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Expression, isolation and characterisation of tetrameric haemoglobin as a model to study the Root effect

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Abbreviations

Å	Ångstrøm
Abs	Absorbance
AFGP	Antifreeze glycoproteins
AFHb	Haemoglobin of Antarctic fish
ATP	Adenosine triphosphate
2,3-DPG	2,3-Diphosphoglycerate
2,3-BPG	2,3-Biphosphoglycerate
dNTP	deoxyribonucleoside triphosphate
Da	Dalton
EDTA	Ethylene-diamino-tetra-acetic acid
GST	Glutathione S-transferase
E. coli	*Escherichia coli*
FPLC	Fast Protein Liquid Chromatography
IPTG	Isopropyl β-D- thiogalactopyranoside
Hb	Haemoglobin
HbA	Human haemoglobin
Hb1Tn	Haemoglobin 1 of *Trematomus newnesi*
Hb1Tb	Haemoglobin 1 of *Trematomus bernacchii*
HbCTn	Cathodic haemoglobin of *Trematomus newnesi*
HPLC	High-Performance Liquid Chromatography
MPEG	Monomethyl polyethylene glycol
mya	million years ago
PCR	Polymerase Chain Reaction
PBS	Phosphate buffer saline
RR	Resonance Raman
rmsd	Root mean square deviations
S. cerevisiae	*Sacchoramyces cerevisiae*
SDS/PAGE	Sodium dodecyl sulfate polyacrylamide gel
TB	Terrific broth
TRIS	2-Amino-2-(hydroxymethyl)-aminomethane.

Summary

Haemoglobin (Hb) is one of the most intensively studied proteins, which has resulted in a deep understanding of its structure-function relationships. Hb has been termed the “honorary enzyme”, since the detailed knowledge of its structure and functions has rendered it a valuable model for studying allosteric interactions in other proteins (1).
Hbs are highly sensitive to temperature and, being a direct link between the exterior and body requirements, experienced a major evolutionary pressure to adapt and modify their functional features. Thus, their properties mirror the relationships between thermal conditions and adaptation (2).
Research on vertebrate Hbs, and on fish Hbs in particular, has continued to reveal exciting new aspects of molecular and cellular control mechanisms.
The O_2 affinity of all vertebrate Hbs is strongly pH-dependent. This phenomenon is known as the *alkaline Bohr effect*.
In many Hbs from teleost fishes, when the pH is lowered, the O_2 affinity decreases to such an extent that Hbs cannot be fully saturated even at very high O_2 pressure. In addition, cooperativity is totally lost and the O_2 capacity of blood undergoes reduction of 50% or more of its value at alkaline pH. This feature is known as the *Root effect*, an exaggerated Bohr effect, reviewed by Brittain (3). Root-effect Hbs are so strongly pH-dependent that they are able to unload a large amount of bound O_2 at low pH and against a pressure gradient.
In this context the study of Hb of Antartic fish is very interesting. In the process of cold adaptation, the evolutionary trend of Antarctic fish has led to unique specialisations in many biological features, e.g. freezing avoidance, polymerisation of tubulins and actins, enzyme catalysis, haematological parameters and O_2 transport. Decreased amounts and multiplicity of Hbs are common features. Some of these contribute to making Antarctic fish quite unique.
In the course of this PhD thesis tetrameric Hbs were studied, both to characterise the oxidation process of this protein and to determinate the structural and molecular basis of Root effect.
The oxidation of tetrameric Hbs from different species can lead to the formation of different products. Under physiological conditions, the oxidation of mammalian and temperate fish Hbs leads to the formation of the exogenous aquo-met hexa-coordinated form at both the α and

β subunits, whereas the oxidation of Antarctic teleost fish Hbs (AFHbs) leads to the formation of an aquo-met form at the α subunits and of an endogenous bis-histidine complex, denoted haemichrome, at the β subunits (β– haemichrome) (4).

In this framework, the research activity consisted of the investigation of Hb1Tn β globin alone, to verify the formation of haemichrome and to establish if in solution the tetramer β_4 is formed, as in HbA, or it rather stays as monomers.

Two vectors were constructed to express the β-chain of Hb1Tn: the vector pGEX, to express the Glutathione S-transferase (GST) fusion protein and the vector pET22, to be used in *E. coli* and in *Arctic cells*. The latter system allows the co-expression of the protein with the cold-adapted chaperonins Cpn10 and Cpn60, from the psychrophilic bacterium *Oleispira Antarctica* (5). The recombinant Hb1Tn β globin was obtained in soluble form through co-expression with GroEL/GroES, molecular chaperones of *E. coli* involved in the correct folding of premature proteins in an ATP-dependent manner.

Nevertheless, the trials of refolding *in vitro* with haem were not successful. The UV-VIS spectrum suggested that the haem was not correctly incorporated into β globin.

The β globin synthesized in the pET22 system, in both BL21 and C41 cells, was found to precipitate as inclusion bodies.

Finally, the Arctic cells produced only small amounts of recombinant protein, which could not be purified in reasonable yields.

Another objective of this project was to investigate the structural and molecular basis of the Root effect. For this purpose the major Hbs of *T. newnesi* (Hb1Tn) and *T. bernacchii* (Hb1Tb) represent an ideal model because of their high sequence identity and their very different functional behaviour (6, 7).

Two different approaches were undertaken.

The first approach to study the structure/function relationship in *T. newnesi/T. bernacchii* Hb1 was the site-directed mutagenesis of their α- and β-globin cDNAs, to express and characterise the mutant proteins.

The recombinant Hb1Tn was obtained in high amounts but not in soluble form.

A number of strategies have been tried to increase the solubility of this protein.

Moreover, it was tried a psychrophilic expression system, that uses *Pseudoalteromonas haloplanktis* TAC125 cells (8).

Unfortunately, these trials were unsuccessful.

The Hb1Tn accumulated as inclusion bodies was dissolved by strong denaturing reagents. The recombinant protein remained in solution after removal of the denaturing agent, however the UV-Vis spectrum suggested that the haem was not correctly incorporated.

The second strategy, undertaken to determine which residues are involved in the Root effect, was to study a natural mutant of human HbA, Hb *Potomac* (β101Glu→Asp) (9). The β101 residue is located at the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) interface, the region involved in the switching of the molecule from the R to the T state, its two normal quaternary structures. In fact, the minimal structural requirement for the Root effect, accounting for a two protons release upon oxygen upload, is attributed to the strong hydrogen bond that is formed by the side chains of Asp95α and Asp101β, assisted by Asp99β. This Asp triad is a common feature of all the deoxy structure of investigated Root-effect Hbs (10, 11).. In literature is reported that Hb *Potomac* has nearly pH-independent low oxygen affinity and no Root-effect, despite the presence of the Asp triad (12). These features are also characteristic of Hb1Tn.

The Hb *Potomac* was produced in *E.coli* using the pHE7 expression plasmid. The recombinant Hb was obtained in soluble form, in high yields and it was easily purified.

The optical and Raman spectroscopic properties of Hb *Potomac* were similar to that of human HbA. These results suggest that the conditions used for bacterial expression promote correct folding and produce soluble Hb *Potomac.*

Several trials of crystallisation were performed, however they were unproductive.

Finally, the crystal structure of the Hb1Tn in the T state at pH 6 was obtained at 2.1 Å resolution and it was compared to that of Hb1Tb.

Their R structures were found to be practically indistinguishable, and the basic features of the T structures, as for instance the hydrogen bond at the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) between Asp95α and Asp101β, are also very similar. Although these results should have been largely expected on the basis of their very high sequence identity, the difference in their functional behaviour (the presence/absence of Bohr and Root effect) allow to suppose, at least, the presence of structural modifications at the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) interface with the break of the aspartic-aspartic interaction.

In the case of the two Antarctic fish Hbs, a possible explanation may reside in other differences also present in the T state. These are mainly related to the unusually large thermal fluctuations which characterise the α chain of Hb1Tn, and in particular the CD segment and the two consecutive E and F helices, which bind the α haem moiety.
In all cases, the ambiguity in defining the structural determinants of the Root effect suggests that more than one mechanism may operate in the various Hbs, and that a more precise characterisation of these properties requires a systematic structural characterisation of selected mutants of the two proteins.

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Riassunto

L'emoglobina (Hb) é una proteina tetramerica di fondamentale importanza per lo svolgimento di processi vitali, essendo coinvolta nel trasporto dell'ossigeno.
L'Hb, rappresentando il legame diretto tra le necessità dell'organismo e il mondo esterno, svolge il suo ruolo fondamentale in un vasto spettro di condizioni: le sue caratteristiche allosteriche la rendono una proteina particolarmente interessante per lo studio di struttura e funzione. Essa rappresenta un modello ideale per lo studio del meccanismo di azione delle proteine allosteriche. L'emoglobina è altamente sensibile alla temperatura e, rappresentando un collegamento diretto tra l'ambiente esterno e le richieste dell'organismo, ha subito una maggiore pressione evolutiva per adattare e modificare le sue caratteristiche funzionali. Pertanto, le sue proprietà rispecchiano la relazione tra le condizioni climatiche e l'adattamento (1).
L'affinità con l'O_2 di tutte le Hb dei vertebrati è fortemente dipendente dal pH. Questo fenomeno è conosciuto come effetto Bohr. Molte emoglobine dei pesci teleostei possono presentare, a pH acidi, una diminuzione così forte dell'affinità e della cooperatività con l'ossigeno, nota come effetto Root, tale che la molecola non raggiunge la piena saturazione anche ad alte concentrazioni di quest'ultimo (2).
In questo contesto lo studio di Hb da pesci antartici può risultare di notevole interesse. Nel processo di adattamento al freddo, l'evoluzione delle specie antartiche ha portato a specializzazioni biologiche peculiari che riguardano: abbassamento del punto di congelamento, polimerizzazione di tubuline e actine, catalisi enzimatica, modifiche nel trasporto dell'ossigeno, come minore quantità e molteplicità di emoglobine. Alcune di queste caratteristiche fanno dei pesci antartici delle specie uniche (3).
Scopo principale di questo progetto di ricerca è stato lo studio di emoglobine tetrameriche sia per caratterizzare il processo ossidativo di queste proteine sia per determinare le basi molecolari dell'effetto Root.
E' stato visto che, diversamente dalle Hb di mammifero, che in seguito ad ossigenazione formano acquomet per entrambe le catene globiniche, le Hb dei pesci antartici seguono un diverso processo ossidativo, formando emicromi per le globine β (4).

Con lo scopo di studiare i processi ossidatavi delle emoglobine, è stata intrapresa l'espressione della sola globina beta dell'Hb1Tn. Quest'ultima potrebbe risultare interessante sia per verificare la facilità di formazione dell' emicromo, sia per vedere se, in soluzione, forma il tetrametro β_4, come nell'HbA (5), o se c'è un equilibrio tra il monomero ed il dimero.
Per l'espressione in *E. coli* della globina β sono stati scelti come vettori il pGEX ed il pET22. Con il primo è stato possibile ottenere la globina in forma solubile con la co-espressione delle chaperonine di *E. coli*. Quindi sono state effettuate le prove di "refolding" con l'eme *in vitro*, ma gli spettri UV-VIS hanno evidenziato che l'eme non è stato incorporato correttamente.
Il secondo vettore, pET22, è stato co-trasformato in cellule contenenti il plasmide per l'espressione delle chaperonine Cpn10 e Cpn60 del batterio psicrofilo *Oleispira antartica* (6). Con questo sistema è stata ottenuta la globina beta, ma le basse rese di espressione non hanno reso possibile la purificazione della proteina ricombinante.
Per gli studi delle basi molecolari dell'effetto Root, le emoglobine principali di due teleostei antartici della famiglia dei Nototeniidae, *Trematomus newnesi* (Hb1Tn) e *Trematomus bernacchii* (Hb1Tb) rappresentano un modello ideale, in quanto mostrano nel legare l'ossigeno una dipendenza dal pH molto diversa nonostante l'altissima identità di sequenza (7, 8).
Il primo obiettivo proposto per lo studio della relazione struttura e funzione dei due modelli emoglobinici di riferimento è la preparazione di mutanti dell'Hb1 del *T. newnesi,* da esprimere mediante opportuni vettori plasmidici. E' risultato però che l'Hb1 del *T. newnesi* viene espressa in alte rese ma in forma non solubile, accumulandosi nei corpi di inclusione. Per cercare di migliorare la quantità di proteina solubile sono state effettuate numerose prove in diverse condizioni. Innanzitutto, è stata provata l'espressione variando la temperatura, la concentrazione dell'induttore IPTG (isopropil β-D- tiogalattoside) e dell'emina.
Un altro tentativo è stato la co-espressione di Hb1Tn con le chaperonine di *E. coli*, che dovrebbero indurre il corretto folding della proteina e, quindi, migliorarne la solubilità.
Tuttavia nessuna di queste strategie ha portato al risultato desiderato.
Quindi sono state effettuate prove di solubilizzazione della proteina e successivo "refolding" *in vivo* in presenza di emina. Sebbene la proteina rimanga in soluzione anche dopo avere allontanato l'agente denaturante, gli spettri UV-VIS mostrano che la proteina è denaturata e

che l'eme non è stato incorporato correttamente. Infine, è stato usato il sistema psicrofilo di *Pseudoalteromonas haloplanktis* TAC125, con il vettore pUCRP, che permette l'espressione di proteine eterologhe a freddo e sotto il controllo di un promotore inducibile, mediante aggiunta di L-malato nel mezzo di crescita (9).

Il non corretto "folding" dell'Hb ricombinante e, quindi, il suo accumulo nei corpi inclusi, potrebbe essere dovuto alla mancata acetilazione della globina alfa, caratteristica peculiare delle Hb dei teleostei antartici.

Contemporaneamente è stato studiato un mutante naturale dell'emoglobina umana, l'HbA *Potomac* (β101Glu→Asp) (10).

Va ricordato, infatti, che un parte dell'effetto Root è dovuto al legame idrogeno tra l'aspartico 95 della globina alfa e l'aspartico 99 della globina beta, all'interfaccia $\alpha_1\beta_2$ ($\alpha_2\beta_1$), assistito dall'aspartico 101 della globina beta. Tale triade di aspartici è presente in tutte le emoglobine con effetto Root (11, 12).

Nell'emoglobina umana, all'interfaccia $\alpha_1\beta_2$ ($\alpha_2\beta_1$) sono presenti sia l'aspartico 95 alfa che l'aspartico 99 beta, mentre in posizione 101 è presente un glutammico. La catena laterale più lunga di questo residuo fa sì che i carbossilati dell'aspartico 95 ed il glutammico 101 non vengano in contatto.

Dati di letteratura (13) riportano che l'emoglobina *Potomac* non presenta effetto Root, nonostante la presenza della triade di aspartici.

Lo scopo, quindi, era quello di vedere, tramite la forma deossi dell'emoglobina *Potomac*, se si forma il legame idrogeno tra gli aspartici.

Mediante mutagenesi sito-specifica, è stato ottenuto il costrutto pHE7Potomac. Si è proceduti con l'espressione dell'Hb *Potomac* in *E. coli*. Sono state ottenute buone quantità di proteina ricombinante e solubile.

La proteina ricombinante è stata purificata e caratterizzata tramite spettrometria di massa, spettroscopia Raman e UV-VIS. Gli spettri di massa hanno evidenziato l' omogeneità del campione, che è presente solo nella forma con la metionina N-terminale; inoltre, il valore della massa della globina beta conferma la presenza della mutazione nella posizione 101. Gli spettri UV-VIS e gli spettri Raman presentano le stesse bande di assorbimento caratteristiche dell'HbA. Questo indica che il "folding" della proteina è avvenuto correttamente e che la mutazione (β101Glu→Asp) non influenza i modi di "bending" dell'anello porfirinico. Infine, sono state effettuate diverse prove di cristallizzazione della forma deossi dell'Hb, ma non sono stati ancora ottenuti i cristalli.

Contemporaneamente sono stati ottenuti i cristalli dell'Hb1 *wilde type* del *T. newnesi* nella forma deossi e ne è stata determinata la struttura.
Un caratteristica principale dell' Hb1Tn deossi è l'elevato valore del fattore termico rispetto a quello trovato per le altre emoglobine tetrameriche in forma deossi; questo indica una maggiore flessibilità e disordine della struttura. Tale disordine è anche consistente con una banda Raman del modo Fe-His più larga per l'Hb1Tn che per l'Hb1Tb o HbA, sia in soluzione che nel cristallo. Nonostante questo elevato disordine che caratterizza l'intera struttura, la regione all'interfaccia $\alpha_1\beta_2$ ($\alpha_2\beta_1$), contenente la triade di aspartici, risulta ben definita; i due aspartici, Asp95α e l'Asp99β risultano ad una distanza tale da condividere un protone.
Questo risultato è tuttavia sorprendente. Infatti, se da una parte poteva essere prevedibile data l'elevata identità di sequenza con l' Hb1Tb, dall'altra le diverse caratteristiche funzionali delle due Hb facevano prevedere una modificazione all'interfaccia $\alpha_1\beta_2$ ($\alpha_2\beta_1$), con conseguente rottura del legame idrogeno tra i due aspartici, come avviene nell' HbI della trota (14).
Pertanto, per trovare una possibile spiegazione del diverso comportamento delle due emoglobine sono state fatte diverse ipotesi. La prima potrebbe risiedere nella maggiore flessibilità della struttura; infatti la regione di maggiore disordine è localizzata nella catena alfa, in particolare nel "loop" CD e nelle due eliche E ed F.
In conclusione, l'ambiguità nel definire i determinanti strutturali dell'effetto Root suggerisce che ci sono diversi meccanismi che agiscono nelle differenti emoglobine; maggiori spiegazioni sulla natura dei gruppi che determinano questa proprietà potrebbero richiedere uno studio più puntuale e sistematico di mutanti selezionati delle due emoglobine.

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Chapter 1

Introduction

1.1 Haemoglobin

Haemoglobin (Hb) is one of the most intensively studied proteins, which has resulted in a deep understanding of its structure-function relationships. Hb has been termed the "honorary enzyme", since the detailed knowledge of its structure and functions has rendered it a valuable model for studying allosteric interactions in other proteins. Research on vertebrate Hbs, and on fish Hbs in particular, has continued to reveal exciting new aspects of molecular and cellular control mechanisms (1).

Haemoglobins are haem-containing proteins that share a well-known "globin fold" and reversibly bind molecular oxygen. They are found in various species from bacteria to man. They exist as monomers, dimers, tetramers or even higher molecular weight assemblies. Myoglobin (Mb), neuroglobin (Nb), and cytoglobin (Cb) on the other hand have monomeric structures. In addition to these differences in the quaternary structure, these molecules show slight variations in their tertiary structure with functional consequences. Mb has higher O_2 affinity than Hb and stores O_2 (2).

Haemoglobins of the higher vertebrates are tetramers made up of two α and two β chains, that in humans consist of 141 and 146 amino acid residues, respectively. The "globin fold" of each chain characteristically consists of seven or eight helices (conventionally labelled A–H) linked by non-helical segments e.g. EF, FG) segments, and of N-terminal and C-terminal extensions referred to NA and HC. Each chain has a crevice, the haem pocket, between the E and F helices, that harbours an O_2-binding haem group. The haem, responsible for the distinctive red colour of blood, consists of an organic component, protoporphyrin IX, with four pyrrole rings linked by methene bridges to form a tetrapyrrole ring, and a central iron atom (Fe^{2+}), bound to the four pyrrole-nitrogen atoms. Protoporphyrin IX has two vinyl groups, four methyl groups and two propionate groups. When Fe^{2+} becomes oxidised to Fe^{3+}, Hb is denoted Met-Hb.

Haem is harboured within the globin fold, formed by an eight-helix arrangement of polypeptides. The main helices within the globin fold are

organised into a two layer structure recognised as a “three-over-three” α-helical sandwich (Figure 1).

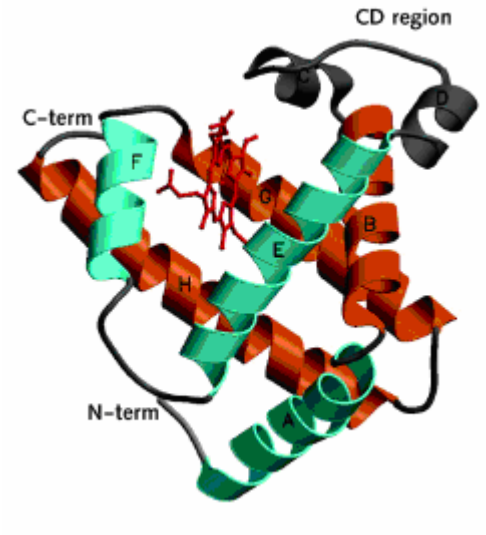

Fig. 1 “Three-over-three” α-helical sandwich (globin fold)

The four chains are arranged tetrahedrally around a two-fold symmetry axis that runs along a water-filled cavity.
Individual amino acid residues are usually referred to their helical positions and/or sequential numbers, starting from the amino termini. Invariant amino acids are HisF8, that anchors the haem to the protein moiety at the *proximal* side of the haem (thus known as the proximal His) and PheCD1, that wedges the haem into its pocket. O_2 binds at the *distal* side of the haem, where a distal HisE7 is highly conserved (figure2).

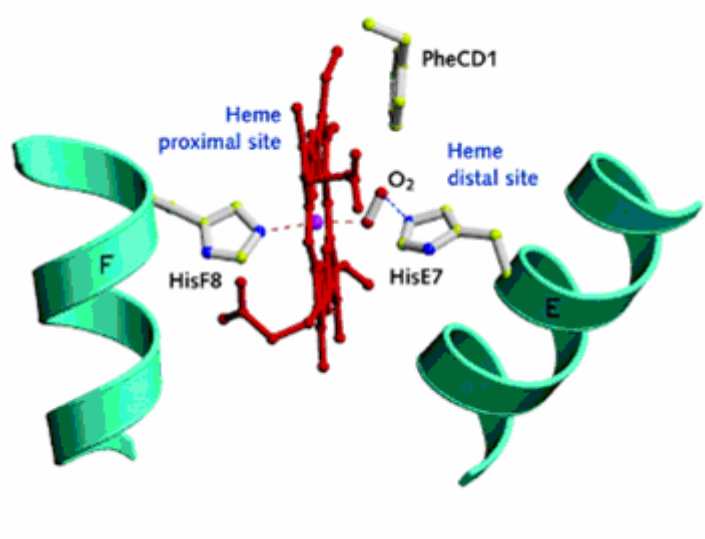

Fig. 2 Haem environment

Binding of O_2 in Mb and Hb occurs on the distal side of the penta-coordinated haem, where O_2 establishes a sixth coordination bond to Fe^{2+}. The binding of O_2 at the sixth coordination site of Fe^{2+} substantially rearranges the electrons within Fe^{2+} so that it becomes significantly smaller, allowing it to move into the porphyrin plane (3).
The physiological functions of vertebrate Hbs are the transport of oxygen to the body tissues, enhancement of carbon dioxide transport in the opposite direction, and regulation of blood pH. Early observations of the oxygenated and deoxygenated Hbs crystallized in different forms suggested that Hb undergoes a structural change upon binding to oxygen. The elucidation of the crystal structure of horse met (ferric) and deoxy and human deoxy Hb, by Perutz and his co-workers, revealed the structural difference between deoxy and oxy Hb. Perutz proposed a stereochemical mechanism of cooperative oxygen binding based on these structures (4) (figure 3) .

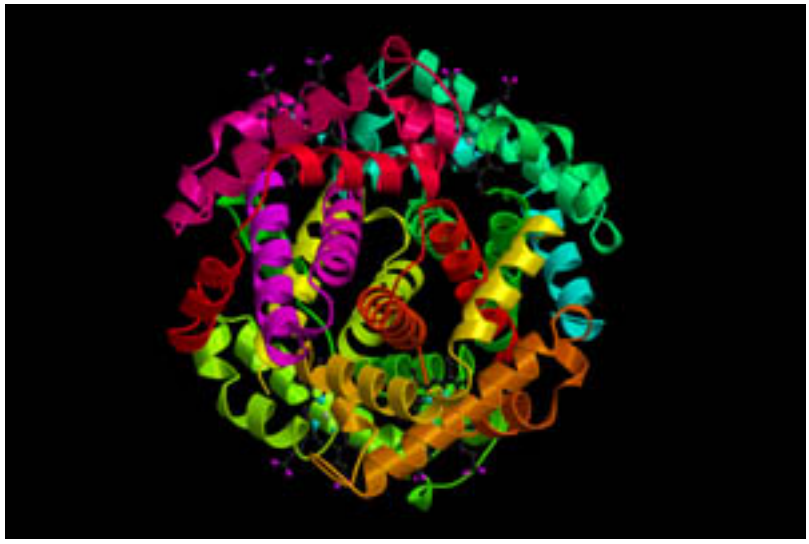

Fig. 3 Quaternary structure of HbA

As a first approximation, the oxygen binding properties of Hb can be described by the two-state allosteric model proposed by Monod, Wyman and Changeux. They proposed that the Hb molecule is in equilibrium between two alternative quaternary structures. They are known as the oxy or relaxed (R) structure, with a high affinity for oxygen, and the deoxy or tense (T) structure, with a low affinity (5).
Uptake and release of oxygen is accompanied by small changes in the tertiary structure of the segments surrounding the haems, and by a large change in the quaternary structure, involving a rotation of one αβ

dimer relative to the other by 15°, together with a relative shift of 1 Å between the dimers.
The rotation widens the cavity between the two β chains so that the cationic groups that form its lining can bind organic phosphates.
When O_2 binds to the haems in the T-state, the iron atom changes its spin state (from high to low) and moves into the plane of the porphyrin ring of the haem group. These movements, that are transmitted via the proximal HisF8 to the F helix, weaken the salt bridges between the two dimers and induce the protein to switch to the R-conformation state as the two αβ dimers rotate relative to each other. Major changes occur between helix Cα1 and the corner FGβ2, a region that is commonly called the switch interface. The T → R shift is basic to cooperativity, the progressive increase in O_2 affinity upon O_2 binding that is reflected by the characteristic sigmoid shape of O_2 equilibrium curves. Cooperativity is conveniently expressed by the Hill coefficient, *n*50, the slope of the Hill plot (log[oxy]/[deoxy] versus log P_{O_2}) at 50% saturation. For human Hb *n*50 is typically close to 2.8. Precise Hb-O_2 equilibria that include extreme (low and high) saturation values allow assessment of the O_2 association equilibrium constants of the T and R states (*K*T and *K*R, respectively) (6).
In mammals, the O_2-transport function of Hb is regulated by interaction with intracellular cofactors. Hb acquires the capability to regulate its O_2 affinity through organic phosphates, i.e. 2,3-biphosphoglycerate (2,3-BPG) or 2,3-diphosphoglycerate (2,3-DPG) in mammals and frogs, adenosine triphosphate (ATP) and/or guanosine triphosphate (GTP) in teleost fish, and inositol pentaphosphate (IPP) in birds (7).
The T state has low affinity for O_2 but high affinity for protons, chloride, organic phosphate and CO_2. In the R state these relative affinities are reversed. Chloride, organic phosphate and CO_2 are allosteric factors that stabilise the T state by forming salt bridges between subunits. These salt bridges are absent in the R state (8).

1.2 Interaction between NO and Hb

Hb binds and releases not only O_2, but also nitric oxide (NO), which is now recognized to be pivotal for an increasing number of key physiological responses, such as vasodilation, neurotransmission, and immune defence (9). The discovery in 1987 that the long-sought

endothelium-derived relaxing factor that regulates vasodilation is indeed NO (10), the ligand with the highest affinity for Hb, posed the puzzle of how NO is able to target and activate the vascular smooth muscle without being scavenged by Hb.

An alternative fascinating hypothesis emerged in 1996, with the discovery that a small fraction of Hb in the blood carries NO bound to the thiol groups of CysF9β as *S*-nitroso adduct (SNO-Hb) (11). Since reactivity of CysF9β in human Hb is controlled by the quaternary state of the protein, it was proposed that the nitrosated form of this residue is sensitive to the O_2-linked allosteric changes of Hb. Indeed, SNO-Hb concentration was originally found to be lower in venous than in arterial blood, suggesting that SNO-Hb may release NO when the O_2 tension decreases, thus increasing vasodilation and blood flow when tissues become hypoxic (12). Recent studies have shown that NO does not appear to react preferentially with vacant haems in partially carboxylated or oxygenated Hb nor to migrate to thiols following repetitive oxygenation–deoxygenation cycles (13).

Although it is unclear whether it occurs in mammals, the SNO-Hb respiratory cycle may be active in other vertebrates. Hb from spot fish appears to show oxygen-linked *S*-nitrosylation at residue CysH16α, suggesting that the conservation of CysF93 is not essential (14). The SNO-Hb content, which is lower in primate blood than in rodent blood, is strongly correlated with a decrease in thiol reactivity. Clearly, further studies are needed to establish the biological significance of *S*-nitrosylation in vertebrate Hbs. Turtles, that show exceptional resistance to hypoxia and have high erythrocytic glutathione concentrations and up to 16–20 cysteine residues per Hb tetramer, seem excellent candidates for further investigations (15).

1.3 Antarctic Region

In the late Paleozoic, about 250 million years ago (mya), land masses were assembled within a single large continent, Pangaea. About 200 mya, Pangaea split into two parts, Laurasia in the northern hemisphere and Gondwana in the southern one. Fragmentation of Gondwana into the modern southern continents initiated 135 mya, and the Antarctic continent reached its current geographic location approximately 65 mya. Final separation of East Antarctica from

Australia and West Antarctica (the Antarctic Peninsula) from South America occurred about 35-40 mya and 22-23 mya, respectively. With the opening of the Drake Passage, the isolation of Antarctica was completed (16). This event produced the Circum-Antarctic Current and the Polar Front, a well-defined, roughly circular oceanic system, running between 50°S and 60°S (figure 4).

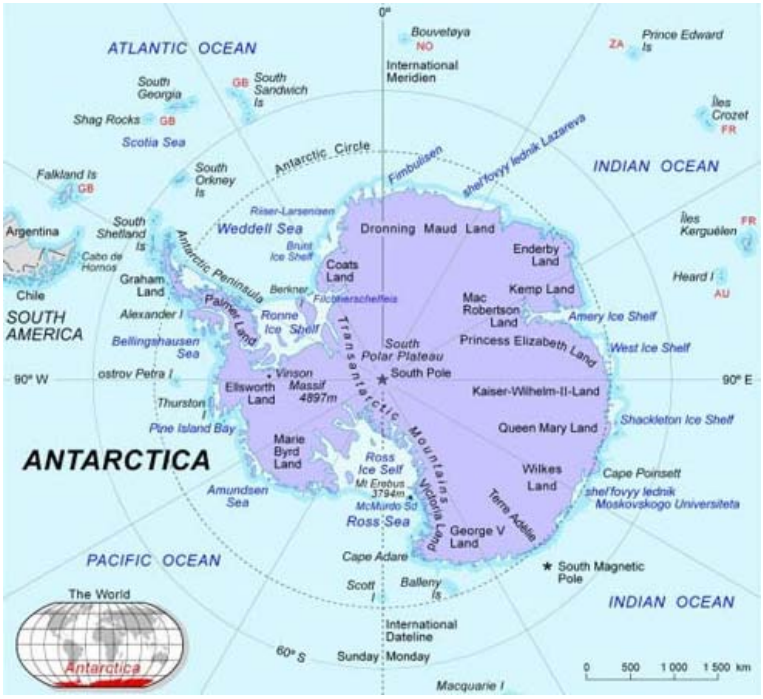

Fig. 4. The Antarctic region

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 about 3°C, a critical factor for ecosystem isolation and adaptation. The Antarctic experienced a slow and discontinuous transition from the warm-water system of the early Tertiary (15°C) to the cold-water system (-1.87°C) of today (17).

When the formation of vast sea-ice areas occurred, the fate of the most temperate species was extinction. One group of teleost fish, the suborder Notothenioidei, became largely dominant, because it was the only one which succeeded in adapting to the challenging environmental conditions, namely low temperature, presence of sea ice, habitat reduction and seasonality of primary production. Antarctic notothenioids live between 2°C and –1.87°C, the freezing point of sea

water, and die at temperatures of 4–6°C. In only ten million years, they have lost the ability to cope with higher temperature (18).

1.4 Antarctic Fish

Organisms living in the Antarctic regions are exposed to strong constraints, among which temperature is often a driving factor. Unlike temperate counterparts, polar fish express their genes and proteins in an extremely cold thermal regime. To compensate for the rate-depressing effect of low temperature on metabolic processes, polar fish have restructured many of their bio-molecular systems through mutation and selection, to preserve biological activity in such an extreme environment.

With the development of cold adaptation, the evolutionary trends of notothenioids have led to unique specialisations, including haematological differences in comparison with temperate and tropical species.

The biosynthesis of AFGPs, which allows polar fish to survive at sub-zero temperatures, is one of the most intriguing evolutionary adaptations discovered in the Antarctic fish. AFGPs are a family of discretely sized polymers composed of a simple glycotripeptide monomeric repeat (19, 20).

In the process of cold adaptation, Antarctic fish developed unique specialisations also in the blood stream. The blood of Antarctic fish contains fewer erythrocytes and less Hb than that of fish from temperate or tropical climates. This decrease in erythrocyte number and Hb content counteracts the increase in viscosity that would otherwise occur with decreased temperature. At the same time, low temperatures reduced the overall metabolic demand for oxygen, while increasing its solubility in the plasma, so that more oxygen can be carried in physical solution, and less needs to be bound to Hb (17).

At the extreme of such evolution, the colourless blood of the 16 species of the family Channichthyidae, the “icefish”, contains a very small number of erythrocyte-like cells and totally lacks Hb. The icefish maintain normal metabolic function by delivering oxygen physically dissolved in blood to the tissues. Reductions of the haematocrit to near zero appears selectively advantageous because it diminishes the energetic cost associated with circulation of highly viscous, corpuscular blood fluid (21).

1.5 Hbs of Antarctic Notothenioidei

Proteins such as Hbs are highly sensitive to temperature, therefore their structural and functional properties mirror the thermal conditions encountered by species during their evolutionary histories. Fish Hbs have been the subject of many studies because of the wide spectrum of structural and functional properties they can exhibit. Their structure/function variations can be considered as evolutionary adaptations that allow fish to take advantage of various habitats or respond to physiological or environmental challenges. Adaptive Hb variations are necessary because internal conditions in fish change markedly in response to environmental variations in oxygen and CO_2 levels, temperature, and solute concentrations. Fish Hbs function over a greater range of in vivo conditions than Hbs of higher vertebrates.

The Hb molecule has evolved structural and functional diversity to adapt and modify its features under selective pressure of all types, but both the predominantly helical structure and a large number of amino-acid residues are well conserved. The primary role of Hb, that of carrying O_2 to vertebrate tissues, is probably the origin of its adaptation to widely different environmental conditions. Its specialised function imposes severe structural constraints on the molecule. Hence, it is not surprising that only a small fraction of the residues of the polypeptide chains is allowed to be replaced during evolution. According to the species-adaptation theory of Perutz, the replacement of few key residues leads to functional modulation. The capacity of fish to colonise a large variety of habitats appears to have evolved in parallel with suitable modulation of their Hb system at the molecular/functional level.

Fish Hbs, similar to other vertebrate Hbs, are tetrameric proteins consisting of two α and two β subunits, each of which contains one O_2-binding haem group. Within different species, the transport of O_2 can be modulated by changes in the Hb structure and allosteric-ligand concentration (ATP for most teleost fish), and by changes in the expression levels of multiple Hbs, likely to display different functional features.

The vast majority of Antarctic notothenioid species have a single Hb (Hb 1), accompanied by minor Hbs (Hb C in trace amounts, and Hb 2, about 5% of the total), having one of the globins in common with Hb 1. The amino acid sequences and the similar functional features of major and

minor Hbs (which, with few exceptions, show pH and organophosphate regulation, (namely the Bohr and Root effects) in a given species, suggest that minor Hbs are vestigial (or perhaps larval) remnants, devoid of physiological importance at least in the adult state (22).

1.6 Bohr and Root effects

The O_2 affinity of all vertebrate Hbs is strongly pH dependent. This phenomenon, known as the *alkaline Bohr effect*, has considerable physiological importance, enabling the animal to exchange O_2 and CO_2 at both pulmonary and tissue levels (23).

Cells actively carrying out metabolism release CO_2 and lactic acid, thus lowering the tissue pH. As the proton concentration increases, more O_2 will be provided to ensure adequate O_2 supply. During oxygenation, the T state is converted to R. A measure of this conversion is given by the *cooperativity* in the Hb molecule, expressed by the Hill coefficient *n* (nHill). In mammalian Hb, the Hill coefficient is independent of pH and remains at or just below 3.

The simplest expression of this effect can be written as:

$$Hb - H_n + O_2 \leftrightarrow Hb - O_2 + nH^+$$

in which the protons released as a consequence of oxygen binding are known as the Bohr protons. For human Hb approximately 2 Bohr protons are released at physiological pH during oxygenation.

The physiological relevance of the Bohr effect is clear when one considers that the metabolism of highly active tissues produces acidic substances which enhance O_2 unloading from Hb. In many Hbs from teleost fishes, when the pH is lowered, the O_2 affinity decreases to such an extent that Hbs cannot be fully saturated even at very high O_2 pressure. In addition, cooperativity is totally lost and the O_2 capacity of blood undergoes reduction of 50% or more of its value at alkaline pH. This feature is known as the *Root effect* (24)

Root-effect Hbs are so strongly pH dependent that they are able to unload a large amount of bound O_2 at low pH and against a pressure gradient (25).

The physiological role of such a pH sensitivity as expressed in Root effect Hbs was for some time a point of contention. It was proposed that the Root effect was associated with the existence of a swim bladder – a structure consisting of a gas containing sac within the fish, which is

variously inflated and deflated to alter the natural buoyancy of the fish and to optimize energy use during swimming at various depths.
It is accepted, however, that Root effect Hbs are found universally in fish which have poorly vascularized retinas which need to be oxygenated to maintain function even at low ambient oxygen concentrations. In both cases of swim bladder inflation and retinal oxygenation, nature has provided the same organ system which drives the gas exchange of the Root effect Hbs. Both the swim bladder and the retina of fish are provided with a small acid producing organ known as "rete" (26). The rete produces lactic acid by normal metabolic means but then excretes this acid into a local counter current circulatory system. The received oxygenated Root effect Hb experiences within the counter current loops a local drop in pH. Under these conditions the oxygen affinity of the Root effect Hb drops precipitously and oxygen is delivered to the organ. The change in oxygen affinity is so great that oxygen can be passed into the swim bladder or retina against opposing pressures of hundreds of atmospheres. In this way, the organ system which drives the Root effect Hb can be used to inflate swim bladders at considerable depths or maintain oxygenation of poorly vascularized retinal tissues even in low ambient oxygen situations.
Antarctic fish lack the swimbladder, and it is worth mentioning that only the few species possessing Hbs without a Root effect, as well as those of the family Channichthyidae, whose blood lacks Hb, are devoid of the choroid rete (27).
Weakening of the Root effect is noticed in lineages where the choroid rete has been lost, whereas the loss of swimbladder O_2 secretion does not affect the magnitude of the Root effect (28).

1.7 Molecular and structural basis of the Root effect

The molecular mechanism of the Root effect has mystified scientists for decades and a number of stereochemical models have been proposed. Comparative sequence analysis, biochemical modifications of Root effect Hbs, biochemical and structural characterisation of mutant recombinant human Hbs with putative Root effect substitutions have all aided in the search. Yet, the complete set of key residues and their relationship to the Root effect remained obscure.

Any interpretation of the Root effect should account both for the *nH+* value (derived from *p50* as a function of the pH), and for the loss of cooperativity (usually measured by *nHill*) (29). Therefore, a structural analysis should focus on two points: 1) the number of residues whose pKa should shift upon oxygenation, with subsequent number of protons released per tetramer, *nH+*; and 2) the reason for the drop in cooperativity (*nHill* approaches or goes below 1 at pH <6.5). Unlike the first point, the second one is dependent on the allosteric model used.

The most common interpretation of the phenomenon has been to identify the Root effect as a super-stabilisation of the low oxygen binding affinity T state of the protein at low pH, relative to the high-affinity R state (30). This inhibits the T→R allosteric transition and causes a drastic reduction in the Hill coefficient. The T→R quaternary transition 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 interdimer $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces, whereas the intradimer $\alpha_1\beta_1$ and $\alpha_2\beta_2$ interfaces remain virtually unaffected (31).

In terms of stereochemical criteria the explanation of the Root effect relies heavily on the crystallographic studies of Perutz and his colleagues. Perutz and Brunori proposed (32) that the single replacement of CysF94β in mammalian Hbs by Ser, in many fish would lead to additional salt-bridge formation in the deoxy T state by additional binding to HisHC146β and its peptide amino group. In addition, in mammals LysHC144β forms a salt bridge with the imidazole group of HisHC146β in the R state. In Root-effect Hbs this Lys is replaced by a non-binding Gln. Thus these two replacements lead to a relative stabilisation of the T state and destabilisation of the R state in the Root effect. Additional support for this idea was the finding that in trout HbI, which lacks the Root effect, the terminal HisHC146β is replaced by Phe (33). Early chemical modification studies on carp Hb also suggested that the terminal His residue was responsible for a significant fraction of the observed Root effect, as enzymatic cleavage of this residues halves the pH sensitivity of the protein.

Perutz and Brunori also noted that the terrestrial frogs *Rana esculenta* and *Rana catesbeiana* and the lungfish *Lepidosiren paradoxa* show no Root effect but nevertheless process the important SerF94β. It was subsequently shown by site-directed mutagenesis that the substitution Cys → Ser is not itself sufficient to change human Hb into Root effect Hb. It appears that a Cys → Ser together with a AspF94β → Glu

substitution may be the minimal requirement to produce a Root effect (34).

An alternative proposal for the basis of the Root effect was made upon examination of the structure of carbomonoxy Hb from spot *Leiostomus xanthurus.* It was suggested that the effect arises from the repulsion of positively charged residues destabilizing the R state at low pH. In the R state of spot Hb, the central cavity is more narrow than in human HbA, due to the presence of some specific residues in the β chains (35).

Tame (36) and co-workers have obtained detailed structures for T state deoxygenated tuna Hb at pH 7.5 and at pH 5.0, together with an R state structure obtained at pH 7.5. This unique data set has shown new light on the origins of the Root effect and is set to change the generally accepted view that we should consider the Root effect only within the context of quaternary structural changes. The major findings can be expressed as follows:

1. The quaternary linked protonation of the Asp–Asp pair is confirmed in tuna Hb (present in both the pH 5.0 and pH 7.5 deoxygenated T state structures). Comparison with other Hb structures identifies the requirement for a "permissive" amino acid at position α96 in order for the Root effect to be expressed by this pair. The tuna Hb has Ser at this position and *Trematomus bernacchii* (*T. bernacchii*) an Ala, both of which allow the formation of a functional Asp–Asp pair, while human haemoglobin has a Val residue and trout HbI a Gly, both of which prevent the formation of an active pair.

2. Comparison of the T state structure obtained at pH 5.0 with that at pH 7.5 identifies a dramatic tertiary structural change as the pH is lowered. At pH 5.0, the distal histidine (Hisα60) of the α chain swings out of the haem pocket and hydrogen bonds with the haem propionate side chain. Reference to previous data, obtained from distal histidine substitution, suggests that this structural change may well be responsible for the characteristic drop in oxygen affinity of the chain between pH 7 and pH 5.

3. In the T state a novel salt bridge is formed between Hisβ69 and Aspβ72 which is dependent on a tertiary structural change induced by a ligand binding. In the case of the tuna protein, deoxygenation of the β haem produces a shift in Leuβ71 and Tyrβ85. The phenoxyl group of Tyrβ85 then moves into and disrupts the E helix. The reverse movement associated with oxygenation breaks the Aspβ72-Hisβ69 interaction and releases Root effect protons. Trout HbI has three of the amino acids

necessary for this structural change, but does not display a Root effect. In the human protein all of these amino acids are substituted.

Another hypothesis is based on overstabilisation of the T state, mainly induced by the inter-Asp hydrogen bond at the $\alpha_1\beta_2$ interface. In fact, the crystal structure of HbC of *T. newnesi* clearly reveals that a strong Asp-Asp interaction, similar to those detected in Hb1Tb and tuna Hb, occurs at the $\alpha_1\beta_2$ interface. A strong hydrogen bond is formed by the side chains of Asp95α and Asp101β. This interaction is assisted by Asp99β, whose negative charge likely increases the pK_a value of Asp95α. Interestingly, this hydrogen bond is not affected by the three substitutions that differentiate the $\alpha_1\beta_2$ interface in Hb1Tb, HbCTn and Hb1Tn. It was previously suggested that the polar side chain of Ser97α, which replaces Ala in Hb1Tb, and the bulky side chain of Ile41α, which repleaces Thr, could prevent the formation of these Asp-Asp interactions.

This inter Asp hydrogen bond, common feature of all the deoxy structures of investigated Root-effect Hbs, is the minimal structural requirement for the Root effect, accounting for a two protons release upon oxygen upload (37).

Mazzarella and his co-workers have recently demonstrated that this overstabilisation of the T state, mainly induced by the inter-Asp hydrogen bond at the $\alpha_1\beta_2$ interface, is modulated by salt bridges involving histidyl residues (38). A detailed survey of the histidine modifications, caused by the change in pH, indicates that at least three hot regions of the molecule are modified (Eβ helix, Cβ-tail, CDα corner) and can be considered to be involved at various levels in the release of the Root protons. Most importantly, at the CDα corner, the break of the salt bridge Asp48α–His55α induced a detailed mechanism that transmits the modification from the CDα corner far to the haem. More generally, the results shed light on the role played by the histidine residues in modulating the strength of the Root effect and also support the emerging idea that the structural determinants, at least for a part of the Root effect, are specific of each Hb endowed with this property.

At present, it is virtually impossible to ascribe the presence or absence of the Root effect to the substitution of few residues, or to invoke a single explanation. Indeed, the situation is much more complex, and is probably linked to the combination and interplay of a number of factors in the architecture of the globin tetramer.

1.8 The major Hbs of *Trematomus newnesi* and *Trematomus bernacchii*: ideal model for the study of the Root effect

Advantages of studying the Antarctic fish Hbs are related to their high sequence identity despite strong function differences. Therefore, the Antarctic fish Hbs constitute a good model to obtain information on the cold adaptation and the structure/function relationship. A weakening in the Bohr and Root effect is generally observed in the Antarctic notothenioid lineage in comparision to non-Antarctic notothenioid species, likely due to their adaptation to life style acquired in the evolutionary history.

Fish of the family Nototheniidae normally have a single major Hb (Hb1), often a second, minor component (Hb2, about 5% of the total) and traces of a third component (HbC, less than 1%) (22).

These Hbs are usually functionally similar and display the Bohr and the Root effects. For istance, *T. bernacchii* (39), a sedentary bottom dweller, has a single major Hb (Hb1, 95-99%), whose oxygen binding is strongly regulated by pH and organophosphates. However, the haematological features of another nototheniid, *T. newnesi*, are remarkably different.

The Hb system of *T. newnesi*, a semipelagic, active fish, is made of HbC (20-25% of the total), Hb1 (70-75%; it has the α chain in common with HbC) and Hb2 (5%). This species is the only one having two major Hbs and in fact is the only species in which HbC is not present in traces, thus not being a vestigial remnant. Only HbC displays pH and organophosphate regulation. Cooperativity of O_2 binding is present in Hb1 and Hb2 in the whole physiological pH range, but is completely lost in HbC at lower pH (a typical feature of Root-effect Hbs). This Hb system can ensure O_2 binding at the gills (*via* Hb1) and controlled delivery to tissues (*via* HbC) also when active behaviour may produce acidosis(40). Even if HbC has not been subjected to selection, its expression can nonetheless be activated as a consequence of needs arising from the fish life style, and high levels of HbC, conceivably redundant in other notothenioids (which have only traces of HbC, but count on Root- and Bohr-effect Hb1 and Hb2), compensate for the lack of regulation of Hb1 and Hb2 by protons and other effectors.

Comparison of *T. newnesi* and *T. bernacchii* Hb1 seems particularly interesting. The two Hbs differ from each other by only four residues in

the α chain and ten residues in the β chain, nevertheless *T. newnesi* Hb1 has nearly pH-independent low oxygen affinity and no Root-effect, while *T. bernacchii* Hb1 has high oxygen affinity and very large Bohr and Root effects. Therefore, these two Hbs constitute an excellent model to gain structural information on the Root effect. Most of the fourteen residues that have been found to be different in the two Hbs are external. Only three are at the $\alpha_1\beta_2$ interface, in position 41C7β, 41C6α, and 97G3α (Tyr, Ile, Ser in *T. newnesi* Hb1, and His, Thr, Ala in *T. bernacchii* Hb1, respectively), close to the Asp cluster identified by Ito et al. Among them, Ile in place of Thr in 41C6α, in the central position of the switch region, is the only one common to all the Antarctic Hbs devoid of Root effect. This substitution could have some effect on the stereochemistry of the $\alpha_1\beta_2$ interface, also considering the greater volume of the side chain of Ile with respect to Thr, which on the T state structure of *T. bernacchii* Hb1 is found to be in *van der Waals* contact with the main chain of Asp99β.

Because the R-state crystal structures of the two Hbs are very similar, new insights could arise from the study of *T. newnesi* Hb1 in deoxy form.

Studies of molecular modelling of Hb1 *T. newnesi* and its mutants were perfomed. In particular was modelled the T-state structure of *T. newnesi* Hb1 and its mutants Ser→Ala in 97 G3α, and the T-state structure of *T. bernacchii* Hb1 with the substitution Thr→Ile in 41C6α, using as template the crystallographic coordinates of the deoxy *T. bernacchii* Hb1.

The results show that, in the deoxy model of *T. newnesi* Hb1, the substitution of Ile for Thr in 41 C6α induces at the $\alpha_1\beta_2$ interface conformational modifications able to account for the lack of protonation experimentally observed in this Hb.

The major role of the missed protonation of Hb1Tn appears to be played by the displacement induced by the more bulky Ile residue (in place of Thr 41 C6α) on both the α carbon at the beginning of the β G helix and the Asp 99 G1β side chain. This event, bringing the carboxylic groups of Asp 99β and Asp 95β far apart, ultimately prevents Asp 99β from raising the pKa of the two other Asp residues to the extent necessary for the protonation event. As a consequence the Asp101β side chain is pushed further into the central cavity allowing the entrance of a water molecule between the two Asp residues. The two other substitutions at the $\alpha_1\beta_2$ interface, although producing different interactions in Hb1Tn,

do not seem to play a role on the displacement of the Asp cluster. The local structure of Asp95α and Asp101β appears influenced by the nearby Ser 97α that, in both the conformations studied, appears to be alternatively H-bonded to one or the other Asp residue. However, the substitution of Ser with Ala indicated that the loss of the Asp-Asp interaction occurs independently from the Ser-Asp interaction.

However, if these hypothesis were true, the lack of Root effect could be ascribed to amino acid replacements in the neighbouring area, either acting directly or not on the Asp cluster. In *T. newnesi* Hb1 model Ile41α is likely to play this role. In trout HbI T state structure, the only structure of fish Hb devoid of Root effect solved in T state, Arg104β (Lys in both *T. newnesi* and *T. bernacchii* Hbs) forms a salt bridge with Asp 101β, preventing its interaction with Asp 95α. However, in Antarctic fish Hbs, which are phylogenetically closely related and have a highly conserved primary structure (even higer than 90%), it is difficult to conceive a T state significantly different from that of *T. bernacchii* Hb1. It is interesting to speculate that functional diversification in these Hbs took place following structural modifications determined by the cold adaptation process. In this particular case, the mechanism that regulates the absence or presence of the Root effect should likely be the consequence of a single point mutation (41).

1.9 Objectives of the PhD project

The main objective of this PhD thesis is the study of tetrameric Hbs, both to characterise the oxidation process of these proteins and to determinate the structural and molecular basis of the Root effect.
The oxidation of tetrameric Hbs in different species can lead to the formation of different products. Under physiological conditions, the oxidation of mammalian and temperate fish Hbs leads to the formation of the exogenous aquo-met hexa-coordinated form both at the α and β subunits, whereas the oxidation of Antarctic teleost fish Hbs (AFHbs) leads to the formation of an aquo-met form at the α subunits and of an endogenous bis-histidine complex, denoted hemichrome, at the β subunits (β – haemichrome) (42).
In this context, the research activity was focused on the investigation of the β globin of Hb1Tn, to verify the formation of the hemichrome and to establish if the tetramer β_4 is formed in solution, as in HbA, or it rather stays as monomers.
The major Hbs of *T. newnesi* and *T. bernacchii* represent an ideal model to investigate the structural and molecular basis of the Root effect, because of their high sequence identity and their very different functional behaviour.
Two different approaches were undertaken. The first approach to study the structure/function relationship in *T. newnesi*/*T. bernacchii* Hb1 was the site-directed mutagenesis of their α- and β-globin cDNAs, and the subsequent expression and characterisation of the mutant proteins.
Another strategy, undertaken to determine the residues involved in the Root effect, was to study a natural mutant of human HbA, Hb *Potomac* (β101Glu→Asp) (43). The β101 residue is located at the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) interface, the region involved in the switching of the molecule from the R to the T state. In fact, the minimal structural requirement for the Root effect, accounting for a two protons release upon oxygen upload, is attributed to the strong hydrogen bond that is formed by the side chains of Asp95α and Asp101β, assisted by Asp99β. This Asp triad is a common feature of all the deoxy structure of investigated Root-effect Hbs. In literature is reported that Hb *Potomac* has nearly pH-independent low oxygen affinity and no Root-effect, despite the presence of the Asp triad. These features are also characteristic of Hb1 of *T. newnesi*.

The X-ray crystal studies of the deoxy form of Hb *Potomac* is worth to provide information about the hydrogen bond between the Asp residues. The first step was to obtain the mutant HbA by site-directed mutagenesis of the α- and β-globin cDNAs, and the subsequent steps were the expression and the purification of the recombinant protein.
Another objective was the crystallisation of the deoxy form of the Hb1 *wild type* of *T. newnesi* . The comparison of the structures of *T. newnesi* and *T. bernacchii* could provide important informations on the structural basis of the Root effect.

1.10 References

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Chapter 2

Expression of β chain of *Trematomus newnesi* Hb1

2.1 Introduction

Concerning Antarctic fish Hbs (AFHbs), the study of β globin of Hb1Tn is interesting to verify the formation of β_4 tetramer, as for β_4 variant of human HbA, or rather there exists an equilibrium between the monomer and the dimer; moreover the investigation of the β globin is appealing about the possibility that it forms haemichrome.

The investigation of the recombinant β chain should increase the understanding of the mechanism of assembly of Antarctic teleost fish Hbs, which has not yet been clarified.

In human HbA, isolated β chains also assemble to form β_4 homotetramers. Previous studies showed that the α chains are in monomer-dimer equilibrium and dissociation into monomers is favored, whereas the β chains are in monomer-tetramer equilibrium and association into tetramers is favored. It is generally assumed that dissociation of these oligomeric subunits into monomers must occur before they can combine to form the αβ dimers. Two αβ dimers then associate to form tetrameric Hb. The Cys β112 plays a critical role for facilitating formation of stable αβ dimers, which then form functional Hb tetramers. It has also been demonstrated that the Asp β112 inhibits formation of stable $\alpha_1\beta_1$ and $\beta_1\beta_2$ interactions in $\alpha_2\beta_2$ and β_4 tetramers, respectively (1).

In the AFHbs, Cys β112 is substituted by an Asn. The study of the single β chain could also contribute to a better understanding of the role of this amino acid in position β112, in the tetramer-dimer stability and in the assembly of Hb.

Under physiological conditions, the oxidation of mammalian and temperate fish Hbs leads to the formation of the exogenous aquo-met hexa-coordinated form both at the α and β subunits, whereas the oxidation of AFHbs leads to the formation of an aquo-met form at the α subunits and an endogenous bis-histidine complex, denoted haemichrome, at the β subunits (β-haemichrome) (figure 1) (2).

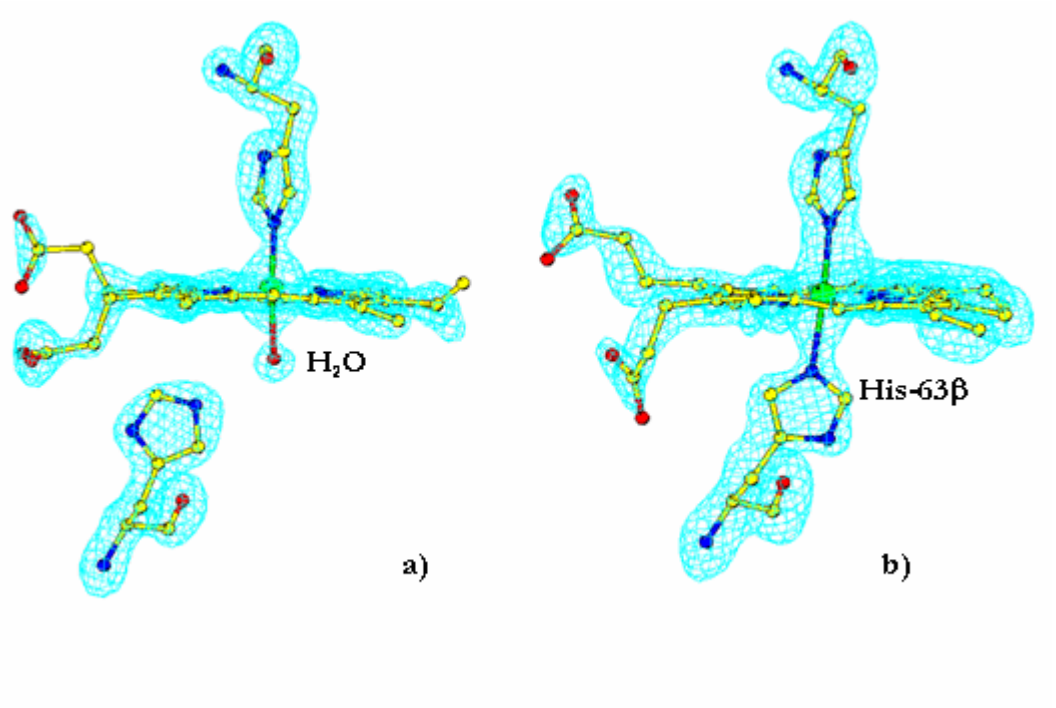

Fig 1 The omit difference maps for the haeme region for alpha (a) and beta (b) subunit.

The physiological role of the haemichrome is disputed. The bis-imidazole complex could be involved in the ligand binding, in the *in vivo* reduction of met-Hb, in the Heinz body formation and in NO scavenging (3). Recently, it has been suggested that haemichrome could be involved in protection of Hb from peroxidative attack (4).

The high resolution crystal structures of the oxidized form of Hb1Tb (5) and of the Hb1Tn (6, 7) provided some clues on the structure and formation of haemichrome in AFHbs. The α-aquo-met β-haemichrome forms of these proteins have a similar quaternary structure, intermediate between the canonical Hb R- and T- states.

This structure demonstrates that the Fe coordination by the distal His, usually associated with denaturing states, may be tolerated in a native-like haemoglobin structure.

The analysis of this structure provides a detailed pathway of haem-haem communication and it indicates that the plasticity of the haem pocket plays a role in the R→T transition of tetrameric Hbs.

The haemichrome formation is associated to a scissor-like movement of the E-F β helices, that makes the distance between the distal and proximal histidines small enough to form the endogenous haemichrome complex. The scissor-like movement of E-F β helices is also associated with a sliding of the β haem plane that moves out of the haem pocket, thus favoring the endogenous hexa-coordination.

Two vectors were constructed to express β chain of Hb1Tn: the vector pGEX, to express the Glutathione S-transferase (GST) fusion protein, and the vector pET22, to be used in *E. coli* and in *Arctic cells*. The latter system allows the co-expression of the protein with the cold-adapted chaperonins Cpn10 and Cpn60, from the psychrophilic bacterium *Oleispira antarctica*.

2.2 Expression in pGEX

2.2.1 Materials and Methods

Materials

The plasmid pGEM7βHSkozak, containing Hb1Tn β-globin cDNA, was previously prepared in the laboratory. The plasmid pGEX and the Glutatione Sepharose 4B were purchased from Amersham. The ArcticExpress™ Competent Cells were purchased from Stratagene. The C41 cells were kindly given by Prof A. Duilio.

Methods

***Construction of pGEXβ- globin of Trematomus newnesi* Hb1**

The Hb1Tn β globin cDNA was amplified by PCR, from the plasmid pGEM7βHskozak, with the primers SP6 (5'-ATTTAGGTGACA CTATAG-3') and T7 (5'-TAATACGACTCACTCACTATAGGG-3'), to be inserted into pDRIVE plasmid. The new construct was then digested with *NcoI* and *XhoI*; the terminals of the obtained fragment were filled in with *Klenow* and then it was ligated into pGEX4T vector to give the construct pGEXbeta (figure 2), that has the β-globin cDNA in frame with the cleavage site of thrombin.

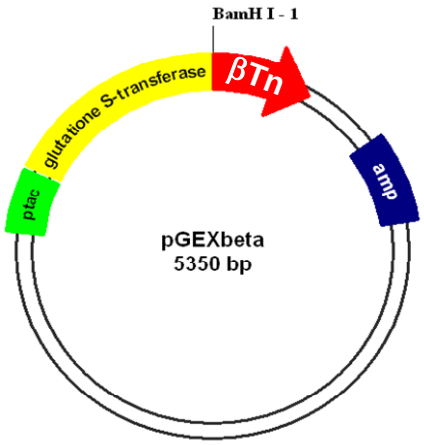

Fig 2 Vector pGexbeta

Expression of β globin of Hb1Tn

E. coli BL21(DE3) cells were transformed with the plasmid pGEXbeta and were grown at 37 °C in 2xYT medium (16 g/l bactotryptone, 10 g/l yeast extract and 5 g/l NaCl) supplemented with 100 μg/ml ampicillin and 20 mg/ml glucose. The expression of Hb1Tn β globin was induced by adding IPTG till 0.6 mM, when the optical density at 600 nm was approximately 0.7/0.8. The growth was continued for at least 4 hours.

The cells were collected at time zero (t_0) of induction and at each two hours intervals. The cells were suspended at 3 ml/g of cells in PBS lysis buffer (Phosphate Buffered Saline 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), sonicated in an ice-bath and centrifuged at 4 °C for 45 min at.14.000 rpm. Hb1Tn β globin was found predominantly in the insoluble fractions.

Co-expression of Hb1Tn β globin with E. coli GroEL/GroES

The co-expression with GroEL/GroES was carried out to obtain recombinant Hb1Tn β globin in soluble form.

E. coli BL21(DE3) cells were co-transformed with the plasmids pGEXbeta and pGro and were grown, as previously described, in 2xYT medium supplemented with 100 μg/ml ampicillin and 100 μg/ml kanamycin.

Purification of Hb1Tn β globin

The cells were suspended in lysis buffer PBS, sonicated in ice-bath and centrifuged at 4 °C for 45 min at 14.000 rpm.
Fusion protein was easily purified from bacterial lysate by affinity chromatography using Glutathione Sepharose 4B. Cleavage of the *β globin* from GST was achieved using a site-specific protease, thrombin, whose recognition sequence is located immediately upstream from the multiple cloning site of the pGEX plasmid.
The lysate was incubated in batch with the Glutathione Sepharose 4B resin and gently agitated at room temperature for 30 min. During this stage the fusion protein was adsorbed on the resin, after which it was washed with PBS to eliminate the unbound proteins.
Subsequently, the resin was incubated with the elution buffer (PBS with 1 unit/μl thrombin) and gently agitated overnight at room temperature.
Then the resin was centrifuged and the surnatant was collected. This contained the Hb1Tn β globin, while the GST remains bounded to the matrix.

2.2.2 Results

The cell lysate was separated into soluble and insoluble fractions. The expression level and the solubility of recombinant protein were determined by SDS-PAGE. A sample of 5 μg of Hb1Tn *wild type* was loaded as standard.
Hb1Tn β globin was found predominantly in the insoluble fractions.
Through the co-expression with GroEL/GroES, the recombinant Hb1Tn β globin was found predominantly in the soluble fractions (figure 3).

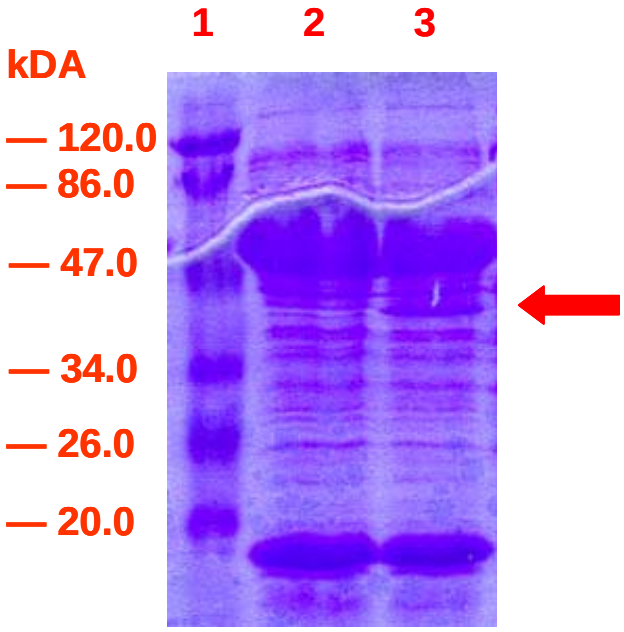

Fig. 3 SDS–PAGE (15%) analysis. Lane 1: molecular weight markers, lane 2: protein background in the host *E.coli* strains, BL21 (DE3) transformed with pGRO, lane 3: protein of the sonicates from *E.coli* strains BL21 (DE3) co-transformed with pGRO and pGEXβ; the intense band at ~45.000 Daltons is the fusion protein (indicates by a red arrow). Each lanes were loaded with 10-15 μg of proteins.

Purification of Hb1Tn β globin

In figure 4 are reported the fractions containing the purified Hb1Tn β globin. The globin obtained by this procedure is not pure for the presence of the chaperonins into the purified fractions.

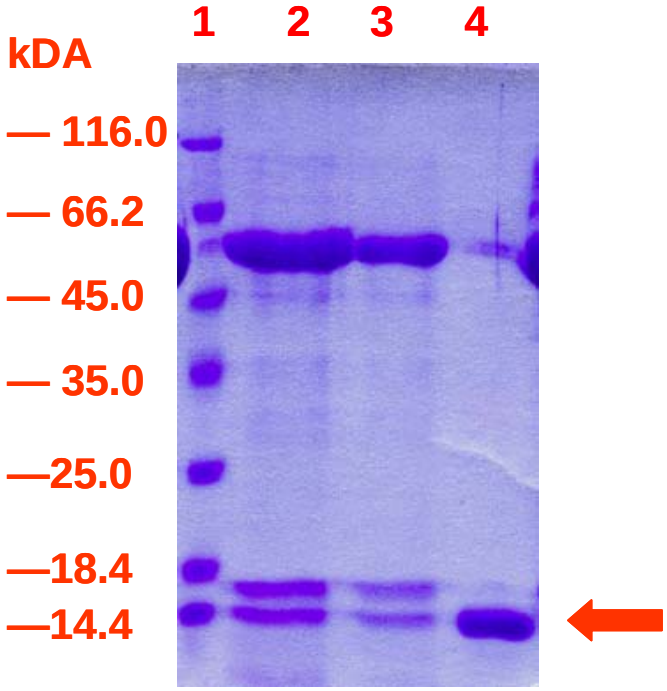

Fig. 4 SDS–PAGE (15%) analysis. Lane 1: molecular weight markers, lane 2 and lane 3: fractions containing the purified Hb1Tn β globin, lane 4: Hb1Tn as standard. A red arrows indicates the position of Hb1Tn β globin. Each lanes were loaded with 10-15 μg of proteins.

The yield of soluble purified Hb1Tn β globin was approximately 0.8 mg/litre culture. The sequencing analysis confirms the presence of additional three amino acids at the amino N-terminal of β globin: Gly and Ser, due to the proteolitic site of thrombin, and Met, associated with the expression in *E. coli*.
The Hb1Tn β globin was reconstituted with haemin in 1.2 molar excess (8), and reduced by sodium dithionite under atmosphere of CO gas. Subsequently, the VIS spectrum was recorded: the Soret band is not sharp and the two maxima at 540 nm and 569 nm are not clearly defined. This was probably due to incorrect folding of the recombinant Hb1Tn β globin (figure 5).

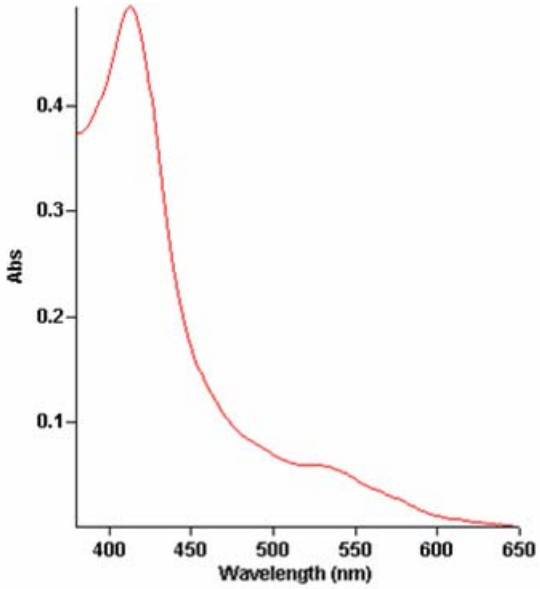

Fig 5 Electronic absorption spectrum of Hb1Tnβ globin in PBS buffer at pH7.3.

2.3 Expression with pET22

2.3.1 Materials and Methods

Construction of pET22β-

Hb1Tn β-globin cDNA was amplified by PCR from the plasmid pGEM7βHSkozak as template and the primers *NdeI*β (5'-GGAATTCCATA TGGTAGAGTGGACTGATAAG-3') and T7 (5'-TAATACGACTCACTCCTATA GGG-3'). The cDNA was digested with *XbaI*, filled in with *Klenow* and then digested with *NdeI*.

The final fragment was ligated into the expression vector pET22, to generate pET22βHb1Tn (figure 6).

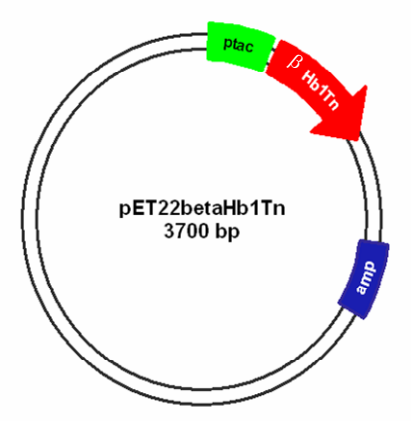

Fig. 6 Vector pET22betaHb1Tn

Expression of Hb1Tn β globin

BL21(DE3) and C41 competent *E. coli* cells were transformed with the pET22β; subsequently cells were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin. The expression of Hb1Tn β globin was induced by adding IPTG till 0.6 mM, when the optical density at 600 nm was approximately 0.7/0.8. In a different trial of expression, the culture was supplemented with haemin (35 μg/ml dissolved in a solution of 15% NH_4OH) and glucose (20 mg/ml), and the growth was continued for at least 4 hours.
The cells were suspended in 3 ml/g of cells of lysis buffer, (20 mM Tris/HCl pH7.5), sonicated in an ice-bath and centrifuged at 4 °C for 45 min at 14.000 rpm.

2.3.2 Results

The cell lysate was separated into soluble and insoluble fractions, and then the expression level and the solubility of recombinant protein were determined by SDS-PAGE. For both the BL21- and the C41-cells, the Hb1Tn β globin was found predominantly in the insoluble fractions (figure 7).

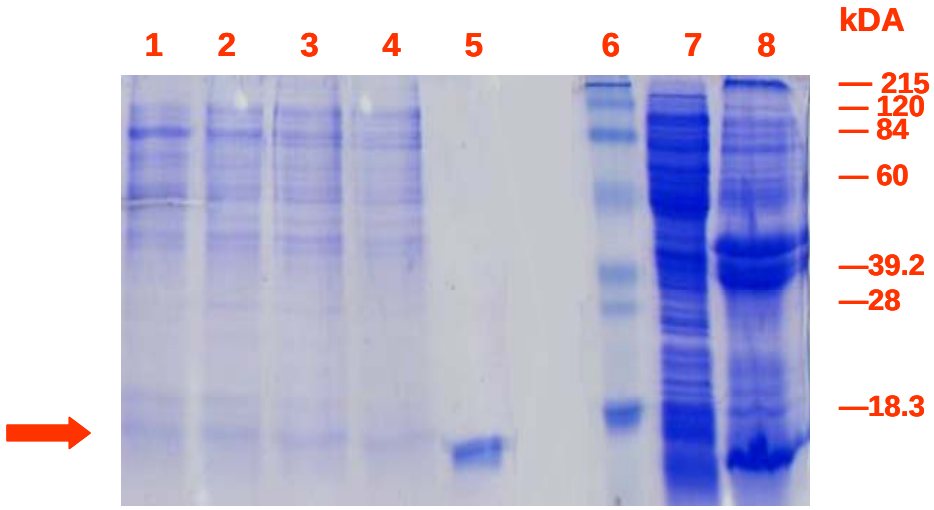

Fig. 7 SDS–PAGE (15%) analysis. Lane 1 and lane 2: patterns of protein expression of the sonicates from E.coli strains BL21 (DE3) at time 2 and time 4, respectively, lane 3 and lane 4: patterns of protein expression of the sonicates from *E.coli* strains C41 at time 2 and time4, respectively, lane 5: Hb1Tn as standard, lane 6: molecular weight markers, lane 7 and lane 8: the soluble and insoluble fractions, respectively, of the sonicated bacteria. A red arrows indicates the position of Hb1Tn. Each lanes were loaded with 10-15 μg of proteins.

2.4 Expression in the Arctic cells

2.4.1 Introduction

ArcticExpress Competent Cells are engineered to address the common bacterial gene expression hurdle of protein insolubility. These cells are derived from Stratagene's high-performance BL21-Gold competent cells, enabling efficient high-level expression of heterologous proteins in *E. coli*.

The mesophilic host *E. coli* is suitable for expression of a wide range of heterologous proteins. At standard cultivation temperatures, however, high level expression of a heterologous protein can impair the ability of cell to properly process the recombinant protein. Low-temperature cultivation represents one strategy for increasing the recovery of soluble protein. An obstacle to this approach, however, is that *E. coli* chaperonins, which facilitate correct protein folding by binding to and stabilising unfolded or partially folded proteins, lose activity at reduced temperatures. Specifically, it has been shown that the activity of the *E. coli* chaperonin complex GroEL/ES retains only about 30% of refolding activity at 12 °C, compared to its activity at the temperature optimum of 30 °C. To overcome this obstacle, ArcticExpress competent cells have been engineered for improved protein processing at low temperatures.

These cells co-express the cold-adapted chaperonins Cpn10 and Cpn60 from the psychrophilic bacterium *Oleispira Antarctica* (9). The Cpn10 and Cpn60 chaperonins from *O. Antarctica* have 74% and 54% amino acid identity compared to the *E. coli* GroEL and GroES chaperonins, respectively, and show high protein refolding activities at temperatures of 4–12 °C. When expressed in ArcticExpress cells, these chaperonins confer improved protein processing at lower temperatures, potentially increasing the yield of active, soluble recombinant protein. This Arctic Express cells were employed to express β globin of Hb1Tn in soluble form in the laboratory of Dr. Raffaele Cannio (Institute of Protein Biochemistry, C.N.R.).

2.4.2 Materials and Methods

ArcticExpress cells were transformed with the plasmid pET22βHb1Tn, and then were grown in LB medium supplemented with 100 μg/ml ampicillin and 20 μg/ml gentamycin. Selection of the *cpn10/cpn60* chaperonin expression plasmid is achieved by gentamycin.

The cells were first grown at 37 °C overnight and then at 30 °C in LB without any antibiotics. The cells were put for 30 min at 10 °C when the optical density at 600 nm was about 0.7/0.8; then the expression of Hb1Tn β globin was induced by adding IPTG till 0.5 mM; the culture was also supplemented with haemin (20 μg/ml dissolved in a solution of 15% NH_4OH) and glucose (20 mg/ml) and the growth was continued for 40 hours.

The cells were suspended at 3 ml/g of cells in lysis buffer (20 mM Tris/HCl pH 7.5), sonicated in an ice-bath and centrifuged at 4 °C for 45 min at 14.000 rpm. The cell lysate was separated into soluble and insoluble fractions. The lysate developed a bright red color when it was saturated with CO gas.

2.4.3 Results

The expression level and the solubility of recombinant protein were determined by SDS-PAGE. Hb1Tn β globin was found predominantly in the soluble fractions, however the level of expression was very low (figure 8).

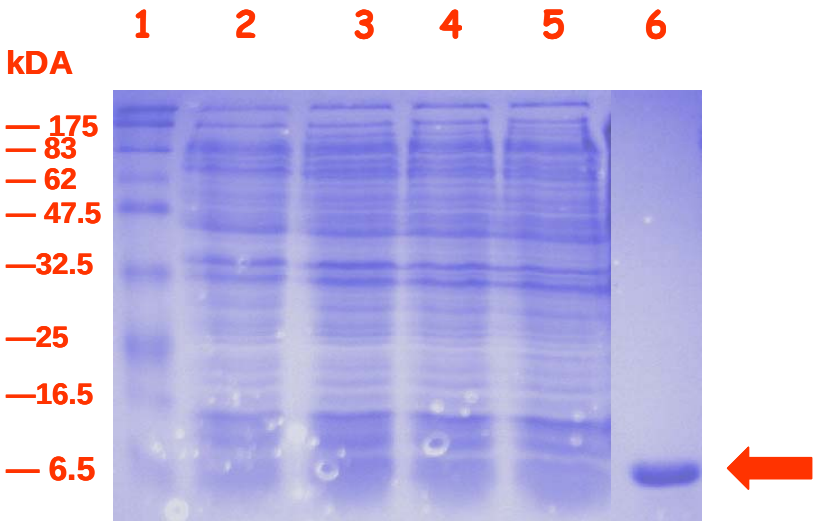

Fig 8 SDS–PAGE (15%) analysis. Lane 1: molecular weight markers; lane 2,3,4,5: patterns of protein expression of the sonicates cells at different times (2, 4, 6...hrs, respectively), lane 6: Hb1Tn as standard. A red arrows indicates the position of the Hb1Tn β globin. Each lanes were loaded with 10-15 μg of proteins.

The absorption spectrum (figure 9) was registered in the visible region between 380 nm and 650 nm on a spectrophotometer VARIAN Cary 300.

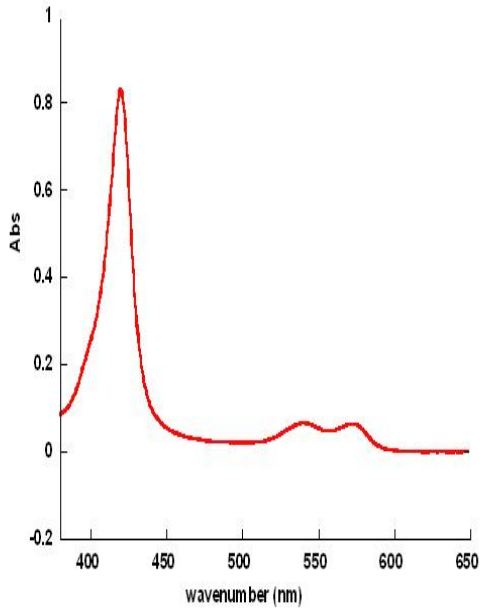

Fig 9 Electronic absorption spectrum of Hb1Tnβ globin in 20 mM Tris pH 7.5.

The absorption maxima of the β globin were nearly the same as the corresponding values for HbA, indicating the correct folding of the recombinant protein.

Several trials of purification for the β globin were performed. However, these did not lead to a significant purification of the protein, due to the low level of expression.

2.5 Discussion

The expressed β chain was primarily insoluble, however by co-expression with GroEL/GroES it was possible to obtain recombinant Hb1Tn β globin in soluble form. Nevertheless, the trials of refolding *in vitro* in presence of haem were not successful. The UV-VIS spectrum suggested that haem was not incorporated correctly into β globin.

On the contrary, the β globin synthesized in the pET22 system, in both BL21 and C41 cells, was found to precipitate into the inclusion bodies, presumably as a partially denatured or improperly folded intermediate. This may be due to hydrophobic interactions between the partially folded polypeptide chains.

Finally, the Arctic cells produced only small amounts of recombinant protein, which could not be purified in reasonable yields.

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Chapter 3

Expression of Haemoglobin 1 of *Trematomus newnesi*

3.1 Introduction

Some clues to the understanding of the stereochemistry of the Root effect may come from studies of site-direct mutagenesis. The Hbs of the *T. newnesi* and of *T. bernacchii* are the best candidates for this investigation because of their high identity of sequence: the α chains differ by 4 residues and the β chains by 10 (1). These changes include neither the residues considered responsible for the Root effect, nor those involved in the binding of organic phosphates. In the following tables are reported the residue substitutions between *T. newnesi* Hb1 and *T. bernacchii* Hb1:

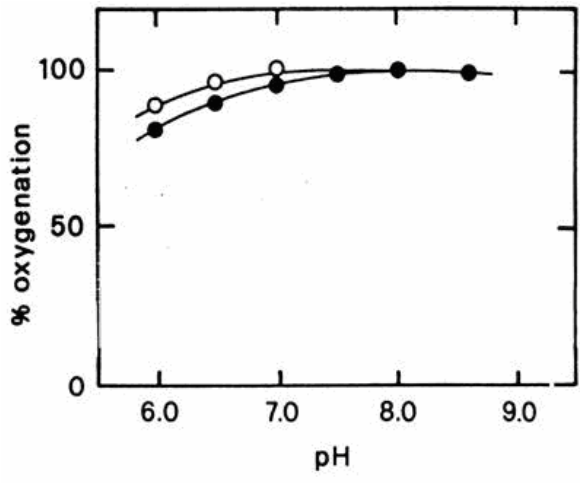
	Alpha chain			
	external		$\alpha_1\beta_2$	
	21B2	55E3	41C6	97G3
Hb1Tn	Ser	Asn	Ile	Ser
Hb1Tb	Ala	His	Thr	Ala

	Beta chain				
	External				
	53D4	56D7	83EF7	84EF8	86F3
Hb1Tn	Gly	Ser	Asp	Ala	Thr
Hb1Tb	Ala	Gly	Ala	Thr	Ala

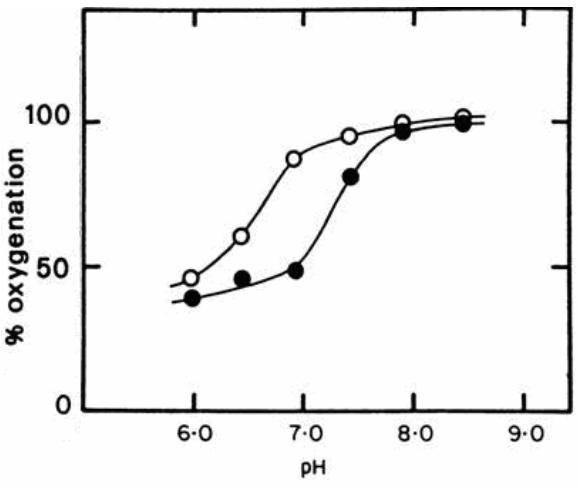
	Beta chain				
	$\alpha_1\beta_1$	$\alpha_1\beta_1$, $\alpha_2\beta_1$	$\alpha_2\beta_1$	**internal**	**centr cav**
	55D6	33B15	41C7	75E19	136H14
Hb1Tn	Met	Val	Tyr	Met	Ala
Hb1Tb	Ile	Ile	His	Val	Val

Table I. Amino acid residues substituted between *T. newnesi* Hb1 and *T. bernacchii* Hb1

Hb1Tb displays Bohr and Root effects, while Hb1Tn shows only a modest Bohr effect and no Root effect. It is worth noticing that *T. newnesi* swims actively and feeds near the surface, whereas *T. bernacchii* is a sedentary bottom dweller. Moreover, the blood of *T. newnesi* contains, in addition to Hb1 and Hb2, a large amount of HbC, which displays Bohr and Root effects. In figure 1 are reported the oxygen equilibrium curves of Hb1Tn (A) and Hb1Tb (B) (2).



A



B

Fig 1 Experimental conditions for determination of the Root effect:-100 mM Tris or Bistris buffer in the absence (*open circles*) and presence (*closed circles*) of 3 mM inositol hexakisphosphate, at 0 °C for all the oxygen saturation curves.

The first approach to study this system has been the site-directed mutagenesis of the α- and β-globin cDNAs, and the subsequent expression and characterisation of the mutant proteins.
In order to carry out these experiments, it is necessary to develop an efficient expression system to produce authentic Hb in high yields.
Both *Sacchoramyces cerevisiae* (*S. cerevisiae*) (3, 4, 5) and *Escherichia coli* (*E. coli*) (6, 7, 8) have been employed for the production of recombinant human HbA. The yeast system has several advantages over the other systems. Firstly, it expresses the proteins with the correct post translational modifications. Another advantage is that recombinant haemoglobin produced in *S. cerevisiae* is able to incorporate haem produced by the yeast haem synthetic patway. The *E. coli* system offers high expression efficiency and the low cost procedures.
Both expression systems were employed for Hb1Tn.
Finally, it was tried a psychrophilic expression system, that uses *Pseudoalteromonas haloplanktis* TAC125 cells.

3.2 Expression in Sacchoramyces cerevisiae

3.2.1 Materials and Methods

Materials

The construct pYESαβ, containing the double expression cassette for the α- and β- globins, was previously prepared in the laboratory. Each expression cassette consists of the GAL 1(9), the hybrid promoter region, the globin cDNA and the transcription termination sequences, CYC 1.
Host strain *S. cerevisiae* INVSc1 was provided by Invitrogen.
Y-PER- S *Yeast Protein Extraction Reagent* was purchased from Pierce.

Methods

Strain was transformed according to procedure described by Invitrogen.
Transformants were selected on minimal medium lacking uracil. INVSc1 cells transformed with pYESαβ were grown in synthetic complete medium SC-U [0.67% (*w/v*) yeast nitrogen base; 0.01% (*w/v*) for adenine, arginine, cysteine, leucine, lysine, threonine and tryptophan;

0.005% (*w/v*) for aspartic acid, histidine, isoleucine, methionine, phenylanalina, prolina, serine, tyrosine, and valine], with 2% destrose as a carbon source, until the exponential phase. Then, the expression of Hb1Tn was induced by adding 2% galactose. This culture was grown at 30° C until the stationary phase. Aliquots of 5 ml of cell culture were harvested at various times after induction. The proteins were extracted according to Y-PER- S protocol from Pierce.

3.2.2 Results

Protein extracts were analysed by SDS-PAGE. Globins were not detected on the gel by staining with Comassie Brilliant Blue. Proteins were transferred to nitrocellulose membranes and globin chains were detected by immunoblot analysis using antibodies prepared against Hb1Tn. Faint bands can be observed on the membrane at the same position as chains from the standard (figure 2).

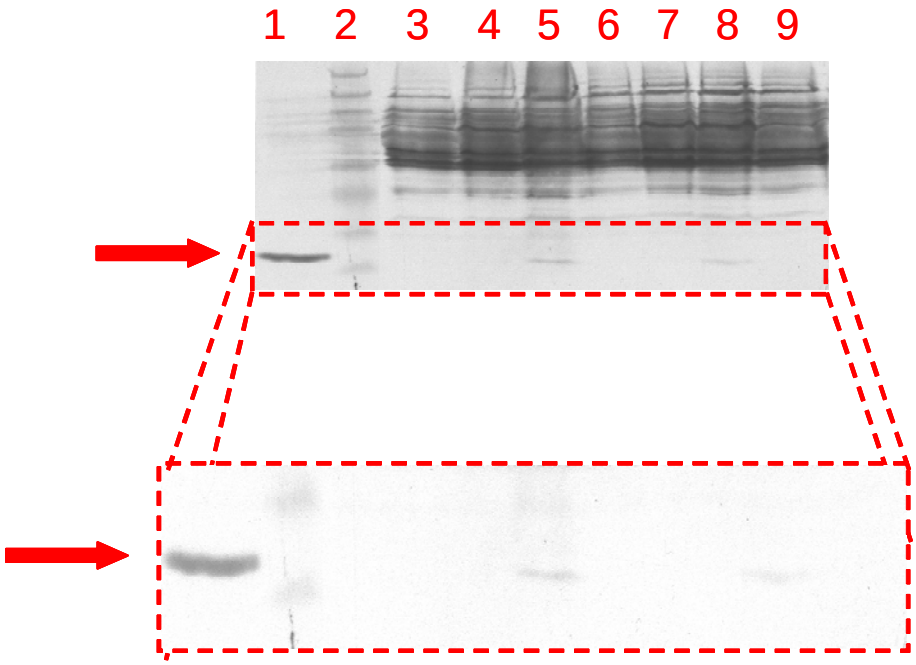


Fig 2 Western blot analysis. Lane 1: Hb1Tn as standard, lane 2: molecular weight markers, lanes 3, 4, 5, 6, 7, 8, 9: patterns of protein expression of the yeast cells strains at different times of inductions (t_0, 2, 4, 6, hrs and over night respectively). Each lanes were loaded with 10-15 µg of proteins.

3.3 Expression in Escherichia coli.

3.3.1 Materials and Methods

Materials

The plasmid pHE7, containing human α- and β-globin cDNAs and the gene for methionine aminopeptidase (MAP) from *E. coli*, was provided by Prof. Chien Ho (10, 11).
Chemicals and restriction enzymes were purchased from Bio-Rad, New England BioLabs, Promega, and Sigma.
The plasmid pBK-CMV, containing *T. newnesi* α-globin cDNA, and the plasmid pGEMβHSkozak, containing *T. newnesi* β-globin cDNA, were previously prepared in the laboratory.

Methods

Construction of Hb1Tn Expression cassette

The α- and β-globin cDNAs were cloned separately, complete of the *Shine-Dalgarno* (SD) ribosome binding site sequence (12), into pGEMT*Easy* plasmid.

α globin expression cassette

The α-globin cDNA was amplified by PCR from the plasmid pBK-CMV and with the primers *Nde*α (5'-GGAATTCCATATGAGTCTCTCCG ACAAAGACAAGG–3') and α*SphI* (5'-ACATGCATGCTTAGCGGTATCTCTC AGC GAG-3'), which contain a *NdeI* site and *SphI* site, respectively. After restriction enzyme digestions, the α-globin cDNA was inserted into pGEMT*Easy* to form plasmid pGEMα.
The SD was obtained by PCR from pHE7 with the primers *Nsi*SDα (5'-CCAATGCATGGTACCCGGGCTACATG-3') and SD*Nde* (5'-GGAATTCCAT ATGTTATTCCTCCTTATTTAAGCG-3'), which contain a *NsiI* site and a *NdeI* site, respectively. After restriction enzyme digestion, the SD was inserted into pGEMα to give plasmid pGEMSDα (figure 3).

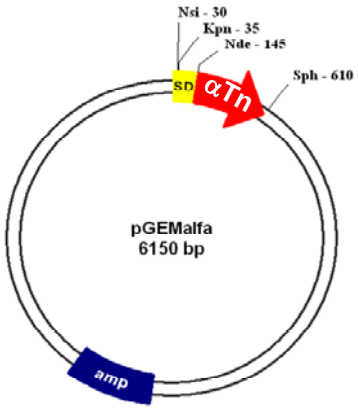


Fig 3 Vector pGEMSDα

β- globin expression cassette

The procedure for the construction of pGEMSDβ was similar to that of pGEMSDα.

The SD region was obtained by PCR from pHE7, with the primers *Sph*ISDβ (5'-ACATGCATGCACTAGAGGGTATTAATAATGTATCGC-3') and SD*NdeI* (5'-GGAATTCCATATGTTATTCCTCCTTATTTAAGCG-3'), which contain a *SphI* site and a *NdeI* site, respectively. After restriction enzyme digestion, the SD was inserted into pGEMT*Easy* to give the plasmid pGEMSD.

The β-globin cDNA was amplified by PCR from pGEMβHSkozak with the primers *Nde*Iβ (5'-GGAATTCCATATGGTAGAGTGGACTGATAAGG-3') and β*NsiI* (CCAATGCATTAGTGGTACTGCTTTCCC), which contain a *NdeI* site and *NsiI site*, respectively. After restriction enzyme digestion, the β-globin cDNA was inserted into pGEMSD to form the plasmid pGEMSDβ.

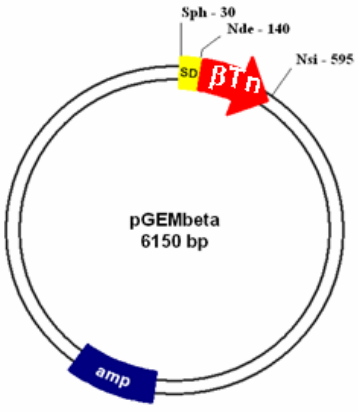


Fig 4 Vector pGEM SDβ

Amplifications were performed using a reaction mixture containing 2.5 u of *Taq* polymerase (EuroClone), 5 pmol each of the above primers, and 0.20 mM dNTPs, buffered with 160 mM $(NH_4)_2SO_4$, 670 mM Tris·HCl (pH 8.8), 0.1% Tween-20, containing 1.5 mM $MgCl_2$. The PCR program consisted of 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and ending with a single cycle of 10 min at 72°C.

pHE7 T.newnesi α /β - globins expression cassette

The α- and β-globin cDNAs were cloned sequentially into the plasmid pHE7. The plasmid pGEMSDα was digested with *KpnI* and *SphI* to give the fragment SDα-globin; plasmid pGEMSDβ was digested with *SphI* and *NsiI* to give the fragment SDβ-globin. These fragments were ligated in a triple ligation with the plasmid pHE7, previously digested with *KpnI* and *NsiI,* to form pHE7Hb*T.newnesi* (figure 5).

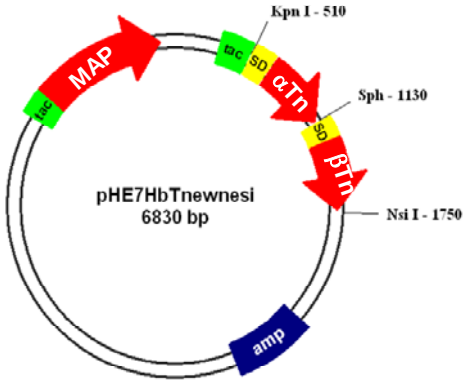


Fig 5 Vector pHE7HbTnewnesi

Standard molecular biological techniques were used in the isolation, restriction, and sequence analysis of plasmid DNA (13).
At each stage of the process, the construction progress was monitored by automated sequencing, to confirm the cloned cDNA fragments.

Expression of Hb1Tn with pHE7HbT.newnesi

E. coli BL21(DE3) cells were transformed with the plasmid pHE7Hb*T.newnesi*; subsequently cells were grown in TB medium (1.2% bactotryptone, 2.4% bactoyeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) supplemented with 100 μg/ml ampicillin. The cells were grown at 37°C; the expression of Hb1Tn was induced by adding

isopropyl β-thiogalactopyranoside (IPTG) till 0.1 mM, when the optical density at 600 nm was about 0.7/0.8. After 30 min the culture was supplemented with haemin (35 μg/ml dissolved in a solution of 15% NH_4OH) and glucose (20 mg/ml); the growth was stopped after 4 hours. The cells were collected at time zero (t_0) of induction and at each two hours intervals. The cells were suspended in 3 ml/g of cells lysis buffer (20 mM Tris/HCl pH 7.5), sonicated in ice-bath and centrifuged at 4 °C for 45 min at 14.000 rpm. The cell lysates were separated into soluble and insoluble fractions. Protein concentration was determined by the standard Bradford procedure (14).

3.3.2 Results

The expression level and the solubility of recombinant protein were determined by SDS-PAGE. A sample of 5 µg of Hb1Tn *wild type* was also loaded as standard.

Hb1Tn was found predominantly in the insoluble fractions.

A number of approaches have been used to try to increase the solubility of the recombinant Hb1Tn (15, 16):

- Modifying the concentrations of IPTG; (figure 6 a and B).

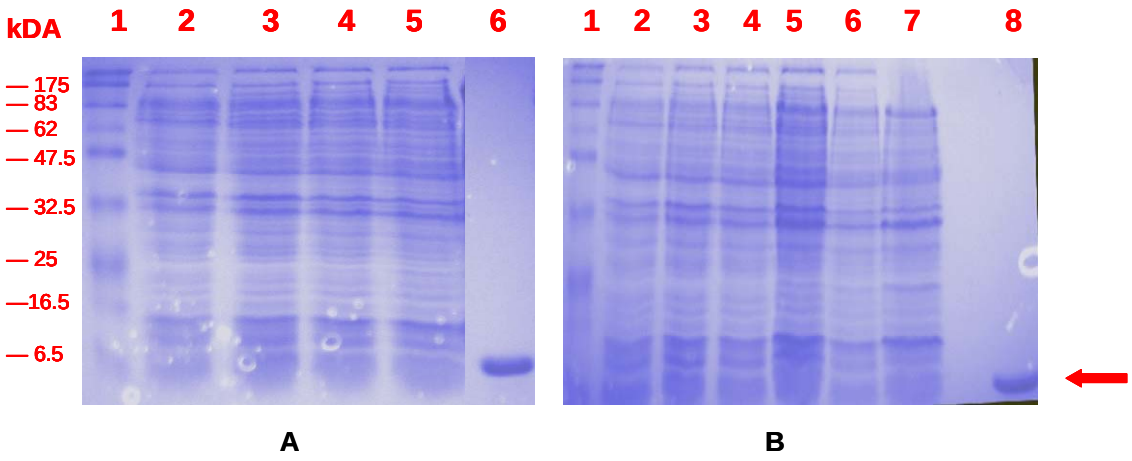


Fig 6 SDS–PAGE (15%) analysis after expression with 0.4 mM IPTG (A) or 0.1mM IPTG (B).Lane 1: molecular weight markers, lanes 2-5 (A) and 2-7 (B): patterns of protein expression of the *E.coli* strains BL21 at different times of inductions (.....hrs, respectively), lanes 6 (A) and 8 (B): Hb1Tn as standard. A red arrow indicates the position of Hb1Tn. Each lanes were loaded with 10-15 µg of proteins.

- By induction at lower temperature (25°-30°C); (figure 7A and B).

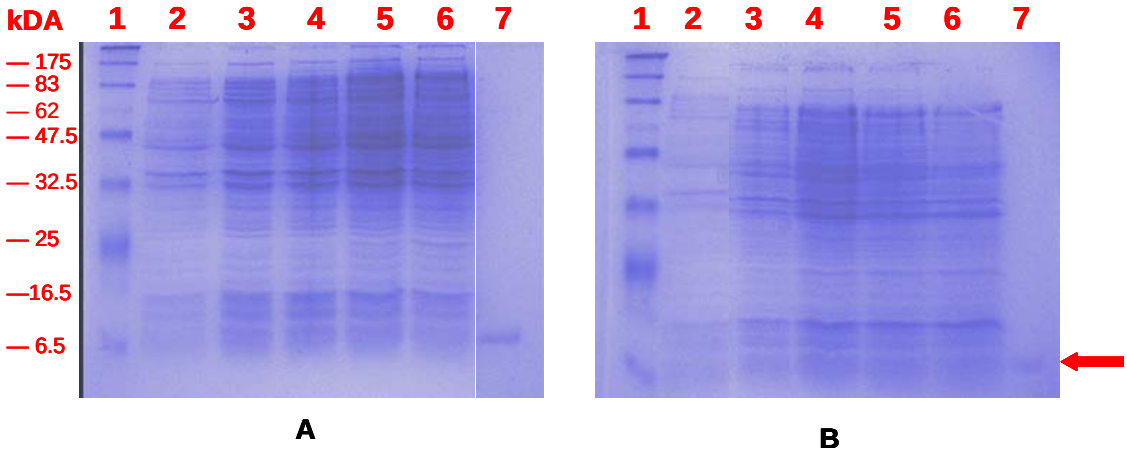


Fig 7 SDS–PAGE (15%) analysis after expression at 25°C (A) and at 32°C (B). Lane 1: molecular weight markers, lanes 2-6: patterns of protein expression of the *E.coli* strains BL21 at different times of inductions, lane 7: Hb1Tn as standard. A red arrow indicates the position of Hb1Tn. Each lanes were loaded with 10-15 µg of proteins.

- By induction for shorter periods;
- By using a specific culture medium.

Unfortunately, all these trials were unsuccessful because an increase of the expression level or of the solubility of the protein was not observed. The best growth conditions were identified to be: 37°C, 0.1 mM IPTG, in TB medium, for 4 hours of induction, as described in the methods section (figure 9 A and B):

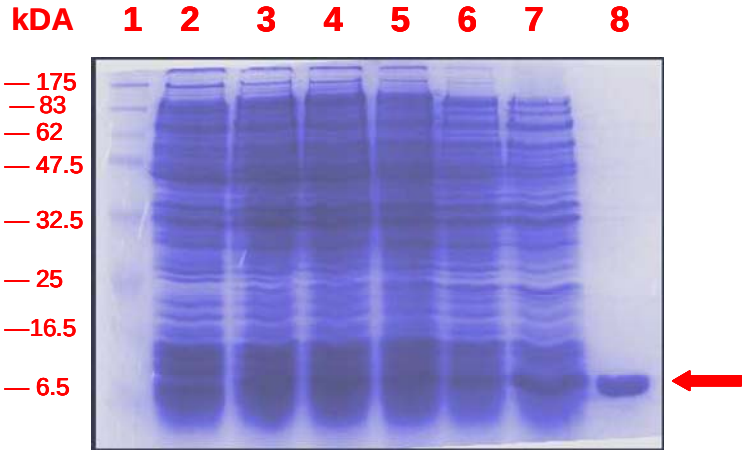


Fig 9A SDS–PAGE (15%) analysis. Lane 1: molecular weight markers, lanes 2-7: patterns of protein expression of the *E.coli* strains BL21 at different times of inductions (t_0, 2, 4, 6, 8 hrs and over night respectively)lane 8: Hb1Tn as standard. A red arrow indicates the position of Hb1Tn. Each lanes were loaded with 10-15 µg of proteins

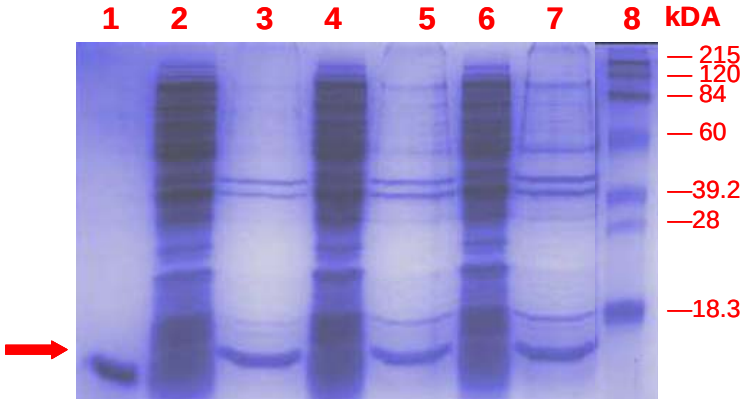


Fig 9B SDS–PAGE (15%) analysis. Lane 1: Hb1Tn as standard, lanes 2, 4, 6: soluble fractions of sonicates *E.coli* bacteria at different times of inductions, lanes 3, 5, 7: insoluble fractions of sonicates *E.coli* bacteria at different times of inductions, lane 8: molecular weight markers. A red arrow indicates the position of Hb1Tn. Each lanes were loaded with 10-15 µg of proteins

3.4 Co-expression of Hb1Tn with E. coli GroEL/GroES

3.4.1 Introduction

Many recombinant proteins expressed in *E. coli* have been obtained in soluble form through co-expression of GroEL/GroES, molecular chaperones of *E. coli*, involved in the correct folding of premature proteins in an ATP-dependent manner.
Therefore, to obtain soluble recombinant Hb1Tn, a system has been developed for the co-expression of Hb1Tn with GroEL/GroES.

3.4.2 Materials and Methods

The plasmid pGro (figure10), containing the genes for the expression of the chaperones GroEL and GroES, was provided by Dr. Takayoshi Wakagi (University of Tokyo) (17). This plasmid contains the resistance to kanamycin.

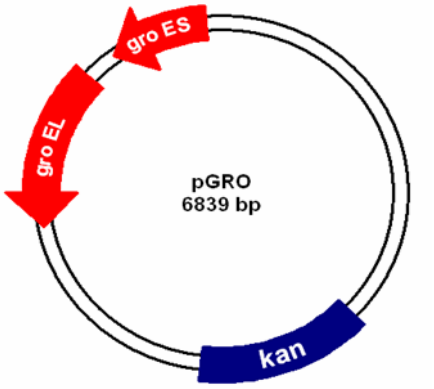


Fig10 Vector pGRO

E. coli BL21(DE3) cells were co-transformed with the plasmids pHE7Hb1*T.newnesi* and pGRO, and cells were grown in TB medium supplemented with 100 μg/ml ampicillin and 100 μg/ml kanamycin. The cells were grown as previously described.
However, even in presence of higher amounts of GroEL/GroES, the recombinant Hb1Tn was found primarily in the insoluble fraction.

3.5 Isolation and refolding of recombinant Hb1Tn

3.5.1 Introduction

Accumulation of recombinant proteins as inclusion bodies during expression can be advantageous due to the very high levels of enriched protein produced and to the protection of the protein product from proteolytic degradation. In addition, inclusion bodies are easy to isolate, as an efficient first step in a purification scheme.
The method used for the isolation of Hb1Tn was based upon the procedure by Nagai *et al.*(18, 19), with some modifications.

3.5.2 Materials and Methods

The cells were suspended in 3 ml/g of cells of lysis buffer, (20 mM Tris/HCl pH 7.5), ultrasonicated in ice-bath and centrifuged at 4 °C for 45 min at 14.000 rpm. After ultrasonication, the pellet containing the recombinant Hb as insoluble inclusion bodies was washed 4 times in a 20 mM Tris/HCl buffer (pH 7.5, 0.5% Triton X-100, 10 mM EDTA and 100 mM NaCl), until a tight pellet was formed. These washing steps are designed to remove contaminants, especially proteins that may have adsorbed onto the inclusion bodies during processing, that could affect the yield of protein refolding. After washing, the pellet was solubilised at a concentration of approximately 0.1 mg/ml in 8 M urea, 20 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM Glycine, 50 mM β-mercaptoethanol, and was stirred at 4 °C overnight. The opaque mixture was first centrifuged at 10000 rpm at 4 °C for 30 minutes and then at 25000 rpm at 4 °C for 2 hours. The Hb1Tn was refolded and reconstituted with haemin at a 1.2 molar excess by dialysis against a buffer containing decreasing concentrations of urea (6 M, 4 M, 2 M, 1 M) until 20 mM Tris/HCl, pH 7.5.

3.5.3 Results

After dialysis, the recombinant protein remained in solution (figure 11):

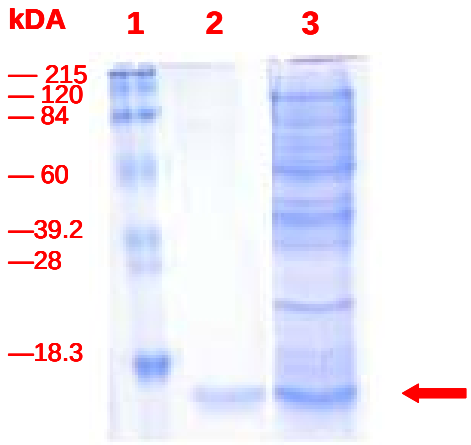


Fig 11 SDS–PAGE (15%) analysis. Lane 1: molecular weight markers, lane 2: Hb1Tn as standard, lane 3: protein solution after dialysis (10 μg). A red arrow indicates the position of Hb1Tn

In the VIS spectrum of the protein solution the Soret band is broad and the two maxima at 540 nm and 569 nm are not clearly defined. This was probably due to incorrect folding of the recombinant Hb1Tn (figure12).

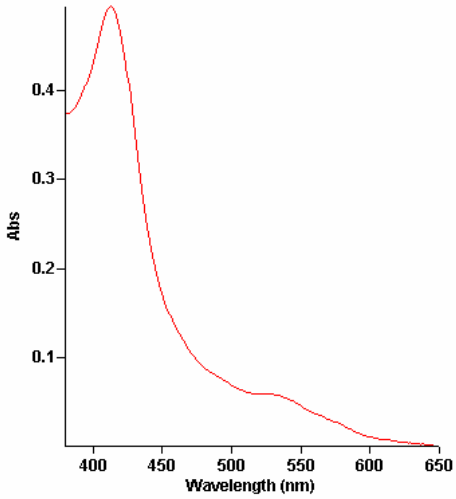


Fig 12 Electronic absorption spectrum of Hb1Tn in 20 mM Tris pH 7.5.

3.6 Expression in Pseudoalteromonas haloplanktis TAC125.

3.6.1 Introduction

To minimize the formation of inclusion bodies, a different experimental approach consists in lowering the expression temperature to the physiological limit allowed for the growth of mesophilic hosts. In fact, lowering the temperature has a pleiotropic consequence on the folding processes, minimizing the hydrophobic effect. Although in some cases this approach has been reported to increase the yield of soluble and active recombinant proteins, in this case it was not successful. A rational alternative to mesophilic organisms is the use of naturally cold-adapted bacteria as hosts for protein production at low temperature. The development of a shuttle genetic system for the transformation of the cold- adapted Gram-negative bacterium in *Pseudoalteromonas haloplanktis TAC125* with a L-malate inducible promoter has been reported (20, 21, 22).
This inducible system was employed to try to express the Hb1Tn in soluble form in the laboratory of Prof Angela Duilio (University of Naples "Federico II").

3.6.2 Materials and Methods

Construction of expression vectors for the production of Hb1Tn in TAC125

T. newnesi Hb1 α-globin cDNA, complete of the SD region, was obtained from the plasmid pHE7Hb*T.newnesi* by digestion with *NdeI*. *T. newnesi* Hb1 β-globin cDNA was amplified by PCR from the plasmid pGEMβHskozak, with the primers *NdeI* β(5'-GGAATTCCATATGGTAG AGTGGACTG ATAAGG-3') and T7 (5'-TAATACGACTCACTCACTATAGGG -3'). Then the β-globin cDNA was digested with *XbaI*, filled in with *Klenow,* and digested with the *NdeI*. These fragments were ligated in a triple ligation reaction involving the expression vector pUCRP (plasmid pUC containing a Regulated Promoter) previously digested with *NdeI* and *SmaI,* generating pUCRPHb1Tn (figure 13).

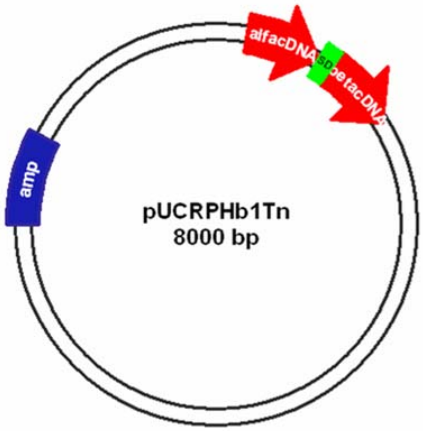


Fig 13 Vector pUCRPHb1Tn

P. haloplanktis TAC125 (*Ph*TAC125), a Gram-negative bacterium, was grown in aerobic conditions at 15 °C in TYP broth (16 g/l yeast extract, 16 g/l bacto tryptone, 10 g/l marine mix pH 7.5). The plasmid pUCRPHb1Tn was immobilized into *Ph*TAC125 by interspecific conjugation, using the transformed *E.coli* 3363 cells as donor. Aliquots of 100 µl of logarithmic cultures of the donor and the recipient strains were mixed and spotted as a drop onto TYP plates. After 16 hours of mating at 15 °C, the cells were resuspended in 200 µl of TYP medium. Psychrophilic transconjugants were selected by plating serial dilutions at 15 °C on TYP plates containing 100 µg/ml ampicillin and 100 µg/ml kanamycin.

Culture of recombinant *Ph*TAC125 cells were aerobically grown in minimal medium, SHATZ (1 g/l KH_2PO_4, 1 g/l NH_4NO_3, 10 g/l NaCl, 0.2 g/l $MgSO_4 \times 7H_2O$, 10 mg/l $FeSO_4$, 10 mg/l $CaCl_2 \times H_2O$, supplemented with casamino acid 0.5% w/v, pH 7) at 15 °C. The expression of Hb1Tn was induced by adding L-malate at 0.2% and haemin at concentration of 35 µg/ml when the optical density at 600 nm was approximately 0.4/0.5. The growth was continued for at least 48 hours.

Cellular pellets were collected during the growth. The cells were suspended at 3 ml/g of cells in lysis buffer (20 mM Tris/HCl pH 7.5), ultrasonicated in ice- bath and centrifugated at 4 °C for 45 min at 14.000 rpm; no pellet was formed.

3.6.3 Results

The cell lysates were loaded on the SDS-PAGE, about 7 µg per line. However no detectable quantities of recombinant Hb1Tn were visible on the gel. To detect the recombinant protein it was necessary to perform an immunoblot analysis of cell lysates, using antibodies prepared against Hb1Tn (figure 14).

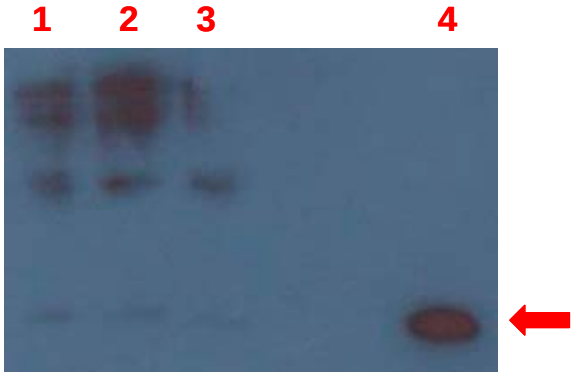


Fig 14 Western blot analysis. Lanes 1, 2, 3: patterns of protein expression of the *Ph*TAC125, lane 4: Hb1Tn as standard. A red arrow indicates the position of Hb1Tn. Each lanes were loaded with 10-15 µg of proteins.

3.7 Discussion

Although the system successfully used for the expression of Hb *Potomac* (see next chapter) was used for expressing Hb1Tn, as well, the latter protein was obtained in high amounts but not in soluble form. This result maybe due to the temperature at which the expression takes place; in fact, Antarctic fish live at significantly lower temperatures than that preferred by *E. coli*. An expression temperature higher than that characteristic of the physiology of an organism could prevent the correct folding of the growing recombinant protein, and cause the precipitation as inclusion bodies.

The temperature is directly related to the molecular motion. This implies that a less stable protein is more likely to fall apart at elevated temperatures and might therefore precipitate as insoluble agglomerate.

Problematic expressions in *E coli* for psychrophilic proteins are quite common: lipases, proteases and amylase (23, 24, 25, 26) from Antarctic psychrophiles were expressed as non-active form or as inclusion bodies. This illustrates the general heat lability of psychrophilic proteins, which is thought to arise from their flexible conformation, allowing functionality at temperatures close to 0°C.

Another possible explanation could be that the alpha chain of Antarctic fish Hb is acetylated, where the acetylation could guide the correct folding, whereas the recombinant form of the protein expressed in E. coli cannot be matured with this post-translational modification.

Acetylation is among the most common types of protein modification, yet its function is incompletely understood. Acetylation of the α-NH2 of N-terminal residues occurs in several human embryonic Hbs and in many fish Hbs. An additional effect of acetylation on human HbF besides lowering the 2,3-DPG response (27), is to promote a decrease in the strength of interactions between dimer pairs at the allosteric interface. A possible explanation for this effect is provided in relation to the known structural features of the N-terminal regions of the α and β subunits. Since the tetramer strength of HbA is decreased about fivefold by the presence of AcSer at the N terminus of its β subunit, it seems very likely that this occurs through weakening of β–β subunits interaction (28). It is possible that in histones, as well, lysine acetylation acts by disruption of nucleosomes through breakage of interactions between a histone tail protruding from a given nucleosome into an

adjacent one, to thereby loosen the assembly and enable efficient access by various transcription factors. In the case of recombinant Hb1Tn, the absence of an acetyl group on the α-NH2 of the N-terminal Ser of the α chain may have an opposite effect, increasing the interactions between the globins of the tetramer, and perhaps acting on the folding.

A number of approaches have been tried to increase the solubility of the recombinant Hb1Tn. Unfortunately, these trials were unsuccessful because was not observed an increase in the expression level or in the solubility of the protein.

Most eukariotic proteins produced in *E. coli* are insoluble in the bacterial cell, and form inclusion bodies that can be dissolved only by strong denaturing reagents. In this case, the recombinant Hb1Tn remained in solution after removal of the denaturing agent, but the haem was not correctly incorporated, as suggested from the UV-Vis spectrum.

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Chapter 4

Expression of Haemoglobin *Potomac*

4.1 Introduction

The Hb *Potomac* (β101Glu→Asp) is a natural mutant of human HbA. According to the Monod, Wyman, and Changuex two-state theory (1) the quaternary structures of the two states, R (relaxed) and T (tense), are different in the number and energy of the noncovalent bonds between the subunits. These stabilizing contacts are known to be primarily at the $\alpha_1\beta_2$ subunit interfaces, where striking differences have been demonstrated to occur upon ligation. Mutant Hbs which have substitutions at this interface generally manifest dramatic changes in their functional properties. Typical examples are mutations that occur at Asp99(G1)β, which result in a shift of the allosteric equilibrium towards the R state, while mutations that occur at Asn102(G4)β result in a T shift.

These changes in the allosteric equilibrium are due to alterations in some of the contacts of these residues, which stabilize either the T form or the R form of the quaternary structure of normal Hb.

Examples of mutant Hbs are Hb *British Columbia* (β101Glu→Lys) and Hb *Alberta*, (β101Glu→Gly), that were found to have high O_2 affinities, and Hb *Rush* (β101Glu→Gln), that was reported to exhibit a normal function (2).

The mutant Hb *Potomac* (β101Glu→Asp) has been detected because of an abnormal O_2 affinity of the blood of the affected individuals rather than by electrophoresis, because its amino acid substitution does not involve a charge difference (3).

This Hb is called *"Potomac"* because the proband lived near that American river.

The minimal structural requirement for the Root effect, accounting for a two protons release upon oxygen upload, is attributed to the strong hydrogen bond formed by the side chains of Asp95α and Asp101β, assisted by Asp99β (figure 1).

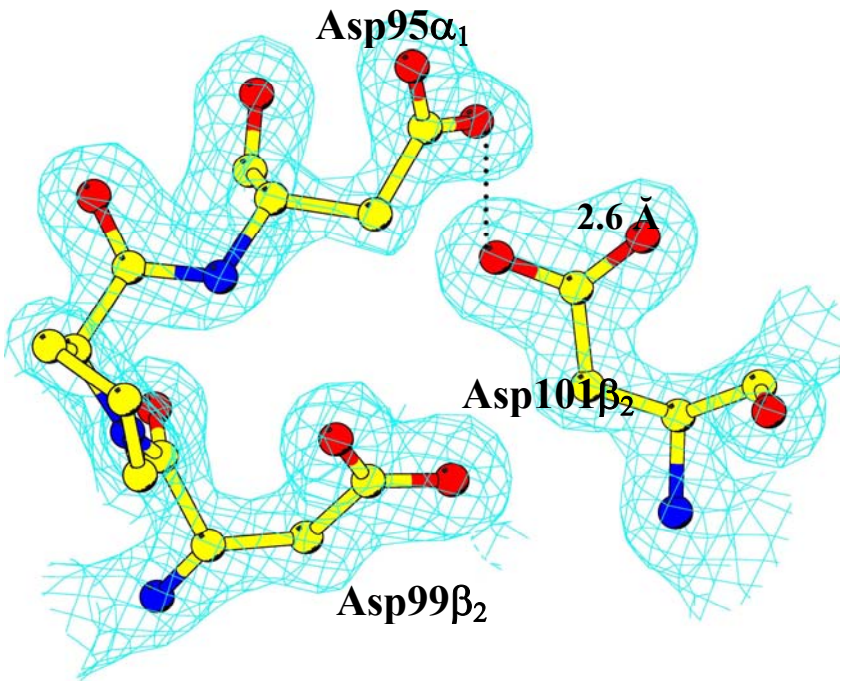

Fig 1 Aspartic triad.

This Asp triad is a common feature of all the deoxy structures of investigated Root-effect Hbs. In literature is reported that Hb *Potomac* has nearly pH-independent low oxygen affinity and no Root-effect, despite the presence of the Asp triad. This feature is common to Hb1Tn. Explanations of these abnormal functional properties and about the role of the aspartic residue in β101 in the Root effect could be obtained from X-ray crystallographic data.

Here is reported the cloning, expression, purification and characterisation of Hb *Potomac*.

4.2 *Materials and Methods*

Materials

The plasmid pHE7, containing human α- and β-globin cDNAs and the gene for methionine aminopeptidase (MAP) from *E .coli*, was provided by Prof. Chien Ho (4, 5). Chemicals and restriction enzymes were purchased from Bio-Rad, New England BioLabs, Promega, and Sigma.

Methods

Construction of Hb Potomac Expression cassette

The vector pHE7 containing human α- and β- globin cDNA was digested with *XbaI* and *NsiI* to extract the fragment with the globin cDNAs completed with the respective SD region. Then this fragment was cloned in the plasmid pGEM7Z, to give pGEM7ZHbA, that was used as template in the next PCR reactions.
The *Potomac* β101Glu(GAG) →Asp(GAT) mutation was introduced using the site-directed mutagenesis procedure called "Overlap Extension".
In figure 2 is represented a scheme of the "Overlap Extension", that is based on multistep PCR reactions that use mutagenic primers:

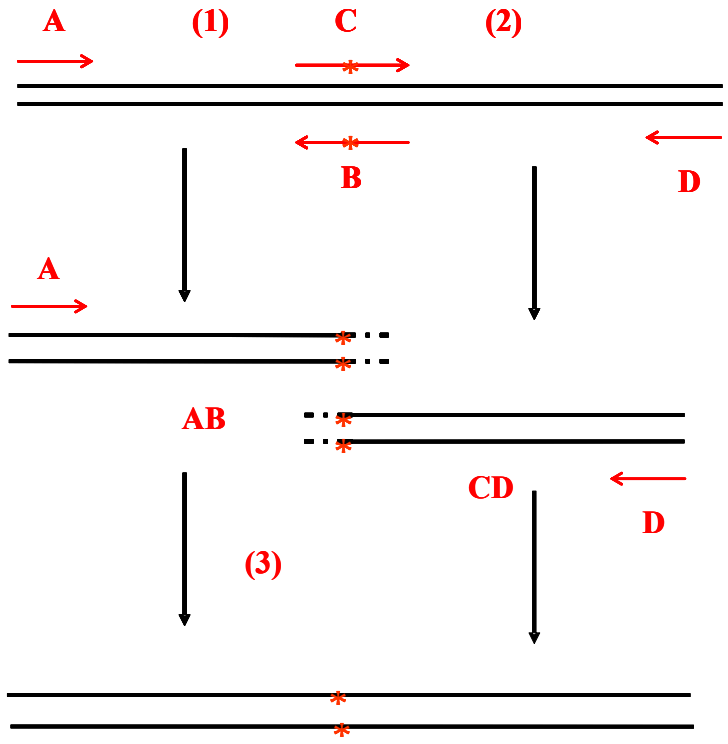

Fig 2 Scheme of the "Overlap Extension". The asterisk represents the mutation and the red arrows represent the primers.

The mutagenic primers are drawn to produce in the AB and CD fragments complementary regions containing the mutation.

The AB fragment was amplified from pGEM7ZHbA with the primers (A) *SphSDbeta* (5'-ACATGCATGCACTAGAGGGTATTAATAATGTATTCGC-3') and (B) *Poto_rev* (5'-GGAGCCTGAAGTTATCAGGATCCACG-3').

The CD fragment was amplified from pGEM7ZHbA with the primers (C) *Poto_dir* (5'-CGTGGATCCTGATAACTTCAGGCTCC-3') and (D) *hs_betarev* (5'-GTGATACTTG TGGGCCAGGGC-3').

Then the AB and CD fragments were combined and used as template in a third PCR reaction with primers (A) and (D), to generate the AD fragment that contains the mutation Glu(GAG) →Asp(GAT).

Amplifications were performed using a reaction mixture containing 2.5 u of *Taq* polymerase (EuroClone), 5 pmol each of the above primers and 0.20 mM dNTPs, buffered with 160 mM $(NH_4)_2SO_4$, 670 mM Tris·HCl (pH 8.8), 0.1% Tween-20, containing 1.5 mM $MgCl_2$. The PCR program consisted of 30 cycles of 1 min at 94°C, 1 min at a temperature between 50÷55°C, 1 min at 72°C, and ending with a single cycle of 10 min at 72°C.

The AD fragment was digested with *BamH I* and *EcoR I* to extract the 60 bp fragment containing the *Potomac* mutation. This fragment was inserted into pGEM7ZHbA to form the plasmid pGEM7ZHb*Potomac*. Standard molecular biological techniques were used for the isolation, restriction, and sequence analysis of plasmid DNA .

The presence of the *Potomac* mutation was confirmed by automated sequencing. Finally the plasmid pGEM7ZHb*Potomac* was digested with *XbaI* and *NsiI* to extract the fragment that was cloned in pHE7 to give the expression plasmid pHE7*Potomac* (figure 3)

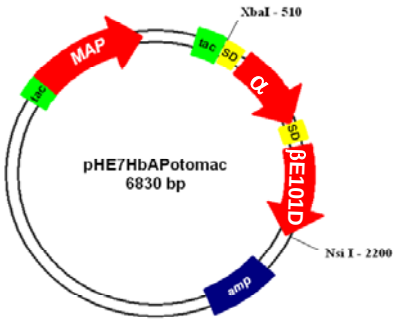

Fig 3 Vector pHE7HbA*Potomac*.

Co-Expression of Hb Potomac with E. coli Met-AP

E. coli BL21(DE3) cells were transformed with the plasmid pHE7*Potomac*; subsequently the cells were grown at 37°C in TB medium (1.2% bactotryptone, 2.4%bactoyeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K2HPO_4) supplemented with 100 μg/ml ampicillin. The expression of Hb *Potomac* and Met-AP was induced by adding isopropyl β-thiogalactopyranoside till 0.1 mM, when the optical density at 600 nm was about 0.7/0.8. After 30 min the culture was supplemented with hemin (35 μg/ml dissolved in a solution of 15% NH_4OH) and glucose (20 mg/ml), and the growth was continued for at least 4 hours. The cells were harvested by centrifugation and stored frozen at -80°C until needed for purification.

Isolation and Purification of mutant Hb Potomac

The cells were suspended in 3 ml/g of cells lysis buffer (20 mM Tris/HCl pH 8.4), sonicated in ice bath and centrifuged at 4 °C for 45 min at 14.000 rpm. The lysate developed a bright red colour when it was saturated with CO gas. The pH was adjusted to 8.4 with lysis buffer.

Protein concentration was determined by the standard Bradford procedure; the concentration of Hb *Potomac* in solution was estimated using published molar extinction coefficients (6).

Fast Protein Liquid Cromatography (FPLC) system was used to purify the Hb *Potomac.* Three steps were employed: 1) anionic exchange chromatography, 2) cationic exchange chromatography and 3) size exclusion chromatography.

1. A Fast Flow Q-sepharose column (18 ml bed volume) was equilibrated with 20 mM Tris/HCl pH 8.4. An amount of about 120 mg of total protein was loaded for each chromatography run at 2 ml/min; after loading the column was washed with 40 ml of equilibration buffer to eliminate the unbound proteins. Subsequently, the fractions containing the Hb *Potomac* were eluted with equilibration buffer containing 0.180 mM NaCl. These fractions were saturated with CO gas, concentrated and dialyzed against the starting buffer of the second crhomatographyc step (20 mM sodium phosphate pH 6.8).

2. A Fast Flow SP-sepharose (10 ml bed volume) was equilibrated with 20 mM sodium phosphate pH 6.8. The sample, about 10 mg, was loaded at 2 ml/min; after loading the column was washed with 40 ml of equilibration buffer to eliminate the unbound proteins. The fractions containing Hb *Potomac* were eluted with equilibration buffer containing 0.180 mM NaCl. These fractions were saturated with CO gas and concentrated until about 2.5 mg/ml. The concentration of Hb *Potomac* at this step was determinated from the UV-VIS spectra by molar extinction coefficients.
3. A Gel filtration Superdex 200 16/60 Hi Load column was equilibrated with 20 mM Tris/HCl pH 7.6 and 0.2 mM NaCl. The Hb *Potomac*, that was visible during chromatography as a thin red band, was eluted in a single peak at 16 ml.

4.4 Results

In figure 4 it is shown the 15% SDS- PAGE of the soluble and insoluble fractions of the cells collected at time zero (t_0) of induction and at each two hours intervals.

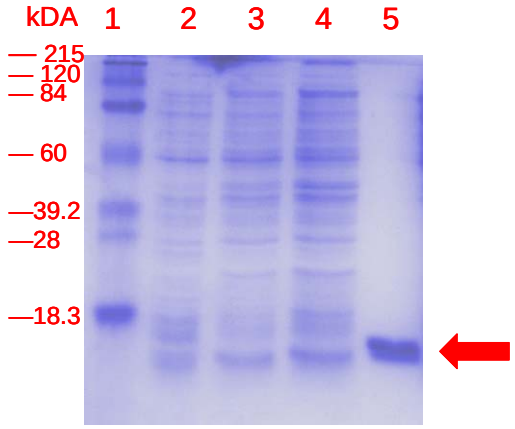

Fig. 4 SDS–PAGE (15%) analysis. Lane 1: molecular weight markers, lanes 2, 3, 4: patterns of protein expression of the *E.coli* strains BL21 at different times of inductions, lane 5: HbA as standard. A red arrow indicates the position of HbA.

Hb *Potomac* was found predominantly in the soluble fractions after four hours from induction.

Cells were collected by centrifugation, resuspended in SDS-PAGE sample buffer and heated to 95°C for 5 min. Each line was loaded with 50 μg of protein, as calculated from cell density. The proteins were visualized by staining with Coomassie brilliant blue. A sample of 5 μg of HbA was also loaded as standard.

The yield of soluble purified protein was approximately 4 mg/litre culture.

In figure 5 it is shown the elution profile of the Q-Sepharose chromatographyc step; the fractions were monitored at 280 nm and at 418 nm: the first wavelength provide a profile of all the proteins, while the second wavelength detect only the presence of haem-containing proteins. The fractions containing the Hb *Potomac* are indicated by the arrow.

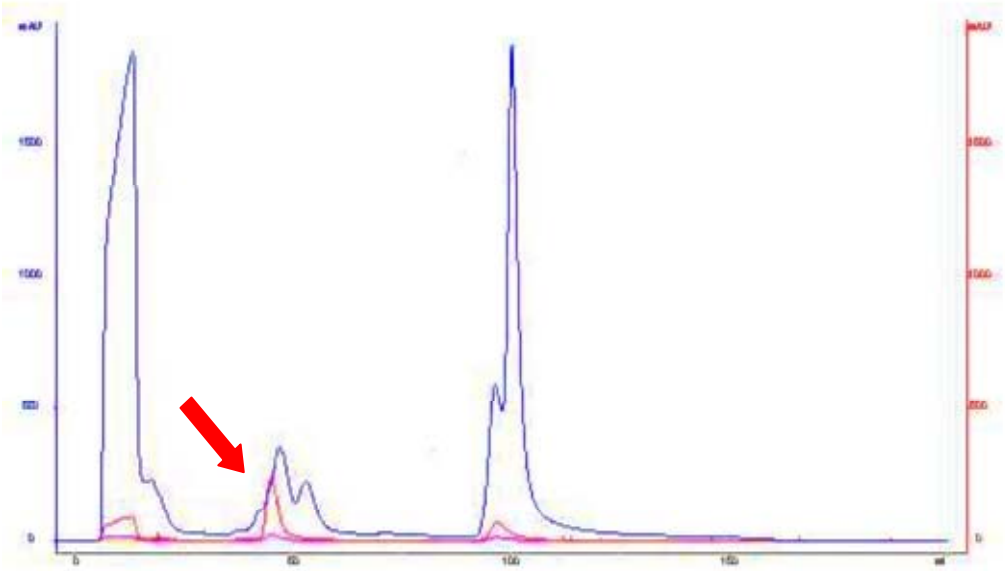

Fig 5 Elution profile of the Q-Sepharose chromatographyc step. The blue profile and the red profile represent the traces at 280nm and at 418nm, respectively. A red arrows indicates the fractions containing Hb *Potomac*.

In figure 6 it is shown the elution profile of SP-Sepharose chromatographyc step; the major amount of the proteins was eluted with the void volume, while the major peak contains quite pure Hb *Potomac*.

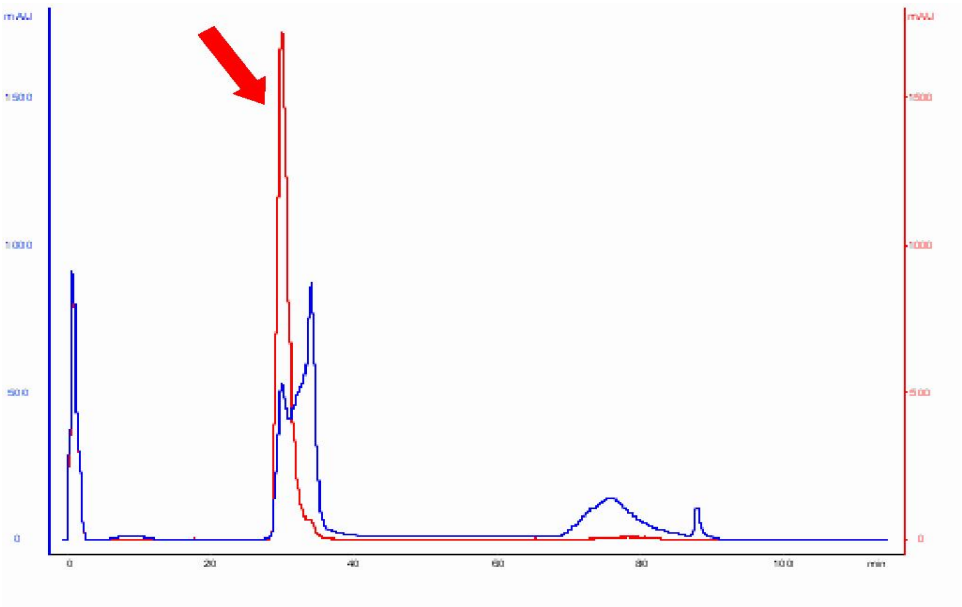

Fig 6 Elution profile of the SP-chromatographyc step. The blue profile and the red profile represent the traces at 280nm and at 418nm, respectively. The fractions containing the Hb *Potomac* are indicated by a red arrow.

In figure 7 it is shown the elution profile of Superdex 200 gel filtration: the Hb *Potomac* is eluted at 16 ml, in accordance with the standard calibration.

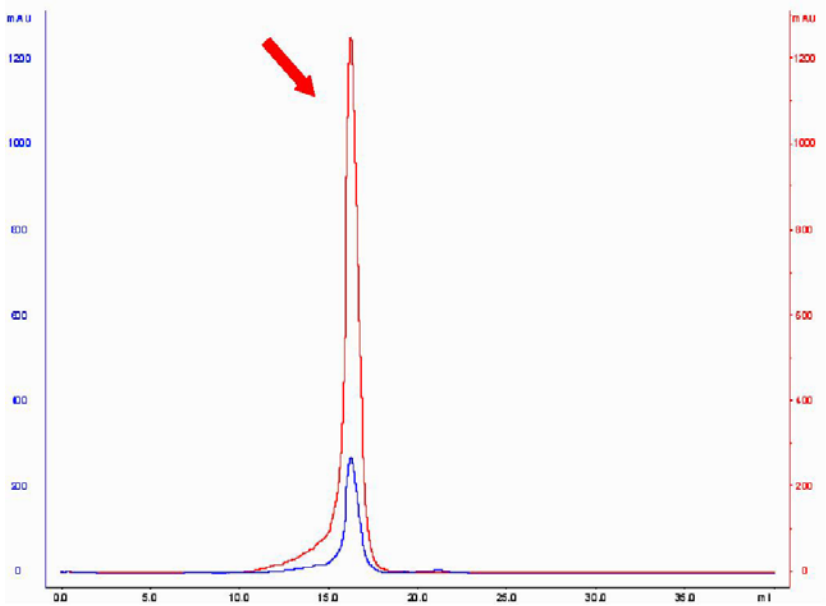

Fig 7 Elution profile of the gel filtration step. The blue and the red profiles are the traces at 280nm and at 418nm, respectively. The fractions containing the Hb *Potomac* are indicated by a red arrow.

The 15 % SDS- PAGE gel (figure 8) shows the protein patterns of the fractions collected during the three chromatography steps, (each line contains a protein amount of 15 μg).

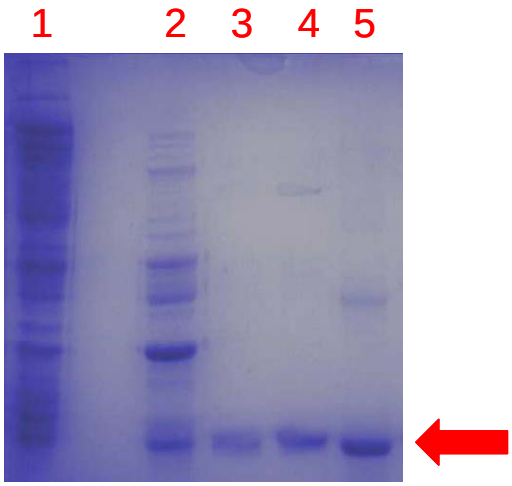

Fig. 8 SDS–PAGE (15%) analysis. Lane 1: pattern of protein expression of the *E.coli* strains BL21, lanes 2, ,3, 4: protein patterns of the samples eluted at 1st, 2nd and 3rd chromatographic step, respectively, lane5: HbA as standard. A red arrow indicates the position of HbA.

4.5 Characterisation

4.5.1 Mass Spectrometry

Methods

The α- and β-globin chains from mutant Hb *Potomac* were separated by reverse phase HPLC on a Vydac C_4 analytic column. A sample of purified Hb *Potomac* was diluted with 0.1% TFA to give a final concentration of 5 µg/µl. The column was equilibrated at 1 ml/min with 57% solvent B (60% CH_3CN 0.1% TFA) and 43% solvent A (20% CH_3CN 0.25% TFA); solvent B was increased to 70% in 35 minutes and subsequently to 95% in 15 minutes.
The single globin chains were analyzed by electrospray, ESI, using a single quadrupole instrument, the ZQ 2000 of Water-Micromass. A ion spray source was used at 3,9 kV using a CONE potential of 38 V. The range of acquisition was from 700-1400 m/z, with scan time of 3 sec.

Results

Electrospray mass spectra of α and β globins are shown in figure 9A and 9B respectively:

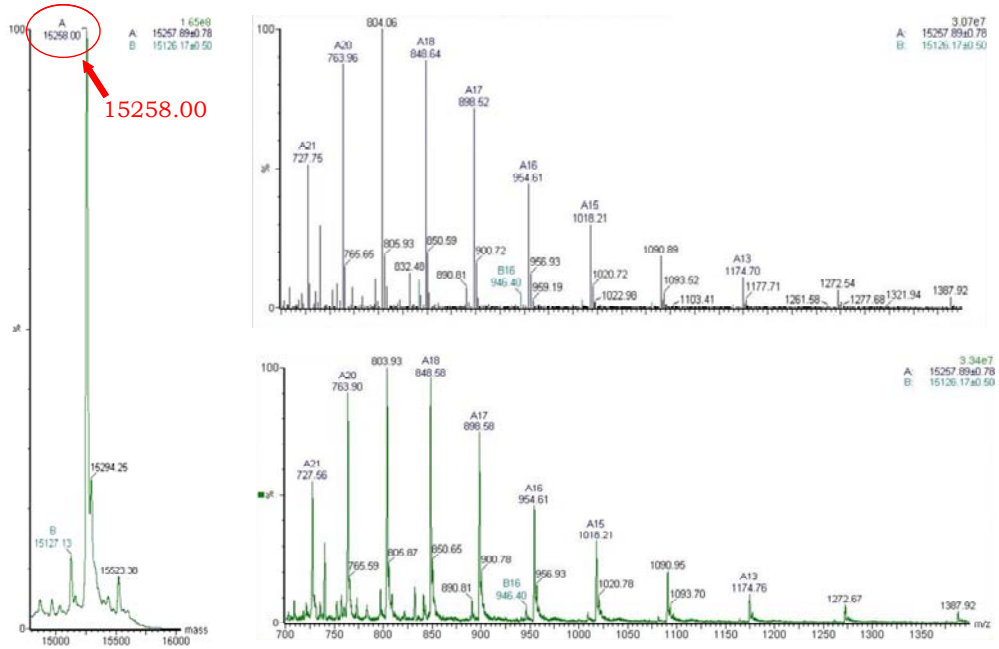

Fig 9A ESI mass spectrum of α globin chain.

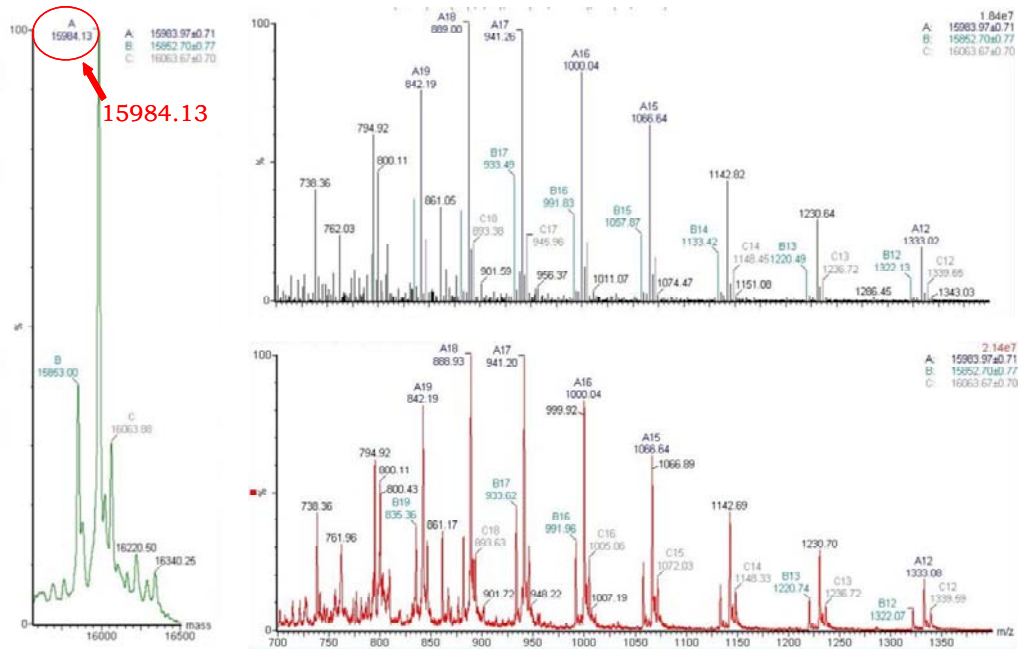

Fig 9B ESI mass spectrum of β globin chain.

The values assigned for α and β globins are 15258.00 ±0.48for α globin and 15984.13± 0.71 for the major component of β globin; however there was a small component (25%) whose mass corresponds to that of β globin devoid of the N-terminal Met. These molecular weights are in accordance with the values predicted from the amino acid sequences of the proteins. The molecular weights found for α and β globins indicated that the N-terminal methionine was not efficiently removed.

Discussion

The finding of a not complete remotion of N-terminal methionine is in agreement with Chen Ho *et al.* (5). In fact, they have observed that there are variable amounts of uncleaved N-terminal Met residues in various fractions of different preparations of Hbs.

The relatively low expression of Met-AP may be the main cause of the failure of the total remotion of the N-terminal Met from the expressed α and β globins. However, it is known that the specificity of Met-AP is influenced by a number of factors, such as the nature and the side-chain size of the penultimate amino acid residue, the nature of neighbouring amino acid residues and the tertiary structure of the protein molecule. The difficulty in processing the N-terminal Met of β globin may also be related to the presence of the His residue in the third position. It is possible that the failure to remove the Met is due to obstruction of the processing enzyme by an early folding intermediate,

formed while the nascent polypeptides are still bound to the ribosome. In addition, both chains have α helical regions that occur within the first 20 amino acids, that could block the enzymatic reaction.

To obtain a homogenous sample of protein it was necessary to inhibit the Met-AP (7, 8).

The Met-Ap is a metalpeptidase, and it is inactive in presence of EDTA.

Therefore, the experiments described above (see method section) have been repeated in presence of 5 mM EDTA in the buffer solutions and in the growth medium.

The molecular weights of the α and β globins were redetermined by mass spectrometry. Hb samples were diluted to give a final concentration of 100 pmol/µl. The electrospray analyses were performed on a Q-star LC-MS equipment.

The α- and β-globin chains from mutant Hb *Potomac* were separated by reverse phase HPLC on a Vydac C_{18} analytic column, with a gradient from 10 % to 70% acetonitrile containing 0.1% formic acid and 0.025% TFA. Subsequently, the separated globins were directly introduced into the electrospray ion source at 50 µl/min.

Mass spectra of α and β globins are shown in figure 10:

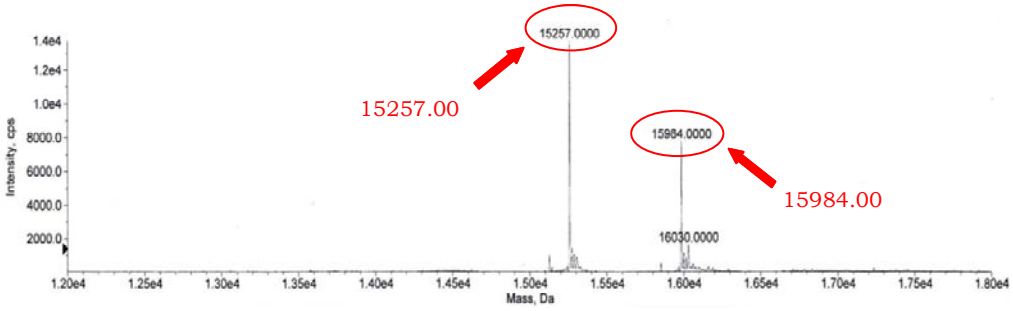

Fig 10 ESI spectrum of α- and β- globin chains.

The obtained molecular weights are 15257.5 for α globin and 15984.4 for β globin. These values are in agreement with those predicted from the amino acid sequences of the proteins and they confirm the presence of the N-terminal Met residue, as well as the mutation *Potomac* β (β101Glu→Asp).

4.5.2 UV Spectroscopy

The absorption spectrum, in the oxy and deoxy form (figure 12), was registered on a spectrophotometer VARIAN Cary 300 in the visible region between 380 nm and 650 nm.

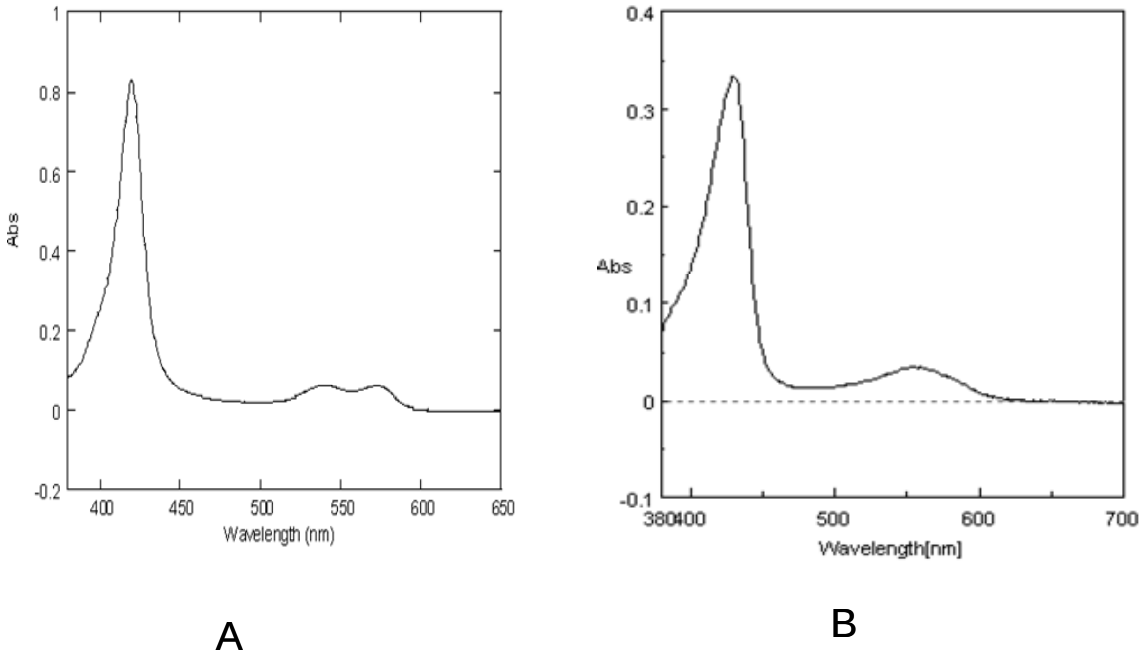

Fig 12 Electronic absorption spectra of Hb *Potomac* in the oxy (A) and deoxy (B) form, in 10mM ammonium phosphate buffer at pH 6.5.

A typical absorption spectrum of HbA in the form HbCO is characterised by a strong maximum at 415-419 nm (Soret) and two maxima at 540 nm (ε = 13.4) and 569 nm (ε = 13.4), while the Hb deoxy form is characterised by a 430 nm maximum (Soret) and a single maximum at 555 (ε = 12.5) (6). Deoxy–Hb *Potomac* was prepared according to two protocols: 1) starting from its carbomonoxy derivatives by exposing the haemoglobin solution to a strong white light under argon; 2) *via* a cycle of oxidation/reduction adding potassium hexacyanoferrate/sodium dithionite, respectively.

The absorption maxima of the Hb *Potomac* were nearly the same as the corresponding values for HbA, indicating that these proteins are indistinguishable with respect to these spectral properties.

4.5.3 Resonance Raman (RR) Spectroscopy

The deoxy form of Hb *Potomac* and of HbA were analysed *via* Resonance Raman (RR) spectroscopy. In the RR experiments the proteins were kept in 10 mM ammonium phosphate buffer pH 6.5. The haem concentration of Hb samples was 2 mM. The starting Hb *Potomac* and HbA deoxy samples were prepared by deoxygenation *in situ* using a solution 2 mM of sodium ditionite.

A confocal Raman microscope (Jasco, NRS-3100) was used to record Raman spectra. The 458-nm line of an air-cooled Ar^+ laser (Melles Griot, 35 LAP 431-220), 125 mW, was injected into an integrated Olympus microscope and focused to a spot size of approximately 2 μm by a 100x or 20x objective. A holographic notch filter was used to reject the excitation laser line. Raman scattering was dispersed through a monocromator (2400 grooves/mm grating) and collected by a peltier-cooled 1024 x 128 pixel CCD photon detector (Andor DU401BVI). Typically, several 10 min solution spectra were recorded and averaged (4 cm^{-1} resolution) by standard software routine. Frequency shifts were calibrated by using indene and CCl_4 as standard.

The high frequency region (1300-1700 cm^{-1}) includes the porphyrin in-plane vibrational modes, that are sensitive to the electron density of the macrocycle, to the oxidation, coordination and spin state of the iron atom (9). The low-frequency region (200-450cm^{-1}) contains bands with contributions from deformation of the various interior angles of the porphyrin ring and of the peripheral substituents, as well as stretching of the bonds from the pyrrole N atoms to the central metal atom.

In figure 13 are shown the spectra obtained at high and low frequency of Hb *Potomac* and HbA; these spectra are indistinguishable, indicating the correct folding of the recombinant protein and that the substitution Glu with Asp not influenced the porphyrin in-plane vibrational modes.

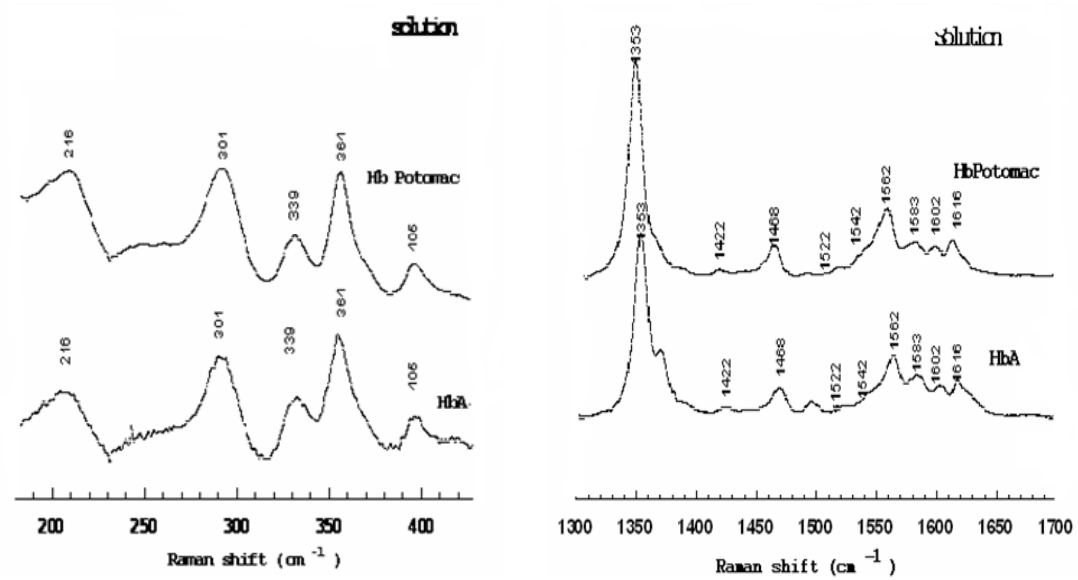

Fig 13 Resonance Raman spectra in solution for the low- (A) and high (B) wave number region of Hb *Potomac* and HbA.

4.5.4 Crystallisation

Crystallisation trials were performed according to the method described by Perutz (10), with several modifications. The Hb *Potomac* was dialyzed into 10 mM ammonium phosphate pH 7.0 and concentrated to approximately 60 mg/ml prior to crystallisation. Deoxy–Hb *Potomac* was prepared as previously described. Crystallisation trials were performed at room temperature in an inert nitrogen atmosphere, provided by a glove box. The precipitant solution was 3.6M sulphate/phosphate buffer pH 6.5 constituted by 0.8 volumes of $(NH_4)_2SO_4$ 4 M, 0.05 volumes of $(NH_4)H_2PO_4$ 2 M and 0.15 volumes of $(NH_4)_2HPO_4$ 2 M. The trials were carried out in batch mixing, in a total volume of 12 µl, the protein solution (1% final concentration) with the precipitant solution, at a final concentration ranging between 2.2 and 2.8 M. To prevent protein oxidation, 8.5 mM Fe (II) citrate was added to all crystallisation trials. The same trials were also carry out varying the pH of the precipitant solution in the range 6.5÷7.5, and using different Hb concentrations. The sitting drop technique was also used mixing 1.5 µl of protein solution and 1.5 µl of precipitant solution. Finally, the trials were performed with a different precipitant solution, 30% (*w/v*) PEG 4000 (11) and 10% (*w/v*) PEG 6000 (12, 13, 14).
All this trials did not lead to crystallisation of the protein.

4.6 Discussion

The experimental results presented in this chapter clearly show that authentic human mutant Hb can be produced in high yields in *E.coli* using pHE7 expression plasmid. The recombinant Hb is obtained in soluble form, in high yields and it is easy to purify.

The molecular weights found for α and β-globins indicated the presence of the N-terminal methionine. As discussed previously, it is unclear why methionine is not efficiently removed from amino termini of recombinant Hb.

In literature it is reported that the efficiency of N-terminal Met removal in E. coli is limited by the side-chain radius of the penultimate residue, that is the second residue after Met (15, 16, 17). Moreover, the efficiency of Met removal from basic penultimate residue (Arg or Lys) is extremely low; the cause could be due to the charge repulsion and steric hindrance of the substrate by the residues of MetAP in the substrate-binding pocket (18).

In addition to the penultimate residue, the removal of N-terminal Met was also influenced by the size and the charge of the antepenultimate residue, that is the third residue after Met, being the smaller and the hydrophilic the residues that favour the elimination of the terminal Met.

The N-terminal sequences of the globin chains of Hb *Potomac* are MVL for the alpha and MVH for the beta. Both the penultimate and the antepenultimate residues of this regions belong to groups of residues that do not favour the elimination of N-terminal Met (6). Another possible explanation is that a big fraction of the recombinant Hbs are folded in such a way that their amino termini are not accessable to MAP (see discussion about the influence of N-acetilation on folding).

The spectrophotometric properties of Hb *Potomac* were similar to that of human HbA.

These results suggest that the conditions used for bacterial expression promote correct folding and produce soluble Hb *Potomac.*

In addition, the Raman spectra of both human and recombinant Hb *Potomac* are indistinguishable, indicating not only the correct folding of the latter protein, but also that the substitution of Glu101 with Asp101 does not influence the “in-plane” vibrational modes of the porphyrin.

4.7 References

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Chapter 5

Crystal Structure of Deoxy Haemoglobin 1 from the Antarctic fish *Trematomus newnesi*.

5.1 Introduction

Over the years, several hypothesis on the structural determinants of the Root effect have been suggested. A variety of possible mechanisms involving the replacement of Cys93β with Ser, the formation of a strong hydrogen bond between the side chains of aspartic residues in the deoxy T state, and the presence of a cluster of charged residues located at the $\beta_1\beta_2$ interface of the liganded R state, have been proposed.

A number of crystal structures of R and T state fish Hbs have been described. The results have demonstrated that the basic features of the mechanism proposed by Perutz and his colleagues for the allosteric transition in human Hb holds remarkably well for fish Hbs too, despite the large difference in amino acid sequence.

In many cases, the functional effects of several heterotropic ligands and/or the mutation of selected residues have also been rationalised in terms of their structural effects.

A detailed definition of determinants for the Root effect requires structural information on R and T states at different pH values. The occurrence of the Root effect, supposed to be linked to an overstabilisation of the T state versus R state at acidic pH, makes it extremely difficult to obtain structural data on the R state at acidic pH.

The Root effect Hb isolated from the Antarctic fish *T. bernacchii* (Hb1Tb) has been used in pioneering investigations in the field. Both the R and the T state crystal structures of Hb1Tb have been reported. A detailed comparison of these two structures has shown that a partial contribution to the Root effect is provided by the unusual interaction between aspartic acid side chains which is present exclusively in the T state. In this structure, three aspartic residues cluster together at the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) interface: in particular Asp95α forms a strong hydrogen bond with Asp101β, implying that one of the two residues must be protonated. The presence of a third aspartic residue at short distance from the first two helps to stabilise the uncharged form of the aspartic residue (probably Asp95α). This structural feature has been confirmed

by investigations carried out on other Root effect Hbs, and, in particular, for the cathodic Hb component isolated from the blood of *T. newnesi* (HbCTn). However, the Asp triad is also present in some non Root effect Hbs such as trout HbI, the human variant Hb*Potomac*, and Hb1Tn. Therefore, it appears that in these Hbs other effects operating at the level of the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) interface may interfere with the Asp-Asp interaction. Indeed, structural data available on trout HbI have demonstrated that the two Asp side chains moves apart of about 0.5 Å, which is sufficient to break the hydrogen bond with an insertion of a water molecule (1). In this case, the sequence homology with Hb1Tb is not very high so that the reasons for this subtle modification are difficult to rationalize on structural grounds. On the other hand, due to the high level of sequence similarity between Hb1Tn and Hb1Tb, the comparison of the T structure of these two Hbs may give clear-cut indications on the nature of the interchain interactions, which are essential for the formation of the Asp-Asp hydrogen bond.
Here the crystal structure of the Hb1Tn in the T state at pH 6 is reported at 2.1 Å resolution and discussed in comparison to that of Hb1Tb. In addition, this study has been complemented by Raman spectroscopy, a powerful technique that provides information about the overall environment of the haem pocket.

5.2 Materials and methods

5.2.1 Crystallisation

Hb1Tn was purified by ion-exchange chromatography on a DE52 column, equilibrated with 10 mM Tris-HCl pH 7.6, and eluted by stepwise with the same buffer (2). Deoxy-Hb1Tn stock solutions were prepared according to two protocols:

A) *Via photolysis*: starting from its carbomonoxy derivates by exposing the Hb solution to a strong white light under argon;
B) *via* a cycle of oxidation/reduction: adding potassium hexacyanoferrate/sodium dithionite, respectively.

The deoxy formation was tested via optical spectroscopy. The absorption spectrum shows the shift of the Soret band from 418 nm (typical of carbomonoxy form) to 430 nm and the disappearance of the α and β bands, with a single band at 555 nm (3). These data confirm the presence of the deoxy form of Hb1Tn. Crystallisation trials were

performed at room temperature, in an inert nitrogen atmosphere provided by a glove box. The free interface diffusion (FID) technique was used: the protein, in a 100 mM NaAc pH 6.0 buffer with 2mM dithionite, with a final concentration of 10 mg/ml, was poured into a capillary containing a solution 20% (*w/v*) MPEG 5000 (2 mM dithionite). Single crystals of deoxy-Hb1Tn, suitable for X-ray diffraction, were grown in about twelve days (4) (figure 1).

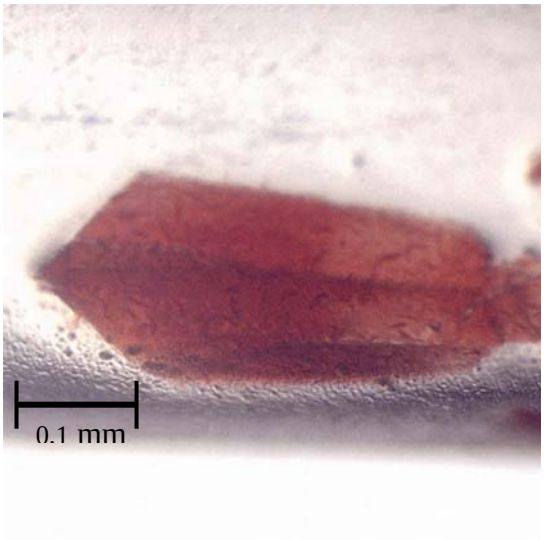

Fig 1 Crystal of deoxy- Hb1Tn

Crystals grown using deoxy-Hb1Tn obtained *via* the two procedures described above are isomorphs.

5.2.2 X-Ray Data Collection and Processing

X-Ray diffraction data were collected on crystals obtained from Hb samples procedure following the protocol A and B, to 2.1/2.2 Å (respectively) resolution with a Saturn 944 CCD (Rigaku) imaging plate, mounted on a rotating-anode generator from one crystal frozen at liquid-nitrogen temperature (100 K) using glycerol as cryoprotectant (22%). The space group of this crystal is tetragonal $P4_1$ with four molecules in the unit cell (one tetramer for asymmetric unit). Diffraction data were processed using the program DENZO and Scalepack (5). The reflections collected in different frames and correlated by crystal symmetry were mediated. The value of the R_{merge} indicates the internal consistence of the collected data:

$$R_{merge} = \frac{\sum_{ij} \left| I_{ij} - I_i \right|}{\sum_{ij} I_{ij}}$$

where:

I_{ij} = intensity of the j^{th} copy of the i^{th} reflection;

$\langle I_i \rangle$ = intensity of i^{th} reflection averaged over the n_i observations of that reflection $I_i = \sum_j \frac{I_{ij}}{n_i}$

The R_{merge} value was 7.9 % and 10.7 % for A and B, respectively. A summary of the processing statistics is reported in Table I

Table I Crystal Data and Data Collection Statistics

	A*	B*
Space Group	$P4_1$	$P4_1$
Cell parameters		
a (Å)	62.022	61.882
b (Å)	62.022	61.882
c (Å)	187.926	187.014
Resolution Range	2.00 (2.07-2.00)	2.2 (2.28-2.20)
Completeness (%)	89.1 (58.5)	90.8 (74.3)
N° measured reflectionss	148652	125230
N° unique reflections	42501	32307
R_{merge} (%)	7.9 (20.8)	10.7 (18.4)
I/σ	17.7 (4.8)	16.7 (5.9)
Redundance	3.05	3.09

*The value in parentheses refers to the highest resolution shell.

In the course of the refinement (see below) it becomes obvious that crystals were affected by merohedral twinning and the diffraction pattern was interpreted as resulting from two lattices correlated by a rotation of 180° around an axis parallel to $\vec{a}+\vec{b}$ diagonal. Therefore the intensity associated with each reflection hkl is the weighted sum of two contributions:

$$I_{obs}\ (hkl) = c_1 I(hkl) + c_2 I(khl)$$
$$I_{obs}\ (khl) = c_1 I(khl) + c_2 I(hkl)$$

where the fractions c_1 and c_2 determine the degree of twinning. The twin fraction for Hb1Tn, determinate by the algorithm implemented in the program SHELX, is 0.38 for the data A and 0.27 for the data B.

5.2.3 Refinement

The structure of deoxy Hb1Tn was solved by molecular replacement using the program AmoRE (6) and the structure of deoxy Hb1Tb (Protein Data Bank code 1h8f) (7) as starting model. The refinement was performed using the program SHELX (8, 9), which contains a straightforward procedure to deal with data derived from merohedrally twinned crystals. The refinement runs were followed by manual intervention, using the molecular graphic program ***O*** (10) to correct minor errors of the side chains. Water molecules were identified by evaluating the shape of the electron density and the distance of potential hydrogen bond donors and/or acceptors.
The structure of deoxy Hb1Tn was refined to an R-factor of 0.19, R-free 0.26 for the data A and an R-factor of 0.20, R-free 0.27 for the data B. A summary of the refinement statistics is reported in Table II. As the two models do not differ significantly, only the model A was used to analyse the results.

TableII.Refinement Statistics

	A	B
Resolution range (Å)	2.02	2.01
R (%)	0.19	0.20
R_{free} (%)	0.26	0.27
No. of residues	42455	42455
No. of atoms	4670	4670
No. of water molecules	145	145

5.2.4 Resonance Raman (RR) spectroscopy and microscopy.

The deoxy form of Hb1Tn, Hb1Tb and HbA in solution were studied *via* Resonance Raman (RR) spectroscopy. In addition, deoxy Hb1Tn and Hb1Tb in the crystals state were analysed via Resonance Raman (RR) microscopy. In the RR experiments carried out in solution the protein was kept in a 100 mM NaAc pH 6.0 for Hb1Tn, in 100 mM phosphate buffer pH 6.2 for Hb1Tb, and in 10mM ammonium phosphate buffer pH 6.5 for HbA. The haem concentration of Hb samples was 2 mM. The starting Hb1Tn and HbA deoxy samples were prepared by deoxygenation *in situ* by a 2 mM solution of sodium ditionite; while the Hb1Tb was prepared *in situ* from its carbomonoxy derivative, by decreasing the pH down to 6.2. Since Hb1Tb is endowed with the Root effect, the acidification process rapidly leads to the transition from the R to the T state and to the CO release.

A confocal Raman microscope (Jasco, NRS-3100) was used to record Raman spectra. The 458-nm line of an air-cooled Ar^+ laser (Melles Griot, 35 LAP 431-220), 125 mW, was injected into an integrated Olympus microscope and focused to a spot size of approximately 2 μm by a 100x or 20x objective. The laser power at the sample was 2 mW. A holographic notch filter was used to reject the excitation laser line. Raman scattering was dispersed through a monochromator (2400 grooves/mm grating) and collected by a Peltier-cooled 1024 x 128 pixel CCD photon detector (Andor DU401BVI). Typically, several 10 min solution spectra were recorded and averaged (4 cm^{-1} resolution) by a standard software routine. Frequency shifts were calibrated by using indene and CCl_4.

Microscopy experiments were conducted on deoxy Hb1Tn and Hb1Tb crystals by using previously reported procedures (11, 12). Deoxy Hb1Tn crystals were grown as previously reported; deoxy Hb1Tb crystals were grown (0.3 x 0.2x 0.2 mm) according to literature protocols, and transferred to a single hanging drop reactor. Raman spectra on several deoxy Hb crystals did not show a significant dependence on crystal orientation. A complete data set on Hb1Tn crystals was registered in 60 s.

5.3 Results

5.3.1 Overall structure

The independent unit of the crystal includes one tetramer, thus the potential twofold symmetry is only local. Although the constraints related to the non crystallographic symmetry were not used in the refinement, the root mean square deviations (rmsd) of the backbone atoms (N, C^{α}, C, O) between the two α and the two β chains are 0.37 Å and 0.36 Å, respectively. Moreover, the corresponding thermal displacement parameters B are also very similar. These results indicate that the molecular symmetry is very well preserved in the crystal state.
The B factors are generally higher than those found for other tetrameric Hbs, indicating a greater flexibility of the skeleton atoms for Hb1Tn. Moreover, the displacement parameters are considerably greater for the α chains with respect to those of the β chains. This data contrasts with what is usually found: the β chain displays a much greater flexibility in almost all tetrameric Hbs. The trend of the B factors, averaged over the main chain atoms, is plotted as function of the residue number for the two α and β chains in figures 1A and 1B, respectively.

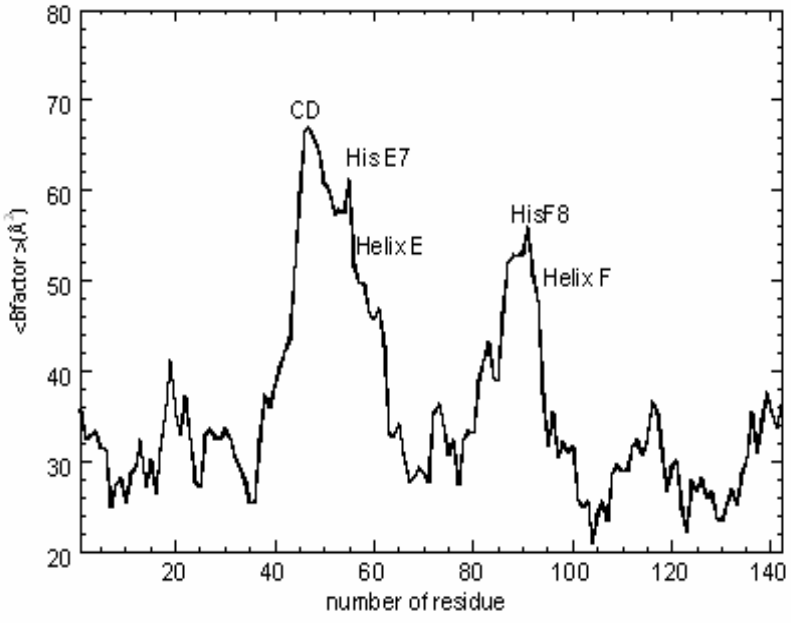

Fig1A B factors, averaged over the main chain atoms in function of the residue number for the α chain.

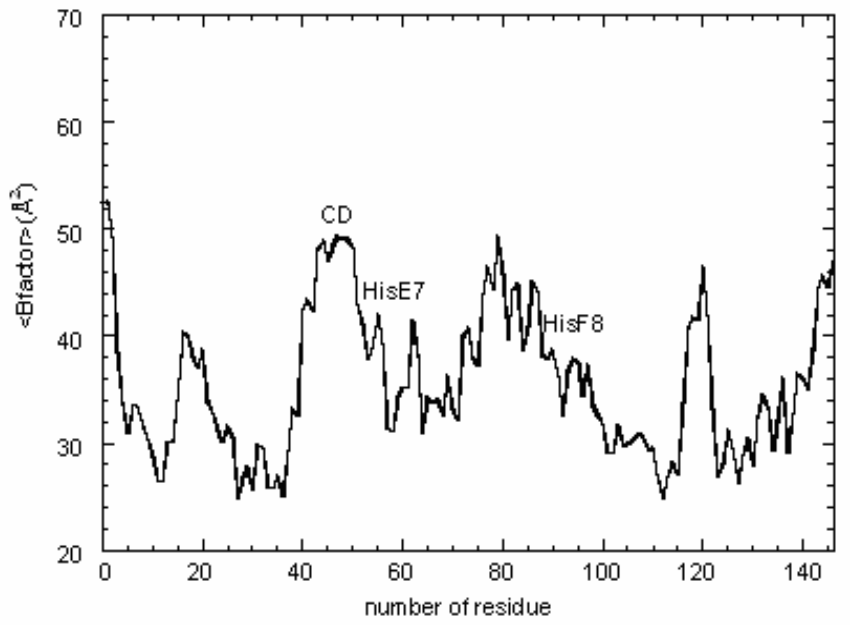

Fig1B B factors, averaged over the main chain atoms in function of the residue number for the β chain.

As clearly shown by the plots, the regions of the α chain which display the greater disorder include the CD loop and the two consecutive E and F helices, that form a V-shaped structure which provides the most important part of the haem binding pocket. As result, also the distal (E7) and proximal (F8) functionally essential histidine residues and the haem group itself do display large temperature factors, and somewhat ill-defined electron density. As usual, the CD region of the β chain and, in part, the helix F too display relatively high temperature factors, but the haem group and the E7 and F8 histidines are better defined, differently from the α chain. Interestingly, the bond length of the proximal histidine to the β haem iron is considerably higher (≈2.48 Å) than the canonical bond length, which usually falls in the range 2.10 – 2.20 Å.

5.3.2 The aspartic triad

As anticipated in the Introduction, the hot point of the molecule regards the organisation of the aspartic triad, which has been considered to be the main source of the Root protons in Hb1Tb and in HbCTn. On the light of the structural results on the non Root effect trout HbI, the Asp-Asp hydrogen bond was expected to be broken also in the T state of Hb1Tn. Despite the average greater disorder of this Hb, the region at the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) interface, which hosts the aspartic triad, is sufficiently well defined. The quality of the density map can be seen in figure 2, where the omit difference maps for the $\alpha_1\beta_2$ and $\alpha_2\beta_1$regions are reported.

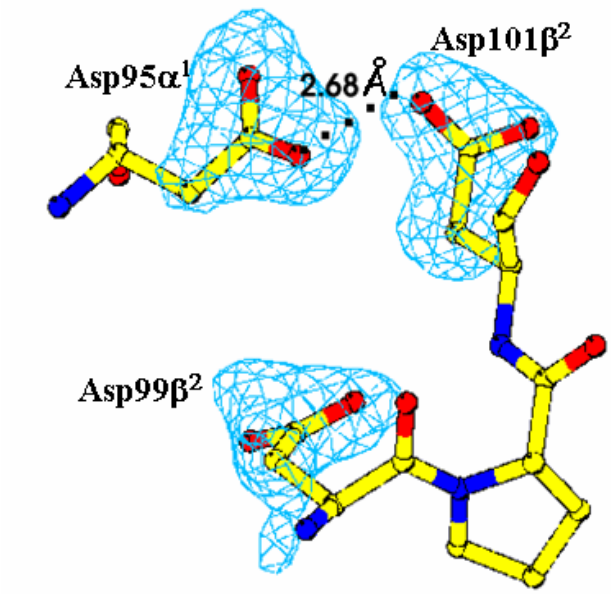

Fig 2A The omit difference maps for the $\alpha_1\beta_2$ region.

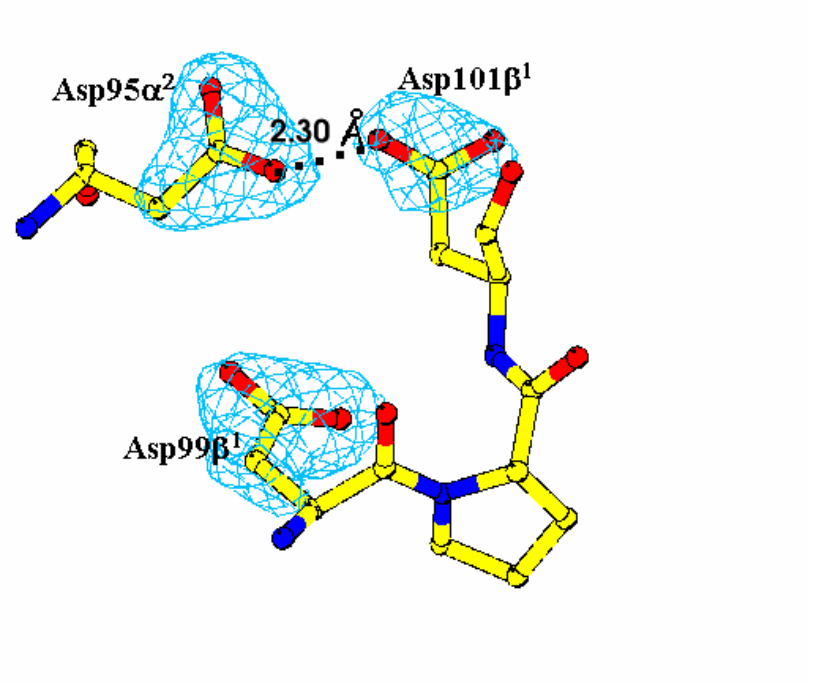

Fig 2B The omit difference maps for the $\alpha_2\beta_1$region.

Surprisingly, the relative position of Asp95α and Asp101β is very much the same as that found in Hb1Tb and in HbCTn. The hydrogen bond length between the carboxylic groups of Asp95α and Asp101β is 2.68 Å for $\alpha_1\beta_2$ and 2.30 Å for $\alpha_2\beta_1$. This result clearly indicates that, at least in the crystal, the T state of the non-Root effect Hb1Tn binds two protons per tetramer, that should be released on the transition to the R structure, where the distance between the two aspartic residues increases to more than 5 Å.

5.3.4 Comparison with Hb1Tb

A more thorough comparison of Hb1Tn and Hb1Tb indicates that the structure of the two proteins in the T state is indeed very similar. On superposition of the tetramers, the rmsd is only 0.50 Å when computed on backbone atoms, and decreases to 0.36 Å and to 0.45 Å when the single α and β chains are compared. As it obvious from these figures, also the quaternary assembly is very similar in the two Hbs. The most significant difference lies in the much greater thermal fluctuation found for Hb1Tn, and in particular for the CD corner and for the E and F helices of the α chain. These differences are clearly evidenced by the plots in figures 3, where the B factors, averaged over the main chain atoms for the two Hbs, are reported as function of the residue number.

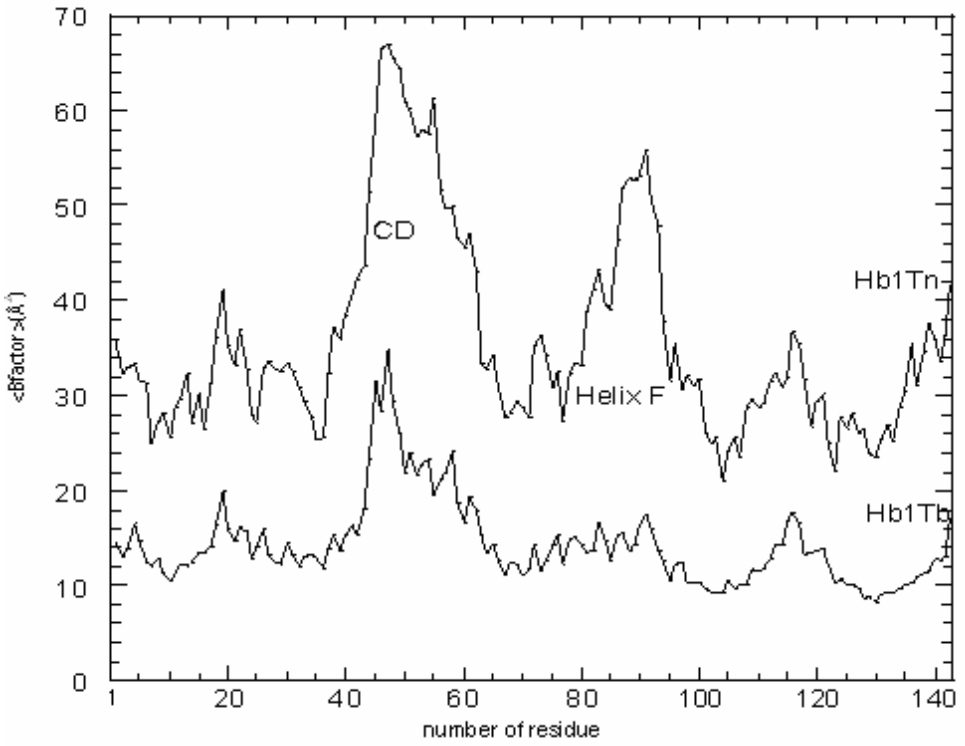

Fig 3A B factors, averaged over the main chain atoms for the two Hbs, are reported as function of the residue number for the α chains.

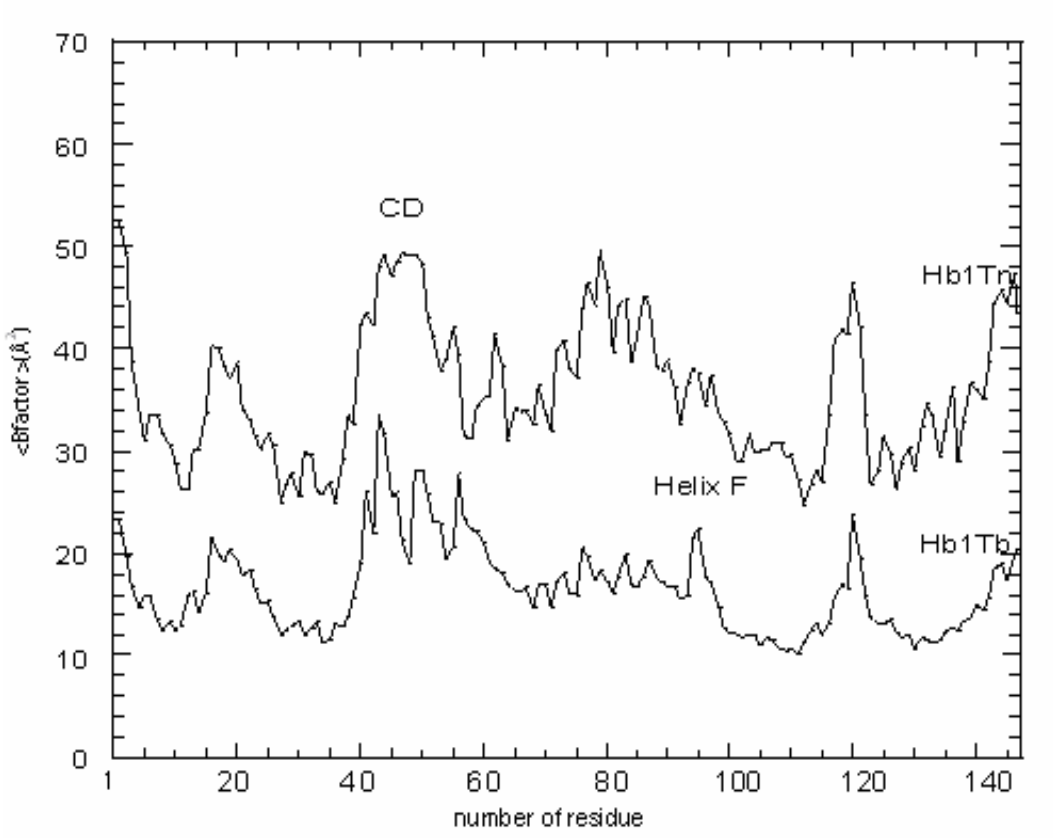

Fig 3B B factors, averaged over the main chain atoms for the two Hbs, are reported as function of the residue number for the β chains.

For a better understanding of these results, the analysis of the packing interactions in the two Hbs is also of interest. Despite the different space group symmetry ($P4_1$ for Hb1Tn and $P2_1$ for Hb1Tb), the two Hbs share a very similar packing organisation. Indeed, both structures are assembled through the stacking of layers parallel to the ac plane in Hb1Tb and to the ab plane in Hb1Tn: the layers are strictly isomorphous and have a practically exact C2 planar symmetry, with the twofold axis coincident with the molecular dyad axis (figure 4).

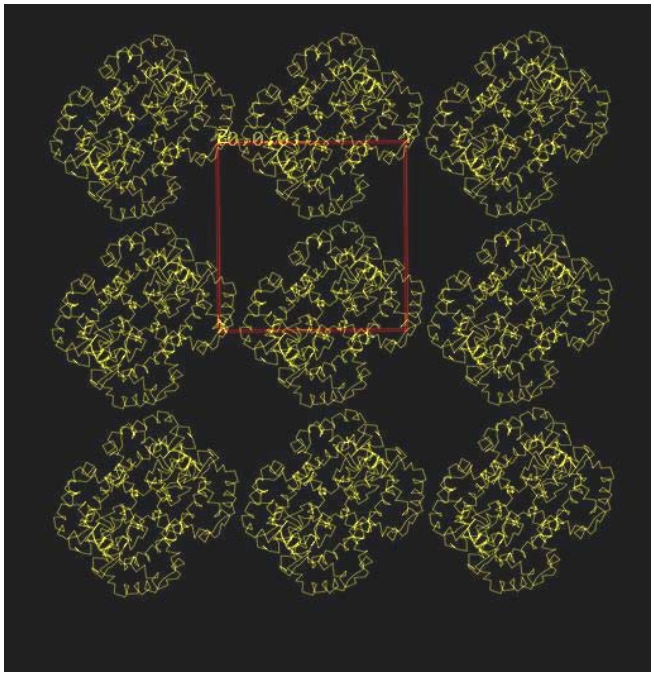

Fig 4 Layer organisation in the crystal packing of the Hb1Tn and Hb1Tb

Packing interactions within the layers are fully conservative in the two crystals. The orientation of the molecules is such that the CD corners of the α and β chain protrude out of the layer and mediate the inter-layer contacts. The differences between the two crystals arise from the manner the layers are repeated in the third direction: in Hb1Tn each layer is rotated 90° with respect to the previous one in the stacking, while in the Hb1Tb it is rotated of 180°. As a consequence, the packing contacts of the layers are different for each species. A comparison of the intra- and inter-layer packing contacts is reported in Tables III and IV, respectively. In the first case, the sequence number of the residues involved in the contacts is the same. Moreover, no mutation occurs in this region.

Table III

Residue	Atom	Residue	atom
Ala 8 α_1	O	Ser 9 β_2	OG
Arg 11 α_1	CB	Ser 12 β_2	OG
Arg11 α_1	NH2	His 17 β_2	NE2
Asp75 α_1	OD2	Asp 13 β_2	OD1

Table III Intra layer packing contacts.

Hb1Tn			
Residue	**atom**	**Residue**	**atom**
Asp 21β_1	O	Tyr 49 β_2	OH
Glu 83α_2	CG	Tyr 49 β_2	CD1
Glu 83α_2	OE1	Tyr 49 β_2	CD1
Glu 83α_2	O	Asn 47β_2	OD1
Glu 86α_2	CD	Asn 47β_2	CD1
Tyr 90α_2	CD1	Ser 56β_2	O
Asp 48α_2	CB	Glu 94β_2	O
Pro 47α_2	O	Thr 90 β_2	CG2

Hb1Tb			
Residue	**atom**	**Residue**	**atom**
Gln 87α_1	OE1	hemeα_2	O2A
Glu 83α_1	OE2	His 55α_2	E2
Pro 47α_1	CB	His 69α_2	NE2
Asn 47β_1	OD1	Glu 52β_2	OE2
Tyr 49β_1	OH	Glu 52β_2	OE2
Gln 87α_1	OE1	hemeα_2	O2A

Table IV Inter layer packing contacts for Hb1Tn and for Hb1Tb.

The inter-layer contacts for Hb1Tn involve the FG and CD loops for the α chain, and the CD loop for the β chain, whereas for Hb1Tb they involve the helices E and F in the α chain and the CD β loop. In this case, the contact regions involve residue that are mutated in the two Hbs.

5.3.5 Resonance Raman (RR) spectroscopy and microscopy.

The high frequency region (1300-1700 cm^{-1}) includes the porphyrin in-plane vibrational modes, that are sensitive to the electron density of the macrocycle, to the oxidation, coordination and spin state of the iron atom (13). The low- frequency region (200-450cm^{-1}) contains bands with contributions from deformation of the various interior angles of the porphyrin ring and of the peripheral substituents, as well as stretching of the bonds from the pyrrole N atoms to the central metal atom.

In the figure 4 are shown the spectra of low and high frequency for Hb1Tn, Hb1Tb and HbA.

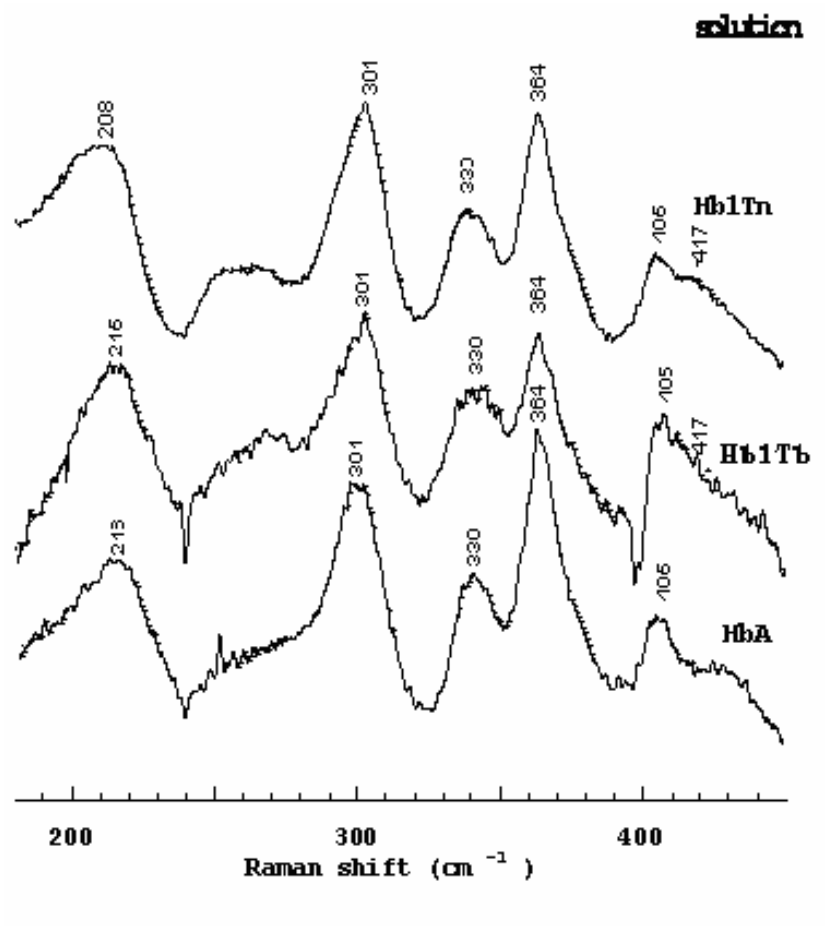

Fig 4A Resonance Raman spectra in solution in the low-wave number region of Hb1Tn, Hb1Tb and HbA.

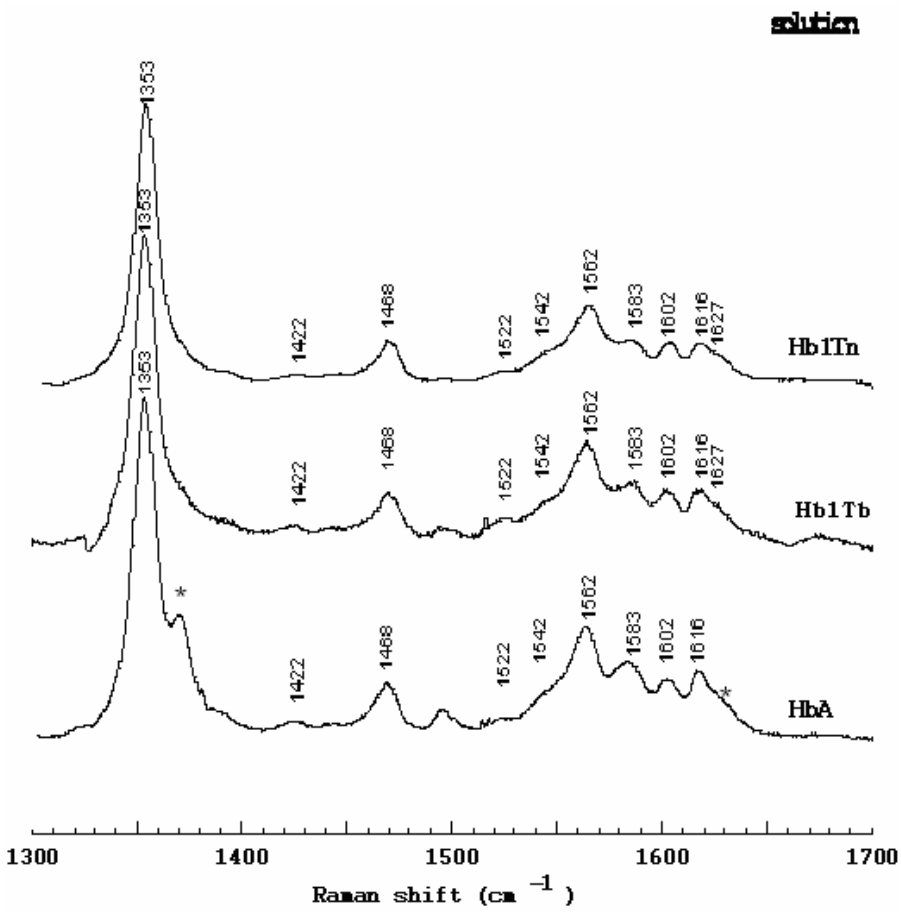

Fig 4B Resonance Raman spectra in solution in the high-wave number region of Hb1Tn, Hb1Tb and HbA.

The spectra are characteristic of a penta-coordinated high- spin haem state. However, unlike in HbA, there is evidence for a second ν(C=C) vinyl stretching mode (at 1625 cm^{-1}) in addition to that at 1617 cm^{-1}. Accordingly, in the low frequency region, two $\delta(C_\beta C_a C_b)$ bending modes of the vinyl substituents are observed at 405 and 412 cm^{-1} (14, 15).
Deoxy Hb1Tn displays an RR ν(Fe-Im) stretching mode at 208 cm^{-1} unlike the normal stretching mode of the ν(Fe-Im), that is usually observed at 215 cm^{-1}, as reported for the HbA, and measured for Hb1Tb. This data is confirmed also in the Raman spectrum of the crystal state (figure 5) of Hb1Tn, where this band is broader than that observed for the normal deoxy Hb.

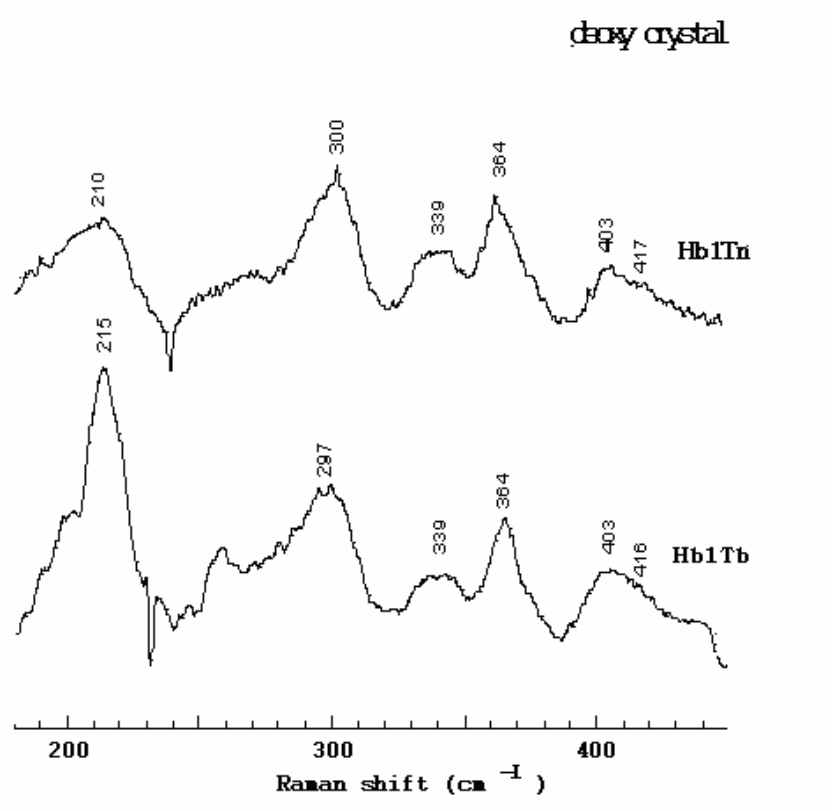

Fig 5A Resonance Raman spectra in the crystal state in the low-wave number region of Hb1Tn, Hb1Tb and HbA.

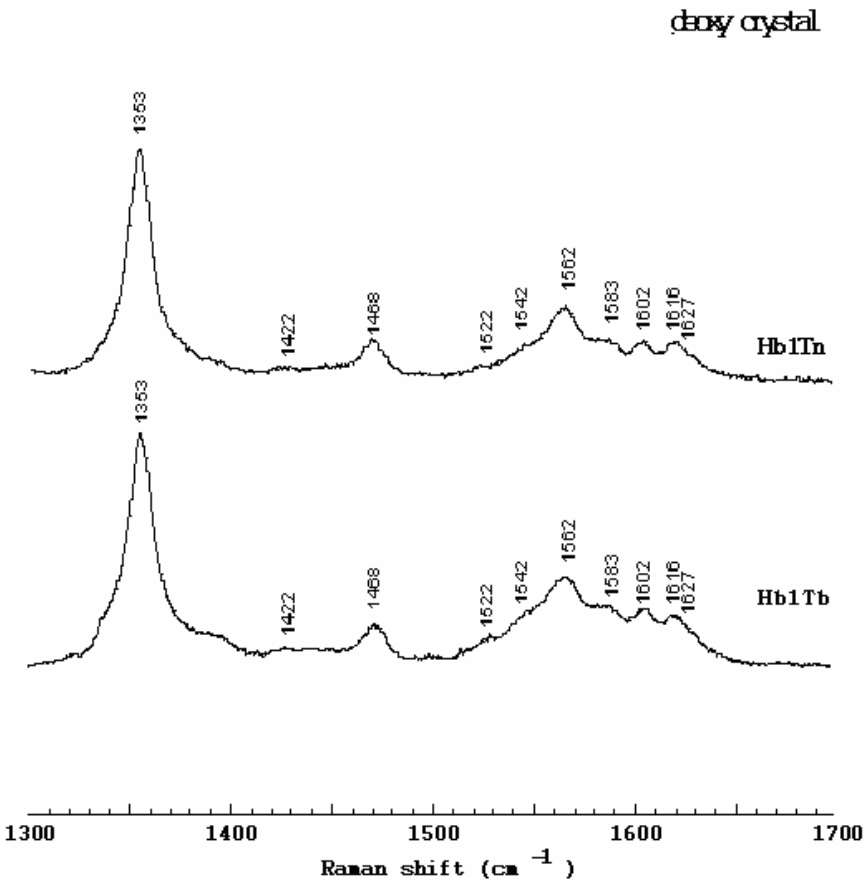

Fig 5B Resonance Raman spectra in the crystal state in the high-wave number region of Hb1Tn, Hb1Tb and HbA.

The downshift of the ν(Fe-Im) stretching mode is a confirmation of the crystallographic data, that indicate a distance of 2.48 Å between the Fe and the imidazole ring of the proximal histidine. In fact this distance is reflected on the vibrational mode and, consequently, there is a shift of the RR mode. Furthermore, the broad band of Hb1Tn in solution and as crystal, compared to Hb1Tb and HbA, suggests that a population of two distinct deoxy forms (corresponding to α and β haems) can be present.
TableV summarizes the wavenumbers and the assignements of the strongest RR bands odserved for Hb1Tn and Hb1Tb.

Table V Mode, simmetry and wavenumbers (ν) of the principal resonance Raman bands observed for deoxy Hb1Tn and Hb1Tb

Mode	Simmetry	Deoxy ν/cm^{-1}
ν(C=C)		1627
ν(C=C)		1618
ν_{10}	B_{1g}	1603
ν_{37}	E_u	1586
ν_2	A_{1g}	1562
ν_{11}	A_{2g}	1542
ν_{38}	E_v	1522
ν_3	A_{1g}	1470
$\delta(=CH_2)$		1422
ν_4	A_{1g}	1353
$\delta(C_\beta C_a C_b)$		415
$\delta(C_\beta C_a C_b)$		405
$\delta(C_\beta C_b C_d)$		364
ν_0		339
ν(Fe-Im)		216 (210)*

*Value in parenthesis corresponds to the Fe-Im mode for Hb1Tn

5.4 Discussion

In many cases the functional properties of a protein in its interaction with ligands can be sufficiently well understood on the basis of the 3D structure of the protein in the free and liganded states. In this respect the Hb molecule was emblematic. At the dawn of the protein structure characterization, the elucidation of the crystal structure of the unligated (deoxy) and ligated (oxy, carbomonoxy) forms provided a firm experimental anchorage for the elegant theory by Monod, Changeux and Wyman of the allosteric equilibrium between a tense T and a relaxed R states. Many properties of Hb could be fitted satisfactorily well in this theory and altogether they furnished a comparatively simple stereochemical model of the cooperative effects despite the complexity of the system. Surprisingly, the Root effect does not appear to be easily inserted in this framework. While the general features of the R and T states, first described for mammalian Hbs, hold very well for all fish Hbs, whose structure has been studied, the exaggerated pH dependence of the oxygen affinity displayed by some fish Hbs still await an adequate explanation. In this context, the structure similarity in both the liganded and unliganded state between the Root effect Hb1Tb and the non Root effect Hb1Tn is somewhat disarming. Their R structures are practically indistinguishable, and the basic features of the T structures, as for instance the hydrogen bond at the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) between Asp95α and Asp101β, are also unchanged. In principle these results should have been largely expected on the basis of their very high sequence identity. However, due to the differences in their functional behaviour (Bohr and Root effects), structural modifications at the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) interface, with the break of the aspartic-aspartic interaction, were also expected. This was indeed found to occur for trout HbI, whose oxygen affinity is practically insensitive to pH, confirming the importance of this unusual interaction for the Root effect.

In the case of the two Antarctic fish Hbs, a possible explanation may reside in other differences present in the T state. These are mainly related to the unusually large thermal fluctuations which characterise the α chain of Hb1Tn, and in particular the CD segment and the two consecutive E and F helices which bind the α haem moiety. In Hb1Tb, His55α was suggested to play an important role for the ordering of the

CD region, and also to contribute a Root proton. In Hb1Tn the replacement of this His with an Asn may well cause the high level of disordering displayed by this region, as well as the break of the hydrogen bond between His45α and one of the haem propionate. This hydrogen bond is present in both the T and R state of Hb1Tb, and in the R state of Hb1Tn. Thus, in going from the R to the T structure of the Hb1Tn, the break of this hydrogen bond should free a proton which would compensate the proton immobilised by the Asp-Asp bond. The analysis of the packing suggests that the disorder is a genuine molecular properties of Hb1Tn and not caused by a different crystal organisation with respect to Hb1Tb.

Alternatively, but less likely, it may be suspected that the T structure of Hb1Tn is in fact an artefact of the crystal packing, and that in solution it predominates a form in which the Asp-Asp hydrogen bond is broken.

As a third hypothesis somewhat related to the previous one, the insensitiveness of H1Tn oxygen affinity to pH may be the result of a long living deoxy intermediate, which ultimately evolves to the structure found in the solid state. Some interesting intermediates have been found in the study of the autoxidation pathway of Antarctic fish Hbs, which do present different structural features for Hb1Tb and Hb1Tn.

In all cases, the ambiguity in defining the structural determinants of the Root effect, suggests that more than one mechanism may operate in the various Hbs and that a more precise characterization of this properties requires a more systematic structural characterisation of selected mutants of the two proteins.

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Chapter 6

Conclusions

6.1 Conclusions

In the course of this PhD thesis tetrameric Hbs were studied, to characterise the oxidation process of these proteins and to determine the molecular basis of Root effect.

There is not yet an adequate explanation of the autoxidation pathway of Antarctic fish Hbs. It is not clear why the α and β subunits display a different oxidation process: the α subunit produces an aquo-met form and the β subunit forms an endogenous bis-histidine complex (β haemichrome). Therefore, one of the objective of this project was to express the β chain of Hb1Tn to verify the formation of the haemichrome and to establish whether it do assemble in a tetrameric form, as for istance in the β_4 variant of human HbA, or it rather stays as monomer.

Two vectors were constructed to express the Hb1Tn β chain: the vector pGEX, to express a Glutathione S-transferase fusion protein, and the vector pET22, to be used in *E. coli* and in *Arctic cells*. The latter system allows the co-expression of the protein with the cold-adapted chaperonins Cpn10 and Cpn60, from the psychrophilic bacterium *Oleispira antarctica*. The recombinant Hb1Tn β globin was obtained in soluble form by co-expression through GroEL/GroES, molecular chaperones of *E. coli*, involved in the correct folding of premature proteins in an ATP-dependent manner.

Nevertheless, the trials of refolding of Hb1Tn β chain with haem *in vitro* were not successful. The UV-VIS spectrum suggested that the haem was not incorporated correctly into β globins.

The β globin synthesized in the pET22 system, in both *E coli* BL21 and C41 cells, precipitated as inclusion bodies.

Finally, the Arctic cells produced only small amounts of recombinant protein, which could not be purified in reasonable yields.

Another objective of this PhD project was to investigate the molecular basis of the Root effect. For this purpose a first approach was the expression of Hb1Tn, the major Hb of *T. newnesi*.

The second strategy, undertaken to determine which residues are involved in the Root effect, was to study a natural mutant of human HbA, Hb *Potomac* (β101Glu→Asp).

Although the recombinant Hb1Tn was obtained in high amounts, it could not be obtained in soluble form. This result might be due to the temperature at which the expression took place, in fact Antarctic fish live at a significantly lower temperature than that preferred for *E.coli*.

The Hb *Potomac* was produced in high yields in *E. coli* using the pHE7 plasmid expression vector. The recombinant Hb was obtained in soluble form, in high yields and it was easily purified. The optical and Raman spectroscopic properties of Hb *Potomac* were similar to that of human HbA. These results suggest that the conditions used for bacterial expression promote correct folding and produce soluble Hb *Potomac.* Several trials of crystallisation were performed, however they did not lead to crystallisation of the protein.

Another objective described in this thesis has been the determination of the crystal structure of Hb1Tn, not endoved with the Root effect, in the T state. The crystal structure of Hb1Tn in the T state at pH 6 was obtained at 2.1 Å resolution and it was compared to that of Hb1Tb. Should be recall that the carbomonoxy form of the two Hbs, in the R structures, were found to be practically indistinguishable, also in the T state are indeed very similar. At the level of the features of the Root effect, it is difficult to explain the functional differences on the basis of the molecular structures. At least for the region important for the Root effect, the hydrogen bond at the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) between Asp95α and Asp101β, were also unchanged. Although the differences in their functional behaviour (the presence/absence of Bohr and Root effects) allow to suppose the presence of structural modifications at the $\alpha_1\beta_2$ ($\alpha_2\beta_1$) interface, the presence of Asp-Asp interactions should have been largely expected on the basis of their very high sequence identity.

In the case of the two Antarctic fish Hbs, a possible explanation of the diverse functional properties, may reside in other differences also present in the T state. These are mainly related to the unusually large thermal fluctuations which characterise the α chain of Hb1Tn, and in particular the CD segment and the two consecutive E and F helices, which bind the α haem moiety.

However, no clear and unequivocal explanation was found for the molecular and structural basis of the Root effect. The ambiguity in

defining the structural determinants of the Root effect suggests that more than one mechanism may operate in the various Hbs.

6.2 Suggestions for further research

In future research, a additional purification step should be performed for the Hb1Tn β globin, after which refolding of the protein *in vitro* in presence of haemin may have better chances to take place.
An other objective for further research should be to obtain highly purified Hb *Potomac* and to determine the crystal structure of the deoxy form, that should provide interesting information about the structural basis of the Root effect.
Moreover, different systems could be used for the expression of the Hb1Tn β globin and of the Hb1Tn , for example fission yeast (*S. pombe*), mammalian or insect cells.
Furthermore, it emerges clearly from the present results that a full understanding of the molecular basis of the Root effect requires a systematic structural characterisation of selected mutants of the Hb1Tn and Hb1Tb.