12_12_1$ (Ortho). As frequently observed (see, for example, Tilton *et al.*, 1992; Merlino *et al.*, 2005), the X-ray data sets of the Ortho crystals at room temperature and at 100 K gave similar unit-cell parameter values. Matthews coefficient calculations suggested the presence of one $\alpha\beta$ dimer in the asymmetric unit for the Hexa crystals and one tetramer ($\alpha_2\beta_2$) in the asymmetric unit for the Ortho crystals.

The phase problem was solved by molecular replacement using the program *AMoRe* (Navaza & Saludjian, 1997) with the structure of partially oxidized HbTb (PDB code 3gkv; Merlino *et al.*, 2009) as a search model. For both crystal forms, a clear solution was easily obtained.

The preliminary electron-density maps were of excellent quality. Integrated automated and manual model-building sessions, aimed at defining the complete structures, were performed using the programs *ARP*/*wARP* (Perrakis *et al.*, 2001) and *O* (Jones *et al.*, 1990). Refinement of the structures is in progress.

Raman microscopy experiments were conducted on Hb1EmCO crystals (both Ortho and Hexa) as well as on their ferric and deoxy forms. The high-frequency region (1300–1700 cm^{-1}) of the RR spectrum includes the porphyrin in-plane vibrational modes (which are sensitive to the electron density of the macrocycle and the oxidation, coordination and spin state of the iron ion). High-frequency RR spectra of Hb1Em crystals of the Ortho and Hexa forms together with those of the corresponding ferric and deoxy forms obtained with 514.5 nm excitation are shown in Fig. 2. The ferric form contains a hexacoordinated low-spin haemichrome (bands at 1505, 1559, 1588 and 1640 cm^{-1} assigned to ν_3, ν_{38}, ν_{19} and ν_{10} modes, respectively), as previously observed in HbTb crystals (Merlino, Verde *et al.*, 2008). The deoxy forms of both Ortho and Hexa are pentacoordinated high-spin states (ν_4, ν_{19}, ν_{37} and ν_{10} at 1355, 1548–1549, 1582 and 1602–1607 cm^{-1}, respectively). However, after long laser exposure times (about 10 min), the Ortho but not the Hexa form appears to be unstable under laser irradiation, since it irreversibly converts to a hexacoordinated low-spin haemochrome state (haemo, with ν_4, ν_3 and ν_{19} at 1361, 1496 and 1587 cm^{-1}, respectively).

Even at low laser power (0.1 mW), the degree of photolysis of both carbomonoxy forms of Hb1Em was high, as was apparent from the relative intensity of the ν_4 bands of the CO and photolyzed species (1374 and 1355 cm^{-1}, respectively) and the presence of bands arising

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Hexa, T = 100 K	Ortho, T = 100 K	Ortho, T = 298 K
Space group	$P6_122$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters			
a (Å)	91.702	58.175	58.473
b (Å)	91.702	88.075	89.888
c (Å)	168.716	123.194	125.278
Asymmetric unit	$\alpha\beta$ dimer	$\alpha_2\beta_2$ tetramer	$\alpha_2\beta_2$ tetramer
Resolution range (Å)	30–1.49 (1.53–1.49)	50–1.45 (1.50–1.45)	50–2.05 (2.12–2.05)
No. of total reflections	212199	384398	108997
No. of unique reflections	66710	109738	40284
Completeness (%)	96.4 (92.5)	97.3 (90.8)	95.2 (95.2)
Multiplicity	3.2 (2.6)	3.5 (2.5)	2.7 (2.6)
R_{merge} (%)	0.095 (0.440)	0.075 (0.384)	0.095 (0.580)
$I/\sigma(I)$	8.0 (2.1)	37.5 (3.3)	7.4 (2.2)
$R_{p.i.m.}$ (%)	0.057 (0.295)	0.039 (0.284)	0.075 (0.400)
B value from Wilson plot (A^2)	23.8	16.1	34.1

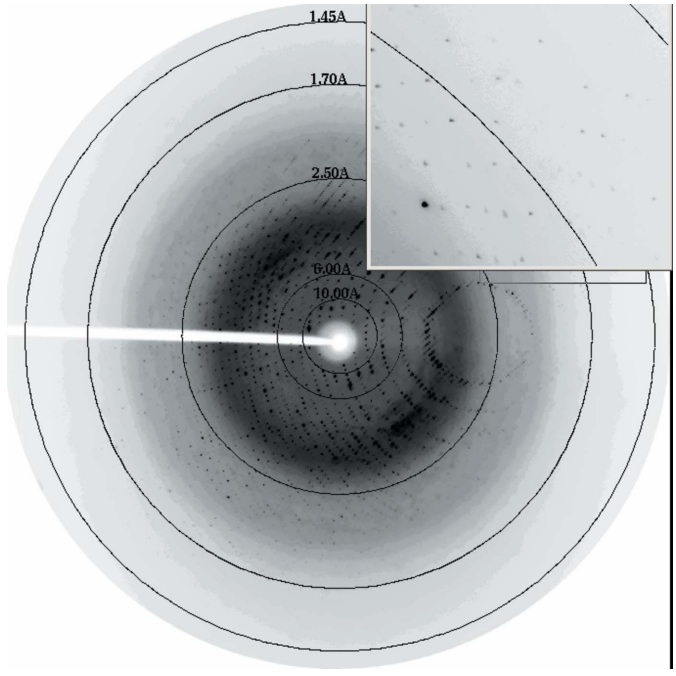

Figure 1
Diffraction pattern of the Hb1EmCO crystal in the Hexa form. Diffraction spots are detectable up to 1.45 Å resolution.

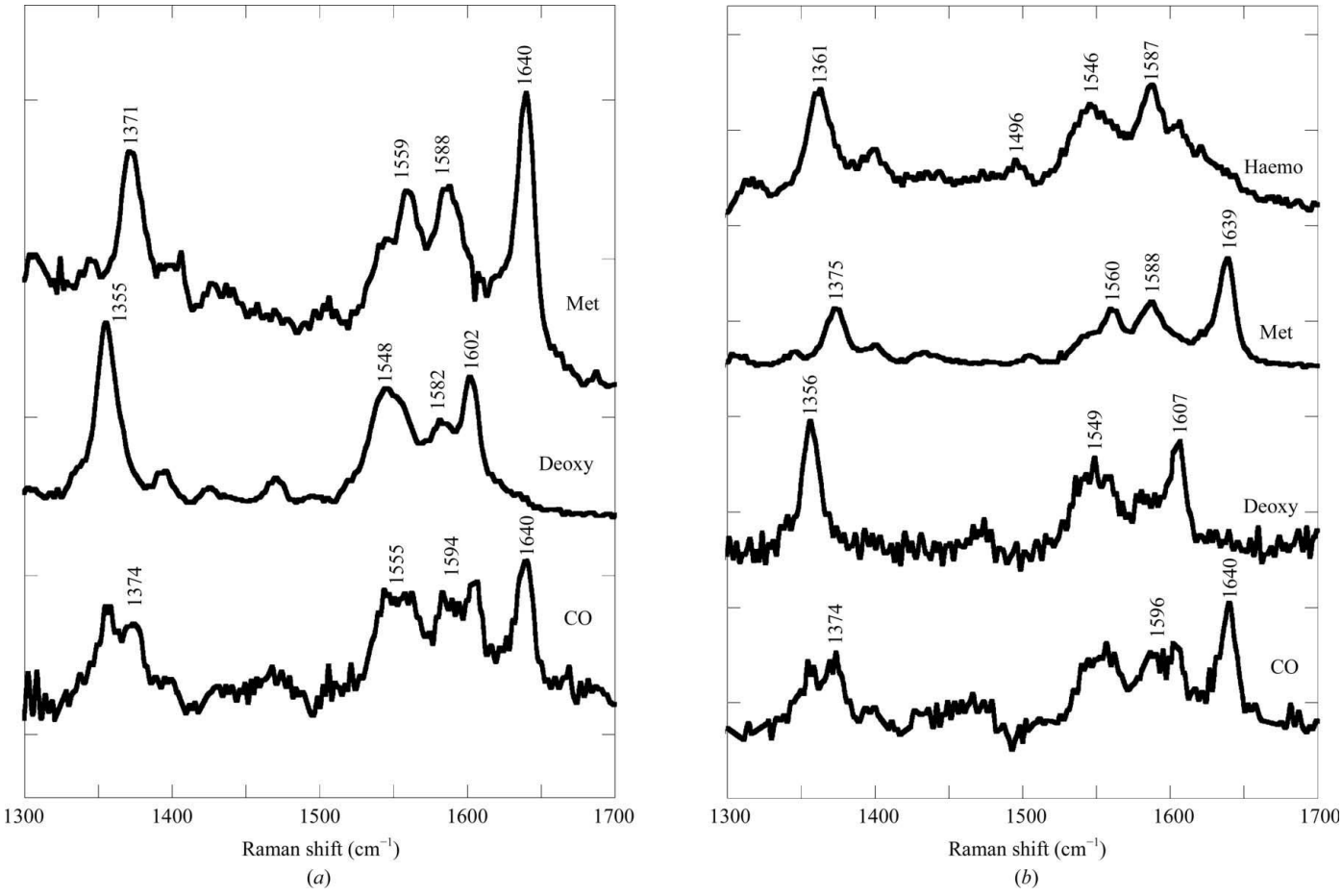

Figure 2
Resonance Raman spectra of crystals of Hb1Em in 100 m*M* Tris–HCl buffer pH 8.0 at room temperature in the carbomonoxy (CO), deoxygenated (Deoxy) and ferric (Met) forms for Hexa (*a*) and Ortho (*b*) crystals. The Ortho deoxygenated form (*b*) converts quickly into haemochrome (Haemo) after 10 min laser exposure. Excitation wavelength, 514.5 nm; laser power at the sample 2 mW for the ferric and deoxy forms and 0.1 mW for the carbomonoxy form. All spectra were an average of at least six spectra with 2 min integration time.

from ν_{19} and ν_{10} of both the deoxy form and the CO complex (deoxy bands at 1548–1549 and 1602–1607 cm^{-1}, respectively).

Raman microscopy, as previously demonstrated (Vergara *et al.*, 2008; Merlino, Verde *et al.*, 2008; Merlino, Sica *et al.*, 2008), is a valuable tool for the preliminary investigation of protein single crystals. Indeed, apart from the different stability in the laser beam, no significant difference was observed using Raman spectroscopy and the respective spectra of the two forms of Hb1Em (Hexa and Ortho) in the carbomonoxy, deoxy and ferric forms are indistinguishable within the experimental noise.

Hb1Em is the first sub-Antarctic fish Hb to be crystallized. Determination of the Hb1EmCO structure will allow detailed comparative analyses with the structures of its psychrophilic (HbTb; Merlino *et al.*, 2009) and mesophilic (tuna; Yokoyama *et al.*, 2004) counterparts. These analyses will provide insights into Root-effect adaptation at low temperature.

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EVOLUTIONARY ADAPTATIONS IN ANTARCTIC FISH: THE OXYGEN-TRANSPORT SYSTEM

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ABSTRACT

Understanding molecular adaptations evolved in response to environmental temperature changes is essential, because temperature affects the kinetic energy of molecules and modifies molecular interactions, macromolecular stability/functioning and membrane features. Environmental oxygen availability may also 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. This review summarises the current knowledge on the structure and function of hemoglobins of fish living in Antarctic habitats. The variety of adaptations underlying the ability of Antarctic fish to survive at temperatures permanently close to freezing is unique among teleosts. The dominant perciform suborder Notothenioidei affords an excellent study group for elaborating the evolution of biochemical adaptation to temperature. The availability of notothenioid taxa living in a wide range of latitudes (Antarctic, sub-Antarctic, and temperate regions) offers a remarkable opportunity to study the physiological and biochemical characters gained and, conversely, lost in response to cold, besides the possibility to reconstruct the likely evolutionary events modulating the ability of these fishes to carry oxygen in freezing habitats.

Although oxygen can be transported in freely dissolved form most animals rely on one or more protein carriers to deliver it to the respiring tissues. Compared to temperate and tropical species, high-Antarctic notothenioids have evolved reduced hemoglobin concentration/multiplicity. The Antarctic family Channichthyidae (the notothenioid crown group) is devoid of hemoglobin. All extant icefish species lack hemoglobin and many have lost myoglobin expression. In these species, oxygen delivery to tissues occurs by transport of the gas physically dissolved in the plasma.
Keywords: Antarctic; cold adaptation; evolution; fish; hemoglobin.

RESUMO

ADAPTAÇÕES EVOLUTIVAS EM PEIXES ANTÁTRTICOS: O SISTEMA DE TRANSPORTE DE OXIGÊNIO. Compreender as adaptações moleculares envolvidas na resposta às mudanças na temperatura ambiental é essencial, pois a temperatura afeta a energia cinética das moléculas e modifica as interações moleculares, a estabilidade/funcionamento das macromoléculas e as características da membrana. A disponibilidade de oxigênio no ambiente pode também ter um importante papel na evolução dos organismos marinhos polares, como indicado pelas estratégias fisiológicas e bioquímicas adotadas por estes organismos para adquirir, transportar e trocar oxigênio. Esta revisão resume o conhecimento atual da estrutura e funcionamento das hemoglobinas de peixes que ocorrem em ambientes Antárticos. A diversidade de adaptações que sustentam a habilidade de peixes antárticos sobreviverem em temperaturas permanentemente próximas do congelamento é única entre os teleósteos. A dominante sub-ordem Perciforme Notothenioidei apresenta-se como um excelente grupo de estudo para melhorar o conhecimento sobre a evolução das adaptações bioquímicas à temperatura. A ocorrência de nototenióides em uma ampla variedade de latitudes (Antártica, sub-Antártica e regiões temperadas) oferece uma oportunidade notável para estudar as características fisiológicas e bioquímicas obtidas e, por outro lado, perdidas em resposta ao frio, além de tornar possível a reconstrução dos eventos evolutivos que provavelmente modularam a habilidade desses peixes de transportar oxigênio em ambientes extremamente frios.

Embora o oxigênio possa ser transportado livremente na sua forma dissolvida, a maioria dos animais depende de um ou mais tipos de proteínas carreadoras para entregar o oxigênio aos tecidos. Quando comparadas às espécies temperadas e tropicais, os nototenióides da região Antártica, propriamente dita, desenvolveram reduzida concentração/multiplicidade de hemoglobinas. A família de peixes Antárticos Channichthyidae (*crown group* nototenióide) não apresenta hemoglobina. Todas as espécies de *icefish* (peixes-do-gelo) não possuem hemoglobinas e muitas também não produzem mioglobinas. Nessas espécies, o transporte de oxigênio aos tecidos ocorre através do gás fisicamente dissolvido no plasma.
Palavras-chave: Antártica; adaptações ao frio; evolução; hemoglobina.

RESUMEN

ADAPTACIONES EVOLUTIVAS EN PECES ANTÁRTICOS: EL SISTEMA DE TRANSPORTE DE OXÍGENO. Comprender las adaptaciones moleculares que han evolucionado en respuesta a los cambios de temperatura del medio ambiente es esencial, porque la temperatura afecta la energía cinética de las moléculas y modifica las interacciones moleculares, la estabilidad de las macromoleculares, sus características y el funcionamiento de la membrana. La disponibilidad de oxígeno ambiental desempeña un papel importante en la evolución de los organismos marinos polares, como se evidencia en las estrategias fisiológicas y bioquímicas adoptadas por estos organismos para adquirir, gastar y usar oxígeno. Esta revisión resume el conocimiento actual sobre la estructura y función de la hemoglobina de los peces que viven en hábitats antárticos. La amplia variedad de adaptaciones que permiten que los peces antárticos tengan la capacidad para sobrevivir de forma permanente en temperaturas cerca de la congelación es única entre los teleósteos. El suborden Notothenioidei, perteneciente a los Perciformes, es un excelente grupo para el estudio de la evolución y adaptación bioquímica a la temperatura. La gran variedad de taxones de nototénidos que viven en una amplia variedad de latitudes (Antártida, sub-antárticas, y las regiones templadas) ofrece una oportunidad extraordinaria para estudiar las características fisiológicas y bioquímicas adquiridas y perdidas por este grupo en respuesta al frío, además de la posibilidad de reconstruir los eventos más probables que direccionaron la evolución de la capacidad de transportar oxígeno en hábitats polares.

Aunque el oxígeno puede ser transportado en su forma libre disuelta, la mayoría de los animales dependen de una o más proteínas para entregarlo a los tejidos para la respiración. En comparación con especies de zonas templadas y tropicales, los nototenoideos de la alta Antártida han evolucionado reduciendo la concentración y multiplicidad de hemoglobina. La familia antártica Channichthyidae (el grupo con corona de los Nototénidos) carece de la hemoglobina. Todas las especies de peces existentes que viven en el hielo carecen de hemoglobina y muchas han perdido la expresión de la mioglobina. En estas especies, el aporte de oxígeno a los tejidos se produce por el transporte del gas físicamente disuelto en el plasma.
Palabras clave: Antártida; adaptaciones al frio; evolución; hemoglobina.

THE ANTARCTIC NOTOTHENIOIDEI

During many million years, the Antarctic biota, both on land and in the sea, has 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 (Clarke & Crame 1992). Over geological time, environmental conditions and habitats changed dramatically; consequently, many groups of organisms became extinct (Eastman 1993).

Nowadays the growing interest in polar marine organisms is closely related to the impacts of current climate change. The impacts of climate change will depend on the rate of ongoing temperature changes and on the genotype and ecology of species. More negative impacts are expected in species physiologically specialised with respect to temperature and with limited acclimation capacity (Pörtner 2010). Species living in thermostable environments are highly specialised within a narrow temperature range. Antarctic marine invertebrates may be more vulnerable to warmer temperatures (Peck *et al.* 2009a, 2009b), while thermal acclimation capacity still exists among Antarctic fish in the short/medium time range. The rate of impact of current changes in relation to the capacity of extant species to acclimate or adapt is a crucial study area for future management of polar ecosystems.

Over the past million years, the Antarctic shelf has been subjected to tectonic and oceanic events that began to alter the composition of the fish fauna and to initiate the process of faunal replacement (Clarke & Crame 1992). Fragmentation of Gondwana into the modern southern continents and the displacement of the Antarctic continent to its current geographic location have been the most significant events responsible for these changes. The crucial opening of the Drake Passage between southern South America and the Antarctic Peninsula occurred 23.5-32.5 million years ago (mya) (Thomson 2004), and possibly even as early as 41 mya (Scher & Martin 2006). The Drake Passage lead to the development of the Antarctic Circumpolar Current (ACC) and this in turn was at least partially responsible for the cooling of Antarctic waters from near 20°C to the present extreme values near -1.9°C, the equilibrium temperature of ice and sea water (Clarke 1983).

The Antarctic Polar Front (APF), the northern boundary of the ACC, is a well-defined, roughly circular oceanic system, running between 50°S and 60°S. Along the Front, the surface layers of the north-moving Antarctic waters sink beneath the less cold and less dense sub-Antarctic waters, generating virtually permanent turbulence. Just north of the Front, the water temperature has an abrupt rise of approx. 3°C, a critical factor for the isolation and adaptation of the ecosystem. The APF greatly limited the opportunities for migration, forcing the shallow-water fish fauna to either adapt to the changing climate or become extinct (Clarke 1983, Eastman 1993).

One group of teleost fish, the perciform suborder Notothenioidei, became largely dominant as a consequence of success in adapting to the challenging environmental conditions, for example low temperature, sea ice, habitat reduction and seasonality of primary production (Clarke & Johnston 1996). The ancestral notothenioid stock probably arose as a sluggish, bottom-dwelling teleost species that evolved some 40-60 mya in the shelf waters (temperate at that time) of the Antarctic continent and experienced extensive radiation, dating from the late Eocene, approx. 24 mya (Near 2004).

The high-Antarctic notothenioids, distributed south of the APF, live at the freezing point of sea water (near -1.9°C), and die at temperatures of 4-6°C (Somero & DeVries 1967). Non-Antarctic notothenioids, which comprise sub-Antarctic as well as temperate species, are found north of the APF and compose 22% (28 of 129 species) of notothenioid biodiversity (Eastman 2005).

Bovichtidae, Pseudaphritidae, Eleginopidae, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae and Channichthyidae are the families of the suborder (Eastman 2005). All Bovichtidae (except one species), monotypic Pseudaphritidae and Eleginopidae and some species of Nototheniidae inhabit waters north of the APF. These species encounter water temperatures of approximately 5°C-15°C (Eastman 2005, di Prisco *et al.* 2007).

Molecular phylogeny has recently begun to provide indications about the time of radiation in the Antarctic. Initial divergence took place about 40 mya during the Eocene (Near 2004).

Bovichtidae, Pseudaphritidae and Eleginopidae essentially never experienced near-freezing water temperatures because they presumably diverged and became established in waters around areas corresponding to New Zealand, Australia and South America before Antarctica became isolated. The phyletically basal bovichtids, pseudaphritids and eleginopids do not possess antifreeze glycoprotein (AFGP)-gene sequences in their genomes, indicating that they diverged well before the tectonic isolation and cooling of Antarctica (Cheng *et al.* 2003). The Antarctic clades thus probably evolved after the complete separation of Antarctica and the following isolation of the Southern Ocean from the surrounding water masses due to the formation of the APF. The subsequent cooling of Antarctic waters and expansion of ice sheets led to the extinction of the original temperate fauna (Eastman 2005). Notothenioids, in the absence of competition, filled all ecological niches made available. As a consequence of their extensive radiation, Antarctic notothenioids are considered one of the few examples of marine species flocks (Eastman & McCune 2000).

MOLECULAR ADAPTATIONS IN NOTOTHENIOIDEI

The variety of adaptations underlying the ability of modern Antarctic fish to survive at the freezing temperatures of the environment represents the extreme of low-temperature adaptations among vertebrates.

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). Comparative analyses of transcriptome profiles of the Antarctic notothenioid fish *Dissostichus mawsoni* and temperate/tropical fishes showed that evolution in the cold produced genomic expansions of specific protein gene families involved in physiological fitness of Antarctic notothenioids under the extreme polar conditions (Chen *et al.* 2008).

Differences that characterise notothenioids (Table 1) include efficient microtubule assembly at temperatures as low as -1.9°C (Detrich *et al*. 1989, 2000), apparent loss of inducible heat-shock response (Hofmann *et al*. 2000, Place *et al*. 2004, Place & Hofmann 2005) still possessed by some non-Antarctic notothenioids (Hofmann *et al*. 2005), enhanced enzyme-structural flexibility (Field & Somero 1998), changes in membrane fluidity (Romisch *et al*. 2003), constraints in aerobic energy supply, mitochondrial functioning and capacity of anaerobic energy production (Pörtner 2006), higher levels of ubiquitin-conjugated proteins in tissues as evidence for cold denaturation of proteins *in vivo* (Todgham *et al*. 2007). Some of these differences are not adaptive in a strict evolutionary sense, for example, loss of heat-shock response, gene expression loss of hemoglobin and myoglobin, higher level of ubiquitinated proteins (Somero 2010).

Table 1. Some peculiarities typical of Notothenioidei.

Feature	Consequence	Reference
Membrane change	Increasing fluidity of membranes	Romisch *et al.* 2003
Heat-shock response	Apparent loss of inducible heat-shock response	Clark & Peck 2009
Tubulin structure	Microtubule assembly	Detrich *et al.* 1989, 2000
Protein/enzyme structure	Maintaining high catalytic efficiency	Fields & Somero 1998
AFGP biosynthesis	Avoiding body-fluid freezing	Cheng & DeVries 1991
Genome plasticity	Up-regulation of specific genes involved in the anti-oxidant functions	Chen *et al.* 2008
Globin-gene loss	Reduction of blood viscosity	di Prisco *et al.* 2002
Aglomerular kidney	Urinary conservation of small antifreeze molecules	Eastman & DeVries 1986
Mitochondrial changes	Increase content in muscle	Guderley 2004

Interesting results showed that *Bovichtus variegatus* (family Bovichtidae) expresses heat-shock proteins in response to heat stress, whereas *Notothenia angustata*, a non-Antarctic notothenioid living in New Zealand waters, does not display the stress-inducible heat-shock protein synthesis (Hofmann *et al.* 2005). These results suggest that heat-shock response, the up-regulation of heat-stress sensitive genes, was lost after evolution in the subzero, stenothermic environment of Antarctic waters during the divergence of Bovichtidae from the other Antarctic notothenioid families. In addition, Buckley & Somero (2009) show that in cold-adapted *Trematomus bernacchii* no transcriptional up-regulation of heat-shock genes occurs during heat shock and that the level of up-regulation of gene expression under heat stress is highly changed, compared to expression changes measured in temperate, eurithermal fish.

ANTIFREEZE GLYCOPROTEINS (AFGPs)

The biosynthesis of AFGPs is one of the most important evolutionary adaptations discovered in high-Antarctic fish. AFGPs allow to avoid freezing by binding frozen water, thus preventing growth of ice crystals in the blood and other body fluids (Cheng & DeVries 1991). Produced by pancreatic tissue and the anterior portion of the stomach (Cheng *et al*.

2006), AFGPs are a family of polymers composed of a glycotripeptide monomeric repeat, -Thr-Ala-Ala-, with each Thr linked to the disaccharide galactose-N-acetylgalactosamine (Cheng & DeVries 1991).

High-Antarctic notothenioids have ample gene families for the production of large amounts of AFGPs. In non-freezing environments, where the antifreeze function becomes unessential, the AFGP function is reduced as observed in non-Antarctic notothenioid fishes (in Artic fish, AFGP biosynthesis occurs only in winter). In *N. angustata* and *N. microlepidota*, living in cool-temperate waters, the AFGP system is reduced with very low blood AFGP concentration and only two to three genes showing some replacements in the -(Thr-Ala-Ala)- repeat (Cheng *et al*. 2003). One of the Ala residues is occasionally substituted by Pro. *Dissostichus eleginoides*, a non-Antarctic notothenioid of the family Nototheniidae, appears to have no functional AFGP sequences, consistent with its non-Antarctic distribution. However, the apparent absence of AFGP genes in *D. eleginoides* is intriguing because the AFGP gene was thought to have evolved once, before the Antarctic notothenioid radiation, at the base of the family Nototheniidae. The hypothesis is that the species had the primordial AFGP genotype, lost or mutated following its migration to non-Antarctic habitats (Cheng *et al*. 2003).

The study of freezing avoidance in Notothenioidei is now developing along new perspectives, linked to the recent discovery of AFGP-deficient, but freeze resistant notothenioids in early life stages (Cziko *et al*. 2006). The absence of AFGP production in larvae suggests that suitable freezing resistance may temporarily be afforded by alternative mechanisms.

THE HEMOGLOBINS IN NOTOTHENIOIDEI

Since hemoglobins of all animal species have the same heme group, differences in their properties, including oxygen affinity, electrophoretic mobility and pH sensitivity, must result from the interaction of the prosthetic group with specific amino-acid residues in the primary structure. For this reason, fish hemoglobins have been the object of extensive studies in the last few years, not only for their structural characteristics, but also because they offer the possibility to investigate the evolutionary history of these ancient genes in species living in a large variety of environmental conditions (Weber & Jensen 1988). Fish hemoglobins, similar to other vertebrate hemoglobins, are tetrameric proteins consisting of two identical α and 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 hemoglobin structure and allosteric-ligand concentration (ATP for most teleost fish), and by changes in the expression of multiple hemoglobins 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 hemoglobin.

Unlike most mammals, including humans, fish often exhibit hemoglobin multiplicity, usually taken 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 hemoglobins, variable concentrations of allosteric effectors, differential response of hemoglobins to effectors (Verde *et al.* 2006, di Prisco *et al.* 2007). The capacity of fish to colonise a large variety of habitats appears to have evolved in parallel with suitable modulation of their hemoglobin system at the molecular/functional level.

Specialised hematological features are striking adaptations developed by the Antarctic ichthyofauna during evolution at low temperature. In the seven red-blooded notothenioid families the hemoglobin level is reduced, the erythrocyte number is an order of magnitude lower than in temperate fish, and is reduced by over three orders of magnitude in the 16 "icefish" species of Channichthyidae (Eastman 1993) in which hemoglobin is absent (Ruud 1954). Icefish retain genomic DNA sequences closely related to the adult α-globin gene(s) of its red-blooded notothenioid ancestors and contemporaries, whereas its ancestral β-globin-gene sequences have been deleted (di Prisco *et al*. 2002). The discovery within the icefish family of two distinct genomic rearrangements, both leading to functional inactivation of the *locus*, seems to point towards a multi-step mutational process (Near *et al*. 2006).

In channichthyids, no carrier has replaced hemoglobin and the oxygen-carrying capacity of the blood is only 10% compared to that of red-blooded fish. They compensate the lack of an oxygen carrier with large gills, increased blood volume and higher cardiac output (Egginton *et al*. 2002); moreover, they have highly vascularised, scaleless skin, which favours cutaneous respiration.

The loss of hemoglobin in icefishes is paralleled by the loss of myoglobin in 6 icefish species through at least 4 mutational events (Sidell & O'Brien 2006). Despite the costs associated with loss of these hemoproteins, the constantly cold and oxygen-saturated waters of the Southern Ocean provided an environment in which fish are able to survive even without oxygen-binding proteins.

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 hemoglobins may be reduced. Most Nototheniidae have a single major hemoglobin generally accompanied by minor or embryonic components (Verde *et al.* 2006, di Prisco *et al.* 2007). In most Nototheniidae, embryonic α and β globins are expressed in trace or limited amounts in the adult stage, but in at least three species, namely *Trematomus newnesi* (D'Avino *et al.* 1994), *Pagothenia borchgrevinki* (Riccio *et al.* 2000) and *Pleuragramma antarcticum* (Tamburrini *et al.* 1996), embryonic globins are expressed at significant levels (approx. 25% of the total). Adults of species of the modern families Artedidraconidae and Bathydraconidae lack the minor or embryonic globins.

Further evidence that the role of hemoglobin in red-blooded Antarctic fish may have become reduced, because of development of additional different and specialised functions, comes from molecular studies (Bargelloni *et al.* 1998) providing compelling evidence for positive selection of hemoglobin in *Gymnodraco acuticeps* of the family Bathydraconidae, the sister group to Channichthyidae lacking hemoglobins and their genes. *Gymnodraco acuticeps* (a sit-and-wait predator) showed a significantly higher rate of non-synonymous (amino-acid replacing) than synonymous (silent) substitutions in the β-globin DNA sequence with respect to the majority of notothenioids (Bargelloni *et al.* 1998). The evolutionary development of an alternative physiology based on hemoglobin-free blood may adequately work in the cold for notothenioids in general, and the benefits may include reduced costs for protein synthesis (Pörtner *et al.* 2007).

Unlike most high-Antarctic notothenioids, some sub-Antarctic and temperate notothenioids display higher multiplicity of hemoglobins. These multiple hemoglobins may differentially work in response to temperature differences and fluctuations (much larger than in the Antarctic), presumably to cope with the small or large temperature changes in the respective habitats north of the Polar Front (di Prisco *et al.* 2007). The hemoglobin multiplicity may reflect dynamic life style and different environmental conditions encountered.

The oxygen affinity of hemoglobins of many high-Antarctic notothenioids is quite low (di Prisco *et al.* 2007). This feature is probably linked to the high oxygen concentration in the cold sea. In contrast, the affinity is higher in hemoglobins of non-Antarctic notothenioids. The relationship between high affinity and habitat features remains an open question as far as its structural basis is concerned. Spectroscopic and modelling studies on the hemoglobins of temperate and sub-Antarctic notothenioids have shown that all the non-conservative replacements in the primary structure of the α and β chains leave the conformation and electrostatic field surrounding the heme pocket essentially unmodified with respect to the hemoglobins of high-Antarctic notothenioids (Verde *et al.* 2004).

The decreased oxygen affinity of hemoglobins at lower pH values in the physiological range is known as alkaline Bohr effect (reviewed by Riggs 1988). In many teleost hemoglobins, the complete loss of the subunity cooperativity in binding oxygen and the inability to saturate the ligand sites at low pH, even at high oxygen pressure, is a distinctive property with respect to the Bohr effect. This feature is known as the Root effect (Brittain 2005).

A general reduction in the Root effect is noticed during the evolution of the Antarctic notothenioids (di Prisco *et al.* 2007), corresponding to a variable scenario pertaining to the choroid *rete mirabile*. The physiological role of the Root effect is to secrete oxygen against high oxygen pressures into the swimbladder (when present) and the choroid *rete*

mirabile (Wittenberg & Wittenberg 1961, Wittenberg *et al.* 1964).

Antarctic fish lack the swimbladder. Among high-Antarctic notothenioids, many species have lost the choroid *rete*, although several retain portions of the *rete* and/or small vestigia of the choriocapillaris (Eastman 1993, 2006). Because high-Antarctic notothenioids still have hemoglobins endowed with Root effect also when the choroid *rete* is absent, this function may undergo neutral selection. It has been argued that the possession of the Root effect may undergo neutral selection pressure in the simultaneous absence of *retia mirabilia* and presence of high hemoglobin buffer capacity, as in some basal ray-finned fishes and in the ancestors of teleosts (Berenbrink *et al*. 2005). This may be the case in notothenioids with increased number of His residues (which confer buffer capacity) in the hemoglobin primary structure (Verde *et al.* 2008). In fact, a role for some His residues as modulators of the Root effect has recently been postulated (Mazzarella *et al.* 2006).

The multiple losses of the ocular oxygen-secretion mechanism in notothenioids would not necessarily be associated with degenerated eyes or less visually oriented life styles. In fact, an alternative oxygen-supply route to the retina by a system of hyaloid capillaries is especially well developed in several notothenioid species that have lost the choroid *rete* (for example, Eastman & Lannoo 2004).

CONCLUDING REMARKS

Gene expression patterns and, even more so, loss of genetic information, especially for myoglobin and hemoglobin in notothenioids, reflect the specialisation of Antarctic organisms to a narrow range of low temperatures. These modifications become explicable by exploitation of high-oxygen solubility at the low metabolic rates in the cold, where an enhanced fraction of oxygen supply occurs through diffusive oxygen flux. Conversely, limited oxygen supply to tissues upon warming is an early cause of functional limitation (Pörtner *et al*. 2007).

The evolutionary development of an alternative physiology based on hemoglobin-free blood may adequately work in the cold for notothenioids in general. The benefits due to hemoglobin loss include reduced costs for protein synthesis and oxygen transport. However, as pointed out by Pörtner *et al*. (2007), the shift from hemoglobin-mediated oxygen transport to mechanisms based on diffusion may account for higher vulnerability of icefishes, and of notothenioids in general, to warmer temperatures.

The southern polar environment is experiencing significant climatic change, as shown by sea-ice reductions at the western side of the Antarctic Peninsula (Clarke *et al*. 2007). Antarctic notothenioids that have had a long evolutionary history at constant temperatures may be uniquely vulnerable to Global Warming (Somero 2005).

Novel globins, such as neuroglobin and cytoglobin, have recently been described in many vertebrates (Burmester *et al.* 2000). Neuroglobin is able to bind oxygen and other ligands and it is transcriptionally induced by hypoxia and ischemia (Brunori & Vallone 2007). It is mainly expressed in retinal neurons and fibroblast-like cells and plays a neuroprotective role during hypoxic stress. The recent discovery of neuroglobin in the brain of red-blooded notothenioids and in some channichthyid species opens the question: what is the role of neuroglobin in fishes lacking hemoglobin and myoglobin (Cheng *et al.* 2009a, 2009b). The finding that icefish retain the neuroglobin gene, despite having lost those encoding hemoglobin and myoglobin in most species, is very intriguing.

Recently, Chen *et al.* (2008) have reported genome-wide studies of transcriptional and genomic changes associated with cold adaptation in Antactic notothenioids. Their results strongly suggest that evolution in the cold has produced dramatic genomic expansions and/or upregulations of specific gene families. Many of these up-regulated genes are involved in the antioxidant function, suggesting that augmented capacities in antioxidative defence are important components in evolutionary adaptations in cold and oxygen-rich environment.

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Low affinity PEGylated hemoglobin from *Trematomus bernacchii*, a model for hemoglobin-based blood substitutes

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Abstract

Background

Conjugation of human and animal hemoglobins with polyethylene glycol has been widely explored as a means to develop blood substitutes, a novel pharmaceutical class to be used in surgery or emergency medicine. However, PEGylation of human hemoglobin led to products with significantly different oxygen-binding properties with respect to the unmodified tetramer and high NO dioxygenase reactivity, known causes of toxicity. These recent findings call for the biotechnological development of stable, low-affinity PEGylated hemoglobins with low NO dioxygenase reactivity.

Results

To investigate the effects of PEGylation on protein structure and function, we compared the PEGylation products of human hemoglobin and *Trematomus bernacchii* hemoglobin, a natural variant endowed with a remarkably low oxygen affinity and high tetramer stability. We show that extension arm facilitated PEGylation chemistry based on the reaction of *T. bernacchii* hemoglobin with iminothiolane and maleimido-functionalysed polyethylene glycol (MW: 5000 Da) leads to a tetraPEGylated product, more homogeneous than the corresponding derivative of human hemoglobin. PEGylated *T. bernacchii* hemoglobin largely retains the low affinity of the unmodified tetramer, with a p50 50 times higher than PEGylated human hemoglobin. Moreover, it is still sensitive to protons and the allosteric effector ATP, indicating the retention of allosteric regulation. It is also 10-fold less reactive towards nitrogen monoxide than PEGylated human hemoglobin.

Conclusions

These results indicate that PEGylated hemoglobins, provided that a suitable starting hemoglobin variant is chosen, can cover a wide range of oxygen-binding properties, potentially meeting the functional requirements of blood substitutes in terms of oxygen affinity, tetramer stability and NO dioxygenase reactivity.

Background

Hemoglobin-based oxygen carriers (HBOCs) are a novel therapeutic class consisting of hemoglobin (Hb) derivatives administered intravenously as substitutes for blood transfusions. Modifications of the natural tetramer are required to reduce toxicity, as unmodified, cell-free Hb, once dissociated into dimers, is easily filtered by the kidneys and causes severe nephrotoxicity. Moreover, Hb extravasates through the endothelium, where it scavenges the vasoactive mediator nitrogen monoxide (NO), causing a range of toxic effects that include vasoconstriction and blood pressure increase. The strategies so far explored to avoid such effects mainly aim at increasing the molecular size of the natural Hb tetramers, thus limiting the size-dependent vessel extravasation and renal ultrafiltration. Beside some attempts at designing recombinant Hbs with higher molecular weight or lower dimer-tetramer dissociation constants,[1-5] most products proposed for clinical use consist of Hb purified from whole blood and chemically modified to achieve either intramolecular cross-linking or conjugation with polyethylene glycol (PEG).[6] PEG derivatization usually consists in the reaction between maleimido-functionalized PEG (MAL-PEG) molecules with either solvent-exposed cysteyl residues or thiol groups introduced through the reaction of lysyl side chains with 2-iminothiolane (IMT) under either aerobic[7] or anaerobic[8] conditions. PEG-decorated human Hb (HbA) derivatives have been evaluated in several clinical trials.[9] Adverse effects have so far prevented their application as a replacement of red blood cells.[10]

One of the limits of HBOCs lies in the large differences between their oxygen binding properties with those of red blood cells. As a matter of fact, cell-free HbA cannot bind the intra-erythrocyte allosteric effector 2,3-bisphosphoglycerate, which increases the P_{50} (the oxygen partial pressure required to achieve half saturation) from 10 Torr to around 26 Torr at 37°C, pH 7.4. Moreover, free Hb in the plasma is usually at concentrations low enough to significantly dissociate into

dimers, which do not show cooperativity and exhibit an oxygen P_{50} close to that of R-state Hb. PEGylation itself destabilizes the Hb tetramer and shifts the tetramer-dimer equilibrium towards the latter, with loss of cooperativity and a further increase in affinity.[11] Particularly, the reaction of PEG with Cys β93, conserved in 90% of vertebrates,[12] was associated with tetramer dissociation and increased affinity.[11, 13] As a matter of fact, both PEGylation of HbA in the T quaternary state, where Cys β93 is not reactive [8] and the reversible protection of Cys β93 HbA prior to conjugation [14] result in higher tetramer stability and lower affinity. However, based on experiments on HbA mutants, an increase in oxygen affinity seems to be at least partially independent of the derivatization of Cys β93.[15], suggesting that PEGylation induces changes in the hydration shell of hemoglobin, shifting the conformational equilibrium towards the more hydrated R state, regardless of the PEGylation sites.

In the light of the recent setbacks suffered by PEGylated Hb in clinical trials,[10] a deeper investigation of the relationship between the oxygen-binding properties and PEGylation in Hbs was undertaken. One of the possible strategies focused on the use of non-human PEGylated Hbs, taking advantage of the low immunogenicity of PEGylated proteins in general.[16] Non-human Hbs might greatly differ in terms of PEGylation pattern, oxygen-binding properties and sensitivity to allosteric effectors. A product consisting of bovine Hb decorated with 10-12 units of 5,000 Da-MW PEG was investigated as a possible blood substitute and showed a P_{50} of 10.2 Torr at 37°C,[17] higher than that of PEGylated human Hb but still far from that of human blood (around 26 Torr). TetraPEGylated canine Hb[16] similarly showed a P_{50} of 10 Torr under the same conditions. In view of investigating the relationship between the oxygen affinity of animal Hbs and that of their PEGylation products, Hbs from Notothenioidei, the dominant suborder of teleosts in Antarctica, are particularly interesting, as they show peculiar features that make them potentially less sensitive to the undesirable effects of PEGylation. The oxygen affinity of these Hbs is exceptionally low,[18] an evolutionary consequence of

the high oxygen concentration in the cold Antarctic waters. Moreover, unlike HbA, fish Hbs show little or no dissociation of the tetramer into dimers, even in the ligated form.[19] Finally, Cys β93, present in the great majority of vertebrate Hbs and known to greatly perturb the properties of PEGylated Hbs and to scavenge NO,[11] is missing in Hbs of almost all teleosts. The remaining cysteyl residues are all buried inside the protein matrix,[20] suggesting that PEG conjugation can be carried out regardless of the quaternary or ligation state. We therefore decorated Hb from *Trematomus bernacchii (Tb*Hb*)* with PEG and characterized the reactivity with oxygen and NO. The results were compared with those obtained for PEGylated HbA.

Methods

Reagents

2-iminothiolane (IMT), HEPES buffer, ethylendiaminotetraacetic acid (EDTA), phosphate buffered saline solution (PBS), sodium ascorbate, catalase and the reagents for the Hayashi enzymatic reducing system[21] were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and maleimido polyethylene glycol (MAL-PEG) (5600 Da-MW) from Nektar Molecule Engineering (Nektar Therapeutics, San Carlos, CA, U.S.A.). All other reagents were of the best available commercial quality.

Collection of specimens and Hbs purification

Specimens of *Tb*Hb were collected by gill nets or hook-and-line in the vicinity of Terra Nova Bay "Mario Zucchelli" Station (74°42'S, 164°07'E), Ross Sea, Antarctica, and kept in aquaria supplied with running, aerated sea water. Blood was withdrawn with heparinised syringes from the caudal vein. Hemolysates were prepared as described previously.[22] Saline-washed erythrocytes were frozen at -80°C until use. Purification of *Tb*Hb at 98% was carried out as described previously.[23] HbA was purified as described elsewhere.[24]

Cysteine titration

Preliminarily to PEGylation experiments, the reactivity of the cysteyl residues of *Tb*Hb was evaluated in both the deoxy- (T) and carboxy- (R) states using 4,4'dithiodipyridine (4-PDS).[25] For the titration under anerobic conditions, the protein solution was incubated in a helium flux until the absorption spectrum shifted to the deoxy-Hb form. A separately deoxygenated stock solution of 4-PDS was anaerobically added.

Hbs PEGylation

The PEGylation reaction was carried out following the protocol published for HbA in aerobic conditions.[11, 26] Briefly, *Tb*Hb or HbA were treated in the presence of IMT (80 moles/tetramer moles) and then with MAL-PEG 5600 Da-MW (12 moles/tetramer moles) (Scheme 1), at 10°C to prevent any heme oxidation during the reaction. *Tb*Hb was treated in the presence of CO, subsequently removed under oxygen flow before measurements.The reactions of IMT and MAL-PEG were quenched using lysine and cysteine in excess, respectively. Less than 5% met-Hb was formed during the reaction. To monitor the PEGylation reaction, small aliquots of the reaction mixture were sampled every 10 minutes. The reaction was quenched by addition of lysine and cysteine in excess. The samples were analyzed by sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and the electropherograms were evaluated using the Quantity One software (Bio-Rad). Under denaturing conditions, SDS-PAGE applied to PEGylated Hbs was able to separate PEGylated Hb into unmodified globin chains and globin chains with different PEGylation degree.[11] To evaluate the homogeneity of the final products, electrophoresis under native conditions was carried out in 8-2% gradient gel and analyzed as described elsewhere.[27]

Determination of oxygen-binding curves

Oxygen-binding curves of HbA, *Tb*Hb and their PEGylated derivatives, called PEG-Hb^{oxy} and PEG*Tb*Hb, respectively, were measured with a modified tonometer [24]. Before titration, the stock

solutions of the proteins were diluted in a solution containing 100 mM HEPES, 1 mM sodium EDTA, at either pH 7.0 or 8.0, to a final concentration of 30 μM. Sodium ascorbate and catalase were added to final concentrations of 5 mM and 10^3 U/ml, respectively, to prevent significant autoxidation during the measurement. For *Tb*Hb and PEG*Tb*Hb, experiments were carried out in the presence and absence of the allosteric effector ATP at a final concentration of 3 mM. For the experiments on *Tb*Hb and PEG*Tb*Hb stored in the carbomonoxy form, CO was removed by exposure to pure oxygen for 2 hours prior to titration, taking advantage of the relatively low affinity of TbHb for CO (data not shown). The samples where then exposed to oxygen partial pressures ranging from 0 to 760 Torr generated using an Environics 4000 (Environics inc, Tolland, CT, U.S.A.) gas mixer and pre-mixed helium/oxygen bottles, at 10°C. Spectra were collected in the 350-700 nm range using a Cary 4000 (Agilent Technologies, Lexington, MA, U.S.A.) spectrophotometer. The oxygen saturation at each partial oxygen pressure was determined by deconvoluting the spectra in the 450-700 nm range to a linear combination of the reference spectra of deoxy-, oxy- and met-Hb, plus a baseline. The deoxy reference spectra were obtained for HbA and *Tb*Hb in the presence of sodium dithionite, whereas reference spectra for the oxy forms were obtained in pure oxygen in the presence of the Hayashi reducing system.[21] The Hill's coefficient (n) and P_{50} were calculated by linear regression of the Hill's plots in the saturation range 20-80%.

Flash photolysis experiments

The experimental set up has been described previously.[28, 29] Flash photolysis measurements were performed using the circularly polarized second harmonic of a Q-switched Nd:YAG laser (Surelite II Continuum) and a cw Xe arc lamp as a monitoring beam. The transient absorbance signals were measured at 436 nm with a 5-stage photomultiplier.

NO dioxygenase activity

The rates of the NO dioxygenase reactivity at a single NO concentration were determined for HbA, *Tb*Hb, PEG-Hb^{oxy} and PEG*Tb*Hb by rapid mixing using a stopped-flow apparatus (SX.18MV, Applied Photophysics). The NO solutions were generated by equilibrating a previously deoxygenated PBS solution at pH 7.4 with a gas mixture of NO in nitrogen. The exact concentration of NO was then measured by titration of the solution with deoxygenated HbA under anaerobic conditions and determined to be 12 μM. The protein concentration was 3 μM. The reaction was monitored at 405 nm. Between 5 and 10 traces were collected and averaged. All measurements were carried out under strict anaerobic conditions at 20°C.

Results and Discussion

Cysteine reactivity

Sulfhydryl reactivity towards 4-PDS of carbomonoxy- and deoxy- *Tb*Hb was very slow (data not shown) with the fastest-reacting cysteine completing the reaction in more than 24 hours. The slow reactivity of *Tb*Hb confirmed the structural data,[20] which indicated the absence of exposed cysteyl residues. 2-iminothiolane-generated SH groups are therefore predicted to be the only reactive sites towards MAL-PEG (Scheme 1). For comparison, HbA reacts with a twice equimolar amount 4-PDS within 10 minutes (data not shown) due to the exposed Cys β93. It is therefore expected that *Tb*Hb, unlike HbA, would not react directly with MAL-PEG.

PEGylation

Samples collected at different times of the PEGylation reaction were compared in a SDS-PAGE gel (data not shown) and analyzed by densitometry (Figure 1). The reaction appeared to be completed in 30 minutes. The densitometric analysis[11] showed that about four PEG chains per tetramer are added during the reaction, as compared to the 5-6 PEG chains/tetramer added to HbA under the same reaction conditions. Nevertheless, in native electrophoresis, PEG*Tb*Hb exhibited a slower migration with

respect to PEG-Hb^{oxy}, possibly due to differences in electric charge (Figure 2). Some other noticeable differences emerged with respect to PEG-Hb^{oxy}. Particularly, the *Tb*Hb PEGylated derivative appeared more homogeneous and did not show any traces of unmodified tetramer (Figure 2), which were consistently observed in all preparation of PEG-Hb^{oxy}.[27] It is widely recognized that unmodified Hb is very toxic, as it can extravasate and be filtered at glomerular level. The complete derivatization of *Tb*Hb would therefore be a valuable property for a blood substitute.

Oxygen-binding properties

Oxygen affinity, cooperativity and the Bohr effect of PEGylated Hb derivatives and unmodified Hbs were measured under different conditions (Figure 3). The oxygen-binding curves were determined at pH 7.0 and pH 8.0, at 10°C. The analysis allows calculating P_{50} and Hill coefficient values (Table 1). At pH 7.0, HbA exhibited a P_{50} of 1.7 Torr, which decreased to approximately 0.4 Torr upon PEGylation (Table 1). The derivatization also resulted in loss of cooperativity, with the Hill coefficient decreasing from around 2 to 1.2. *Tb*Hb, under the same conditions, showed a much higher P_{50} of 28.2 ± 0.2 Torr. PEGylation resulted in an increase in oxygen affinity to 19.7 ± 0.3 Torr. However, P_{50} remained 50-fold higher than PEG-Hb^{oxy} under the same conditions. Cooperativity was significantly reduced, with the Hill coefficient decreasing from 2 to around 1.3. The changes in oxygen-binding properties of *Tb*Hb upon PEGylation are therefore similar to those observed for PEG-Hb^{oxy}, in particular showing loss in cooperativity. Considering the stability of the *Tb*Hb tetramer, this effect is likely due to the steric effects of the PEG moieties, which prevent the transition between the T and R states, rather than the dissociation of the tetramer, as seen in HbA. Despite the loss in cooperativity, the P_{50} of PEG*Tb*Hb remains remarkably high. Moreover, ATP at saturating concentrations of 5 mM (data not shown) still acts as an allosteric effector (Table 1), raising P_{50} to 31.1 Torr. These data, combined with those relative to PEGylated Hbs of other species, particularly in bovine[30] and canine Hbs,[16] show that, regardless of their different oxygen affinity, there is indeed a correlation between the

increase in affinity and the PEGylation reaction. The non-specific effect of PEGylation in increasing the oxygen affinity was also demonstrated within the same preparation, through the electrophoretic separation of differently PEGylated HbA derivatives having different affinities.[27]

Flash photolysis experiments

The CO rebinding kinetics measured after laser flash photolysis on the carbomonoxy forms of HbA and *Tb*Hb in the absence and presence of PEGylation is reported in Figure 4. The rebinding traces were converted to fraction N(t) of deoxy-Hb as a function of time. Dependence of the kinetics on the CO concentration allows distinguishing between unimolecular and bimolecular processes (data not shown). As it is well established for HbA, CO rebinding comprises multiple phases: a nanosecond (unimolecular) geminate process due to rebinding from within the heme pocket or the protein matrix, and two second order processes, one in the microsecond time scale, ascribed to bimolecular rebinding to quaternary R state, and one in the millisecond time scale, ascribed to the bimolecular rebinding to proteins that have switched to quaternary T state.[31] In order to reproduce the experimental rebinding curves for HbA, we used a sum of six exponential decays functions, as proposed by Kliger and coworkers.[32] In Figure 4 we also show the results of the global fitting on HbA and *Tb*Hb (both with and without PEGylation), demonstrating a very good agreement between calculated and experimental curves. Besides the two exponential decays which are necessary for describing the geminate phase (10 and 182 ns for HbA; 14 and 452 ns for *Tb*Hb), we detected two processes ascribed to quaternary relaxation from the R to the T states in the micro-millisecond time scale (1 and 140 μs for HbA; 110 μs and 1.5 ms for *Tb*Hb), one phase associated with the rebinding to R state (350 μs for HbA; 7 ms for *Tb*Hb) and one phase associated with the rebinding to T state (7 ms for HbA; 21 ms for *Tb*Hb). As previously shown in HbA,[11] while PEGylation preserves the general features of the dynamics and reactivity of the protein, it partially prevents the R to T relaxation, so that upon PEGylation the

fractional amplitude of the rebinding to R changes from about 27% to 80% in HbA and from 34% to 76% in *Tb*Hb.

NO dioxygenase activity

The scavenging of NO, associated with an extremely rapid deoxygenation reaction with oxy-Hb to form met-Hb and inert nitrate, is significant when Hb is present in blood vessels outside erythrocytes and is likely to be the main determinant of the adverse effects of HBOCs.[33, 34] In order to develop a possible blood substitute, it is crucial to consider not only oxygen-transport properties of these Hbs, but also the influence that these products can have on NO homeostasis. The reaction rates of the NO with unmodified *Tb*Hb and its PEGylated Hb derivatives were measured and compared with those of HbA and its PEGylation product. Analysis of the oxidation kinetics of oxy *Tb*Hb treated with NO (Figure 5) yields a k_{obs} of 7.2 $\mu M^{-1}s^{-1}$, indicating slower reactivity with respect to HbA, with a k_{obs} of 64 $\mu M^{-1}s^{-1}$ under the same experimental conditions, in agreement with literature data.[34] PEGylation of *Tb*Hb seems to only marginally affect the NO dioxygenase reactivity, with a k_{obs} for PEG*Tb*Hb of 7.7 $\mu M^{-1}s^{-1}$. A comparable decrease in the NO dioxygenase activity of HbA was achieved by mutating residues αE11, βE11, and βB10 (2-15 $\mu M^{-1}s^{-1}$).[33] These mutants showed reduced *in vivo* vasoactivity, directly correlated with the *in vitro* NO oxygenation rate. The same pattern is observed in HbA, the PEGylation of which, under aerobic and anaerobic conditions, yields k_{obs} of 83 $\mu M^{-1}s^{-1}$ (see Figure 5) and 86 $\mu M^{-1}s^{-1}$ (data not shown), respectively.

Conclusions

The functional characterization of the PEG-conjugated derivative of the highly stable Hb tetramer of *T. bernacchii* confirms the non-specific effects of PEGylation already observed in human, bovine and canine Hbs, including an increase in oxygen affinity, a decrease in cooperativity and a reduction of the

R- to T-quaternary switching upon flash photolysis. However, these non-specific effects are accompanied by the partial retention of the remarkably low affinity for oxygen, the sensitivity to allosteric effectors and the low NO dioxygenase reactivity. These results indicate that PEGylated Hbs, provided that a suitable starting Hb variant is chosen, can cover a wide range of oxygen-binding properties, potentially meeting the functional requirements of blood substitutes.

Abbreviations

4-PDS: 4,4'dithiodipyridine, ATP: adenosine triphosphate, EDTA: ethylenediaminetetraacetic acid, HbA: human hemoglobin, HBOC: hemoglobin-based oxygen carrier, IMT: 2-iminothiolane, MAL-PEG: maleimido polyethylene glycol, PEG: polyethylene glycol, PBS: phosphate buffered saline, SDS-PAGE: dodecylsulfate/polyacrylamide gel electrophoresis, *Tb*Hb: *Trematomus bernacchii* hemoglobin.

Authors' contributions

GdP initiated this investigation by collecting material from cold-adapted fish in Antarctica. DC, CiV and GdP purified *T. bernacchii* Hb. DC and LR performed Hb PEGylation and electrophoretic characterization, DC and SB investigated oxygen binding and performed cysteyl titrations. SB characterized NO dioxygenase reactivity and wrote the manuscript, CrV and SA performed the flash photolysis experiments. AM contributed to the manuscript preparation.

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physico-chemical properties, vasoactivity and renal filtration. *Biochim Biophys Acta* 2008, **1784:**1402-1409.

Figure legends

Table 1. **Oxygen binding parameters of human and *T. bernacchii* Hbs and their PEGylated derivatives**. Measurements were made in 100 mM HEPES, 1 mM EDTA, 5 mM ascorbate, 10^3 U/ml catalase, at 10°C. The values for PEG-Hb^{oxy} were calculated from published data taking into account the temperature dependence of the oxygen binding parameters.

Scheme 1. Schematic representation of extention arm facilitated PEGylation chemistry.

Figure 1. PEGylation reaction rates Reaction time dependence of PEG conjugation on globin subunits as determined from the densitometric analysis of SDS-PAGE gels: 0 PEG bound per subunit (open circles), one PEG bound per subunit (open inverted triangles), two PEG bound per subunit (open squares), three PEG bound per subunit (open diamonds), four PEG bound per subunit (open triangles).

Figure 2. Size distribution of derivatized hemoglobins. Gradient native PAGE of HbA, *Tb*Hb and their PEGylated derivatives.

Figure 3. Oxygen binding properties of derivatized hemoglobins. Hill plots of oxygen-binding curves of HbA (closed squares), *Tb*Hb (closed circles) and PEG*Tb*Hb (open circles), measured in 100 mM HEPES 1 mM EDTA, 5 mM sodium ascorbate, 10^3 U/ml catalase, pH 7.0, at 10°C. Experimental points are fitted to the Hill equation, with calculated Hill's coefficients and P_{50}'s reported in Table 1.

Figure 4. CO rebinding properties of derivatized hemoglobins. Effect of PEGylation on CO rebinding to HbA and *Tb*Hb and their PEGylated derivatives. The time courses of the deoxyheme fraction are shown for HbA (gray circles), PEG-Hb^{oxy} (black circles), *Tb*Hb (gray solid line) and PEG*Tb*Hb (black solid line), in 100 mM HEPES, 1 mM sodium EDTA, 1 atm CO, pH 7.0 at 10°C. Data were fitted as reported in Materials and Methods. The fitting curves are shown in red.

Figure 5. NO dioxygenase reactivity. Determination of the effect of PEGylation on NO dioxygenase reactivity of HbA and *Tb*Hb and their PEGylated derivatives. The reaction was carried out by rapid mixing under anaerobic conditions at 20°C. 3 μM oxygenated HbA (gray dotted line), *Tb*Hb (gray solid line), PEG-Hb^{oxy} (black dotted line), and PEG*Tb*Hb (black solid line), with a deoxygenated PBS solution containing 12 μM NO. The reaction was monitored at 405 nm and absorbance differences were normalized.

Table 1:

Protein	pH 7.0		pH 8.0	
	P_{50}	n	P_{50}	n
HbA	1.7 ± 0.1	2.05 ± 0.02	0.39 ± 0.3	1.72 ± 0.03
PEG-Hb^{oxy}	0.4[35]	1.2[36]		
*Tb*Hb	28.2 ± 0.2	2.06 ± 0.01	7.3 ± 0.1	1.75 ± 0.03
PEG*Tb*Hb	19.7 ± 0.3	1.33 ± 0.02	5.3 ± 0.1	1.09 ± 0.02
PEG*Tb*Hb + ATP	31.1 ± 0.2	1.09 ± 0.01	4.4 ± 0.3	1.02 ± 0.03

[a]Calculated from temperature dependence

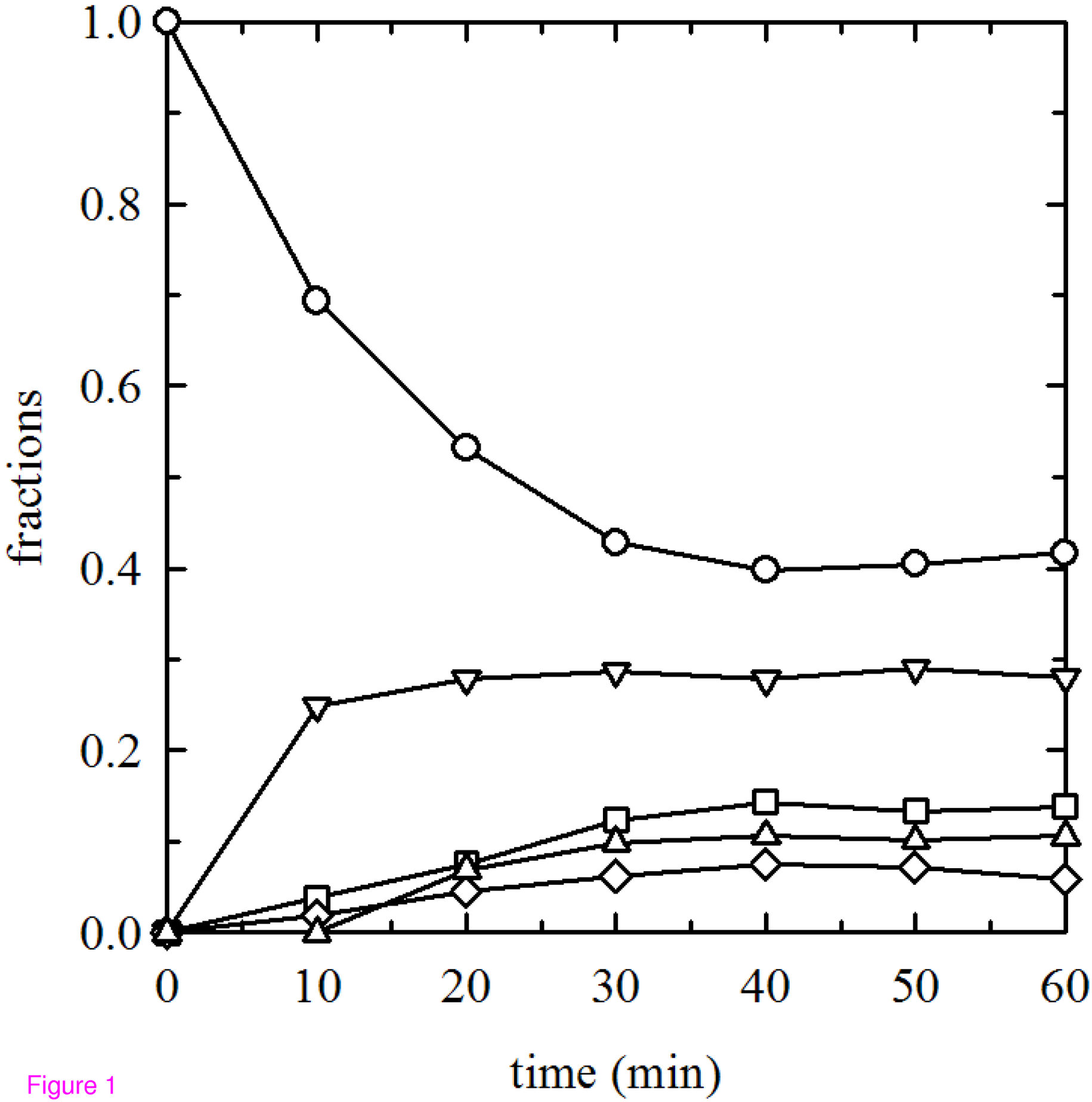

Figure 1

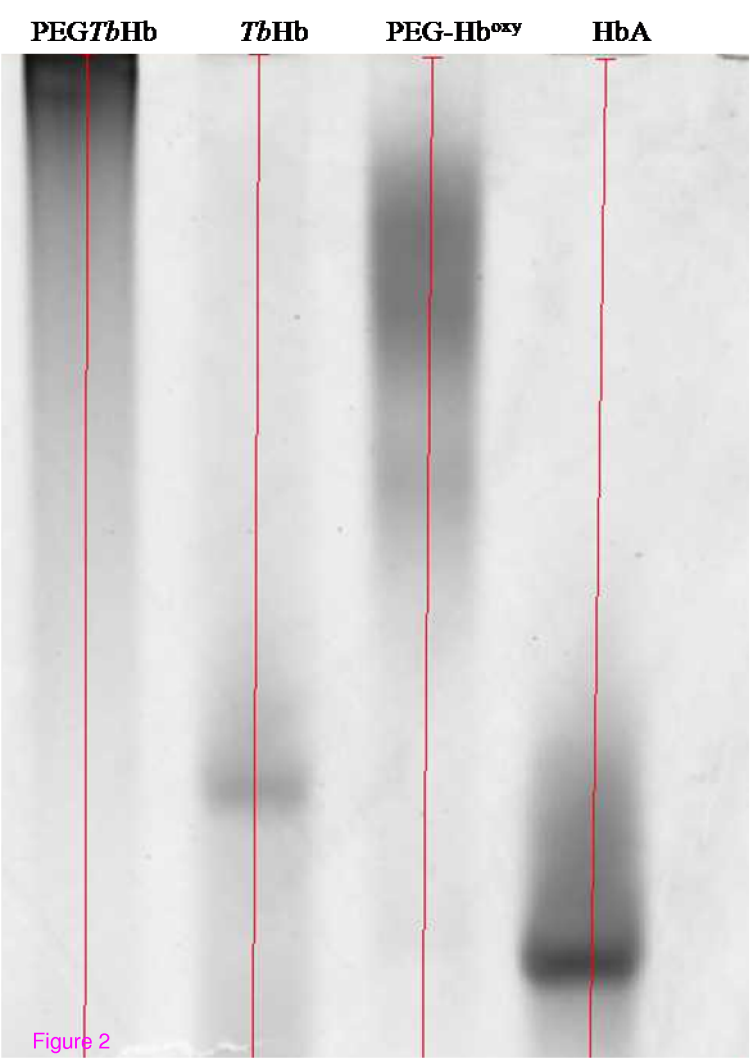

Figure 2

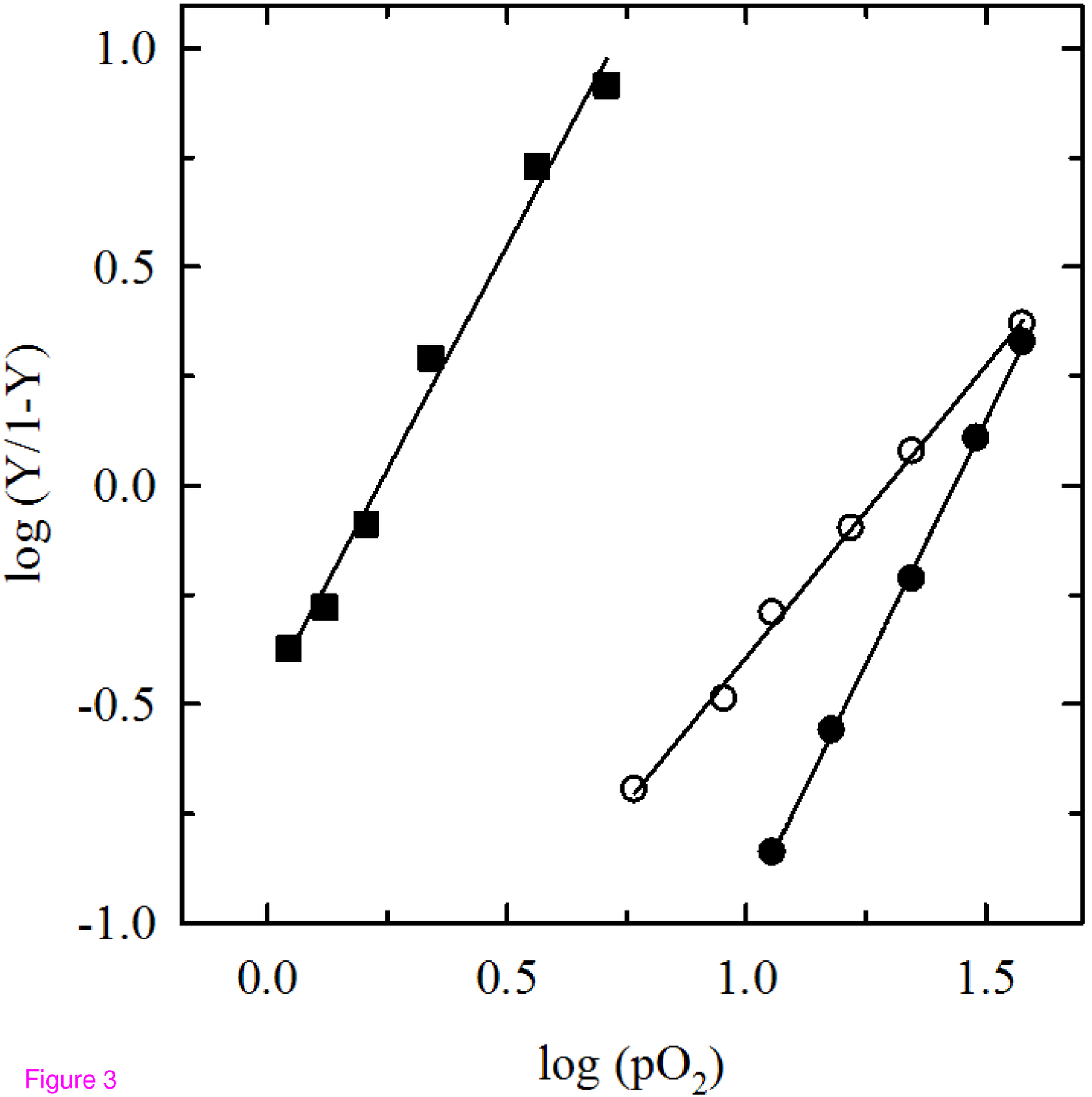

Figure 3

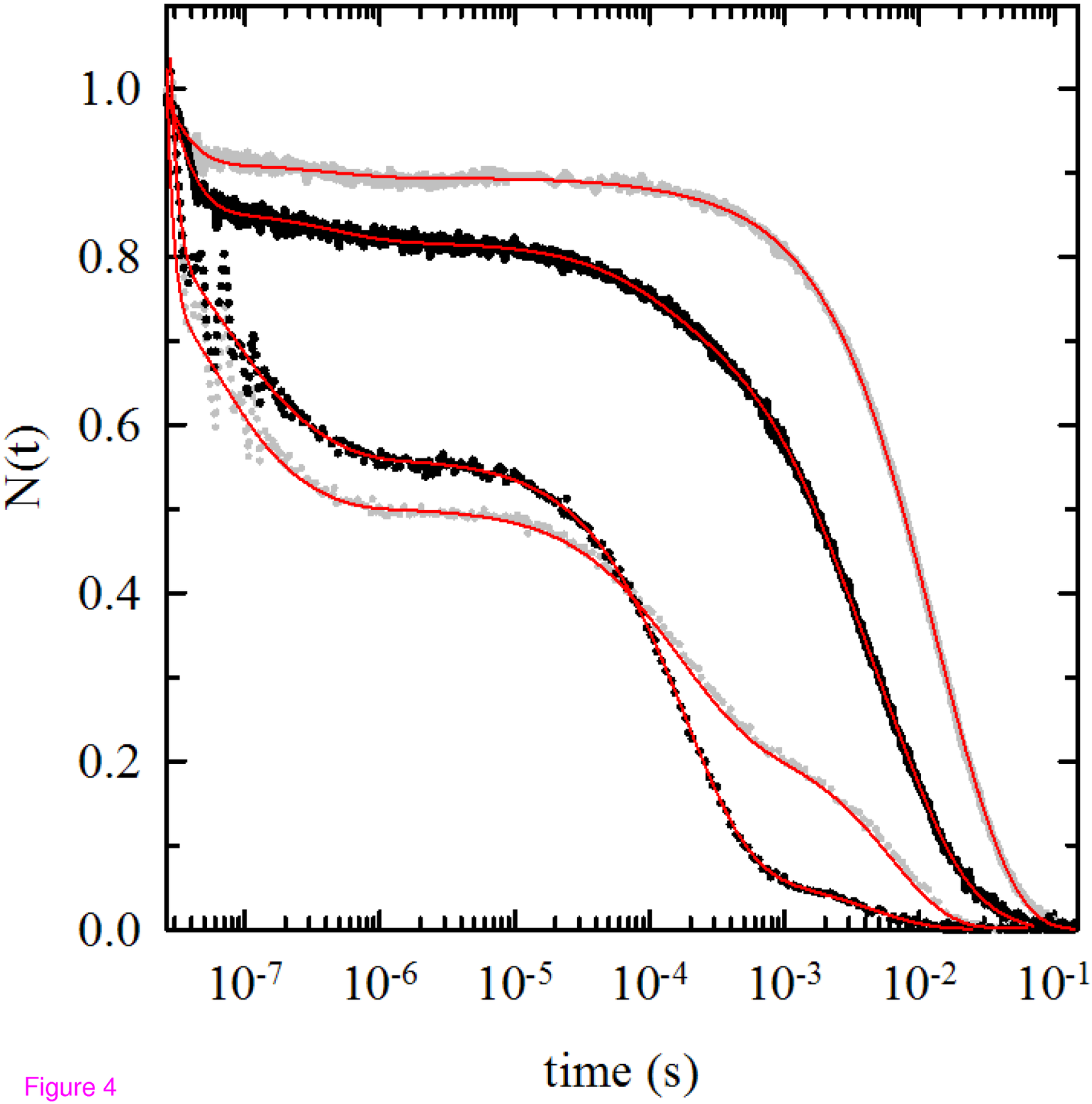

Figure 4

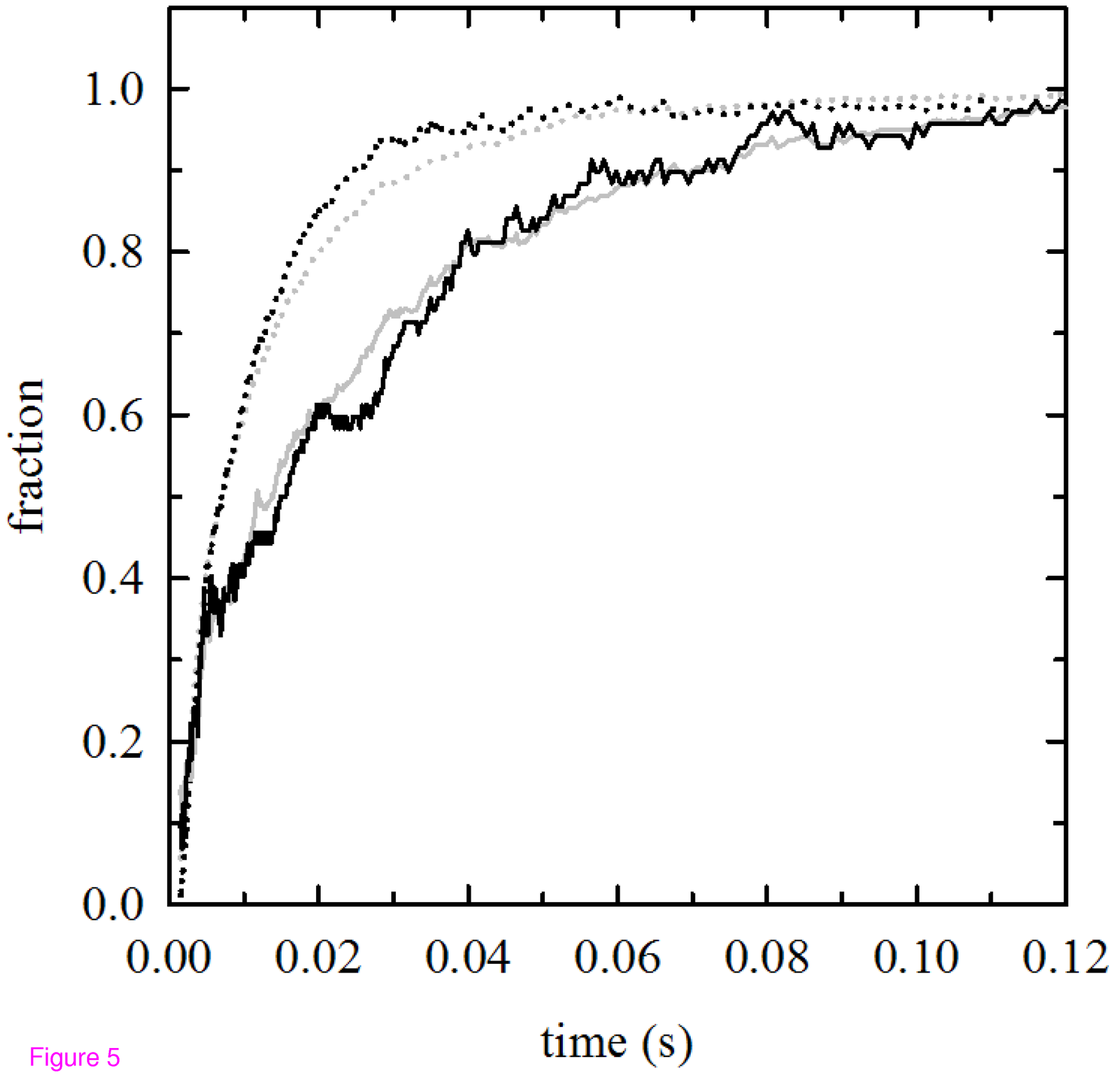

Figure 5

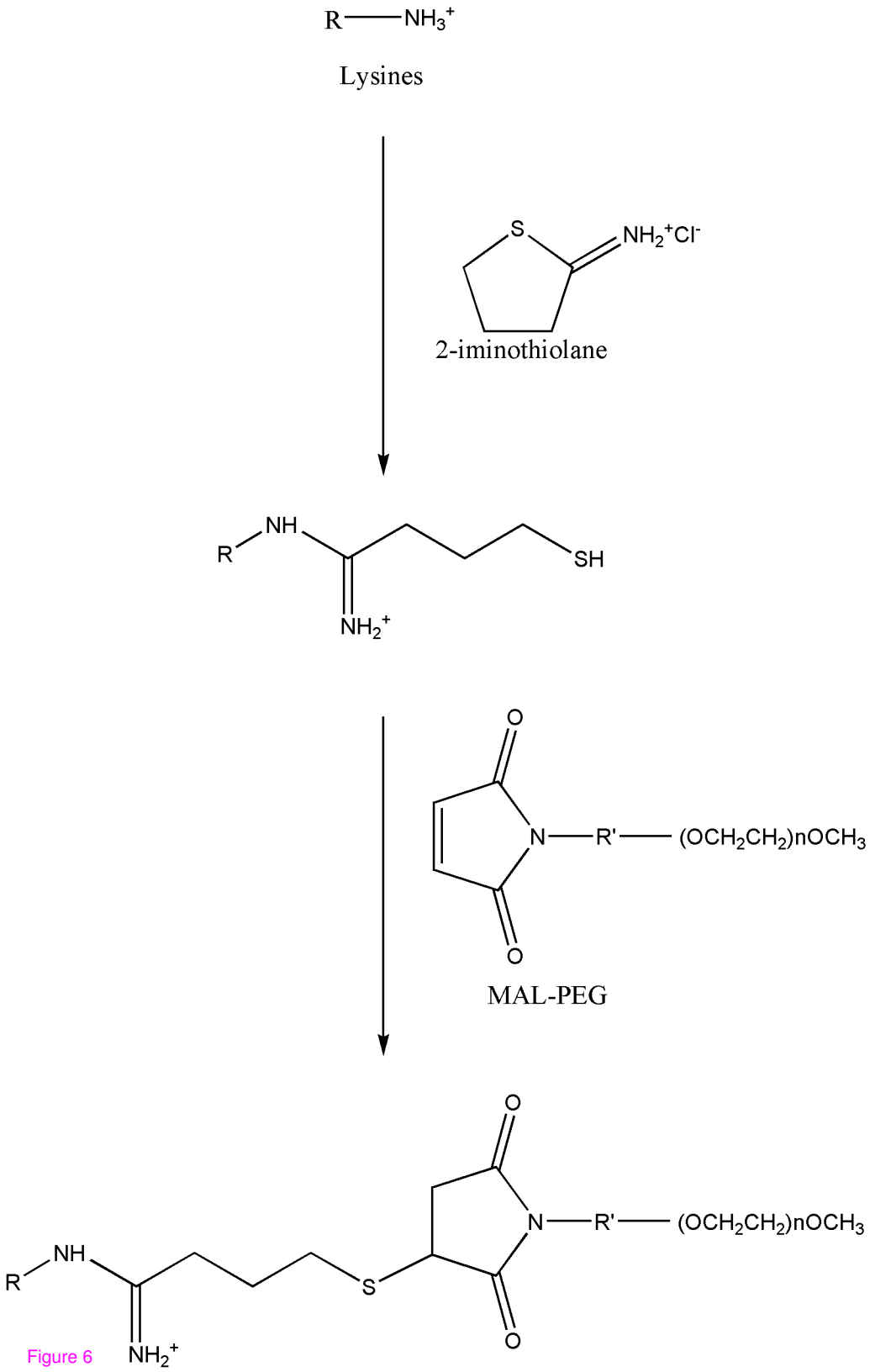
R—NH3+
Lysines
S
NH2+Cl-
2-iminothiolane
R
NH
SH
NH2+
O
N
R'
(OCH2CH2)nOCH3
MAL-PEG
R
NH
S
N
R'
(OCH2CH2)nOCH3
NH2+

Figure 6