Structure and function of the globin and globin gene from the Antarctic mollusc *Yoldia eightsi*

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The mechanism of adaptation of haemoglobin from the Antarctic mollusc *Yoldia eightsi* to its low-temperature environment is a decrease in the oxygen affinity via an increased ligand-dissociation rate. At 2 °C this haemoglobin has an oxygen affinity similar to other haemoglobins at 25 °C. At 25 °C, *Yoldia* haemoglobin shows a low oxygen affinity, resembling that of human deoxyhaemoglobin. The mechanism involves a lower binding energy to oxygen, suggesting a loss or weakening of the usual hydrogen bond, leading to a higher oxygen-dissociation rate. However, *Yoldia* haemoglobin has the usual distal and proximal histidines, so the primary structure alone does not provide an obvious explanation for the low affinity. The CO-binding kinetics are biphasic, with the fraction of slow phase increasing at higher protein concentrations, indicating the formation of dimers or a

higher level of polymerization. The protein–protein interaction appears to be of hydrophobic nature, since it can be partially reversed by addition of ethylene glycol as co-solvent. While the CO-association rates differ by a factor of 10, the oxygen equilibrium data could be simulated with a single affinity. The *Yoldia* haemoglobin gene contains three introns, interrupting the coding region at position NA1.2, B12.2 and G7.0. The conservation of the B12.2 and G7.0 introns is in contrast with the unprecedented NA1.2 intron. Phylogenetic analyses reveal a gene tree where the *Yoldia* haemoglobin gene is separated from other mollusc globin genes, confirming the specific adaptation of the *Yoldia* haemoglobin.

Key words: gill haemoglobin, kinetics, temperature adaptation.

INTRODUCTION

The marine polar environment is very peculiar as far as some environmental parameters relevant to biological systems are concerned. For instance, the temperature of the sea in the Antarctic is very close to the freezing point of water and thermally stable during all the year, ranging from –2 to 2 °C. The low water temperature decreases the metabolic rate of the organisms in it [1,2] and increases the solubility of oxygen: at 0 °C the dissolved oxygen concentration is 1.6-fold higher than at 20 °C. The low temperatures of polar seas imply advantages for marine organisms as far as metabolic demands are concerned compared with those of animals from temperate waters: due to the lower metabolic rate and growth rate [3], the income of external energy can be mostly utilized for reproduction and growth rather than for organism maintenance [2].

The sediments of the sea in the Antarctic represent the usual life environment of the infaunal nuculanid bivalve *Yoldia eightsi*, which is typically sampled in the reduced muddy beds, but also inhabits the coarse sand beds mixed with gravel and sponge spicules [4]. The occurrence of siphons to inhale water allows for oxygen uptake from the upper oxygen-rich water layers overlying the sediments where the organism lives [5]. Although this species lives within a wide range of depths (5–700 m) [6,7], it experiences a very stable environment as far as the temperature and, hence, the oxygen concentration, are concerned. However, due to the lack of selective pressure with respect to the temperature, *Y. eightsi* is strictly stenothermal and cannot tolerate temperatures higher than +6 °C (E. Angelini, unpublished work).

Globins are sporadically distributed among molluscs, which commonly have haemocyanin as an oxygen carrier. They seem to be more common in species that become exposed to low oxygen partial pressure in Nature [8]. Haemoglobins (Hbs) are present in various tissues or in the blood of a number of molluscan species, in the latter case as intracellular or extracellular circulating Hbs. Tissue Hbs are usually found in the radular muscles, nerves and heart tissues of many gastropods and in the adductor muscles, heart and foot of many bivalves [9–11].

Intracellular Hbs located in gills have been found in bivalve molluscs only in few families inhabiting reduced sediments rich in hydrogen sulphide or the deep-sea hydrothermal vents [12–17]. They are found as monomeric, dimeric and tetrameric Hbs. Until now, the amino acid sequences of only the three gill Hbs of *Lucina pectinata* have been elucidated. It has been suggested that Hb may facilitate oxygen and sulphide supply to symbiotic bacteria [18]. No evidence for the presence of bacterial symbionts has been obtained for species of the genus *Yoldia*, such as *Yoldia limatula*, *Yoldia thraciformis* [14,19] and *Y. eightsi* [20].

In *Y. eightsi* two different oxygen-binding proteins were found: a haemocyanin in the haemolymph and an intracellular monomeric Hb of M_r approx. 16000 in the gills [21]. While the concentration of the former protein in the body fluid is so low as to have no biological role, the tissue concentration of Hb is very high (1700–2000 $\mu\text{mol} \cdot \text{kg}$ of wet tissue^{–1}). Upon standing in air, the purified Hb is prone to aggregation.

On account of the peculiar geographic distribution and habitat of *Y. eightsi*, the aim of this work was to study the kinetic binding properties of its gill Hb, to identify the amino acid sequence

Abbreviations used: Hb, haemoglobin; Mb, myoglobin.

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The nucleotide sequence for *Yoldia eightsi* globin has been deposited in the GenBank Nucleotide Sequence database under accession no. AF361744.

through protein and cDNA analysis, and to describe the structure of the gene encoding the globin. Primary structures of the Hbs and their DNA sequences could provide a better understanding of the relationships among molluscan globins, while the position of introns could help to solve questions of convergent and divergent evolution. On the other hand, ligand-binding properties are essential for understanding the internal function of the protein and its mechanisms of adaptation to different environments, in particular to habitats as extreme as the Antarctic.

MATERIALS AND METHODS

Biological material

Y. eightsi samples were collected, in Antarctica, during an Italian ENEA expedition (1995–1996) and during a British Antarctic Survey (Cambridge, U.K.) campaign (1996–1997).

Protein purification

Gills were excised and homogenized in 50 mM Tris/HCl, pH 7.5, containing 1 mM protease inhibitors. Hb was purified from the extract by gel filtration on a Superdex 75 column (Amersham Biosciences), equilibrated and run in 50 mM Tris/HCl/1 mM EDTA, pH 7.5, as described previously [21].

UV/visible absorption spectra

The spectra were acquired using an SLM-Aminco (DW2000) or HP 8354 diode array spectrophotometer. The absorbance difference between 560 and 569 nm was used to follow deoxygenation and oxygenation reactions. The oxidized Hb (met-Hb) forms were obtained by means of the addition of ferricyanide to the sample in 100 mM phosphate buffer at pH 7.4; the spectra of the oxidized form were measured with and without KCN. Oxygen equilibrium curves were measured on a Hemox analyser.

Kinetic measurements

The kinetic measurements were performed in 50 mM Tris/HCl buffer at pH 7.5, with 1 mM EDTA, at different temperatures ranging from 2 to 50 °C. The kinetics were followed by monitoring the absorbance at 436 nm. Association rates for oxygen and CO binding were determined using flash photolysis with 10 ns laser pulses at 532 nm (Quantel, Paris, France). Hb samples were equilibrated under 0.1 or 1 atm (10.13 and 101.3 kPa, respectively) of CO, or under air or pure oxygen. Oxygen-dissociation rate constants were obtained by analysing the replacement reaction by CO, under different mixed CO/O₂ atmospheres. CO off-rates were measured by adding ferricyanide (6 mM) to carboxy-Hb and following the reaction spectrophotometrically. Association equilibrium constants were also calculated from the ratio of the kinetic constants. The autoxidation rate was measured at 37 °C in 100 mM phosphate buffer, pH 7.4. The absorption changes were followed in the range 350–650 nm for 6 h. The data of absorbance versus time were fitted as a first-order reaction.

Mass determination

Electrospray mass spectra were recorded on a VG Quattro II triple quadrupole mass spectrometer (VG, Manchester, U.K.) as described previously [22,23].

Sequencing at the protein level

Purified Hb was cleaved by trypsin and CNBr. The resulting tryptic peptides were separated by reversed-phase HPLC on a C₄

column (Vydac; 25 cm × 4.5 cm) using a 0.1 % trifluoroacetic acid/acetonitrile gradient (0.1 % trifluoroacetic acid/5 % acetonitrile to 0.1 % trifluoroacetic acid/75 % acetonitrile in 45 min; flow rate, 1 ml/min). The resulting CNBr peptides were separated by SDS/PAGE followed by blotting on to PVDF membrane (ProBlott™ membranes, ABI; Caps buffer [3-(cyclohexylamino)propane-1-sulphonic acid], pH 11; 3 h; 50 V). Sequencing of the peptides was performed on a ABI 470-B sequencer as recommended by the manufacturer [22,23]. The blocked N-terminus was unblocked by heating the sample in 30 % trifluoroacetic acid at 55 °C for 3 h according to Fago et al. [24].

Sequencing at the DNA level

Two degenerate primers, YEF1 (5'-ATGACNGCNGAYATH-GAYGC-3') and YER1 (5'-TGRTAYTGNCCDATNCCRTT-3'), were constructed based on the obtained amino acid sequence. They are both 20-mers with redundancies of 192 and 384 respectively. Total RNA was isolated out of the gills using the Total Micro RNA isolation kit from Stratagene. A reverse transcriptase PCR was carried out using the degenerate primers. The amplified product was then cleaned and sequenced according to the dideoxy chain-termination method of Sanger.

Based on the obtained cDNA sequence, three specific primers were designed: the forward primer YEF2 (5'-CCATTGTAACAGCACTGTCTG-3') and the reverse primers YER2 (5'-CGGATGTCCTTTAAGAGCAG-3') and YER3 (5'-CTTGAATTTACTTTGGTAGTCGGG-3'). YEF2 and oligo-dT were used in a PCR to obtain the 3'-end of the cDNA. The PCR was carried out for 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. The amplified product was sequenced as described earlier. To obtain the 5'-end of the cDNA a rapid-amplification-of-cDNA-ends experiment was carried out (Life Technologies). First-strand cDNA was synthesized using YER2. A poly(C) tail was added to the 3'-end of the cDNA with terminal deoxynucleotide transferase. A PCR as described above was then carried out using an oligo-dG adaptor and the nested primer YER3. The sequence of the PCR product was subsequently determined.

Genomic DNA was isolated out of *Y. eightsi* foot tissue using the genomic DNA purification kit from Fermentas. The complete sequence of the *Y. eightsi* globin gene was determined by amplifying three overlapping fragments. A first PCR was set up using genomic DNA as a template and the specific primers YEF3 (5'-GGACTGGCTGTTTCTGCTC-3') and YER4 (5'-GTGGCCGTCCTCAAACTC-3'). It was carried out for 35 cycles of 1 min at 94 °C, 1 min at 53 °C and 5 min at 72 °C. A second PCR was set up for 35 cycles of 30 s at 94 °C, 1 min at 55 °C and 5 min at 72 °C with the primers YEF4 (5'-GCCATCATGAGTTTGTAGTCGG-3') and YER3. The third PCR to pick up the remainder of the gene was done using the primers YEF5 (5'-ACATTGTCTGTTCTCGCTTAGCTT-3') and YER5 (5'-GAAATGCGGTATCCAGCTGC-3') under the same conditions as for the second PCR. All amplified products were purified, cloned and sequenced.

Tree construction

Globin sequences were aligned according to the invertebrate globin template [25]. Based on the globin sequences, neighbour-joining trees [26] were constructed, using the computer programs TREECON [27]. The neighbour-joining trees were derived on the basis of distance matrices calculated using the formula of Poisson as implemented in TREECON, without taking gaps into account. The confidence of trees was tested by bootstrap analyses, running 1000 replicates. According to Hillis and Bull [28], nodes

Figure 1 Temperature dependence of oxygen binding to *Y. eightsi* Hb

Top panel: oxygen equilibrium coefficient (units, molarity). From the plot of $\ln(K_{O_2})$ versus $1/T$, an enthalpy (ΔH) of 10 kcal/mol was determined as compared with 15.5 (Hb A α chain) [29]. Bottom panel: Arrhenius plots for the oxygen-dissociation rate (units, M/s): $\ln(k_{O_2})$ versus $1/T$. The activation enthalpy ($\Delta H^\ddagger$) of 17 kcal/mol for *Y. eightsi* Hb is lower than that from other species, such as the human Hb A α chain (20.5 kcal/mol) [29].

are considered to be reliable if they have a bootstrap value of at least 70 %.

RESULTS AND DISCUSSION

Oxygen-binding characteristics of the *Y. eightsi* Hb

The UV/visible absorption spectra of the met-Hb and met-Hb-CN form were studied. The spectral features and the position of the absorption maxima are typical of other known Hbs and myoglobins (Mbs), having the usual absorption maxima at about 540 and 575 nm [29]. The band near 630 nm for the met-Hb form is less distinct than for other species, possibly indicating a lower pK value for the transition from the aquo-met to the hydroxy-met form.

The ferrous forms were also typical of other Hbs; as for sperm whale Mb there was a red shift in the Soret band of 2 nm relative to human Hb.

The rate and equilibrium constants for ligand binding to *Y. eightsi* Hb are listed in Table 1. It is worth noting that the oxygen k_{off} at 25 °C is very high compared with other Hbs at the same temperature. In fact, among the invertebrates, which display the

Table 1 Rates of ligand binding to *Yoldia* Hb and other representative Hbs

Protein	T (°C)	k_{on} ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{off} (s^{-1})	K_{O_2} ($= k_{on}/k_{off}; \mu\text{M}^{-1}$)	Reference
Oxygen binding					
<i>Yoldia</i> Hb	25	30	440	0.068	This study
<i>Yoldia</i> Hb	20	25	280	0.089	This study
<i>Yoldia</i> Hb	2	10	30	0.333	This study
<i>Lucina</i> Hbl		190	61	3.1	[30]
<i>Lucina</i> HbII		0.39	0.11	3.5	[30]
<i>Lucina</i> HbIII		0.29	0.075	3.8	[30]
<i>Glycera</i> II Hb		186	1800	0.103	[30]
<i>Ascaris</i> Hb		1.5	0.004	375	[33,44]
<i>Ascaris</i> Mb		1.2	0.23	5.2	[44]
Human Hb A α chain	25	40	50	0.8	[29]
Horse heart Mb	20	14	10	1.4	[29]
Sperm whale Mb		15	18	0.83	[11]
CO binding					
<i>Yoldia</i> Hb	20	1.9	0.065	29.23	This study
Horse heart Mb	20	0.5	0.017	29.41	[29]

most varied oxygen rate constants due to different molecular mechanisms, the values at this temperature range between 0.1 s^{-1} for *Lucina* gill Hb and 1800 s^{-1} for *Glycera* blood Hb [30]. Among the vertebrates, the oxygen k_{off} values of fishes are usually quite high, but if considered at their body temperature they have similar values to those of other vertebrates. However, the k_{off} for *Y. eightsi* Hb at 2 °C, the normal temperature in its Antarctic environment, is comparable with those of mammalian Mbs at 25 °C. The oxygen on rate is less influenced by temperature.

As such, the oxygen affinity is quite low, if compared with other Hbs from vertebrates and invertebrates at the same

Figure 2 CO-rebinding kinetics of *Y. eightsi* Hb

Fresh samples show predominantly the rapid CO rebinding (left-hand curve, 2 μM Hb). After aging (1 week) the same sample shows a significant amount of slow phase; the fraction of slow phase depends on the Hb concentration (series of curves on the right). If the co-solvent ethylene glycol (EGOH) is added to the aged sample, the fraction of slow phase decreases (data shown as dots are for 10 μM Hb in 20% ethylene glycol).

Figure 3 SDS/PAGE of *Y. eightsi* Hb preparations

Lane a, freshly prepared *Y. eightsi* Hb; lane b, *Y. eightsi* Hb kept in air for 24 h; lane c, M_r standard.

temperature, due to the high dissociation rate. Lower temperatures offset this effect, so the affinity at about 2 °C is similar to that of mammalian Hbs at 25 °C. In this manner, a good functionality of *Y. eightsi* Hb is achieved at the environmental temperature of Antarctica.

The temperature dependence of the oxygen equilibrium coefficient (Van't Hoff plot) and dissociation kinetics of oxygen (Arrhenius plot) are shown in Figure 1. The energies for *Y. eightsi* Hb are quite low (equilibrium enthalpy, $\Delta H = 10 \pm 1$ kcal/mol, and energy of activation, $\Delta H^\ddagger = 17 \pm 1$ kcal/mol), compared with other Hbs of vertebrates and invertebrates (human Hb A chain, $\Delta H = 15.5$, $\Delta H^\ddagger = 20.5$; Horse Mb, $\Delta H = 13.5$, $\Delta H^\ddagger = 19$; *Aplysia* Mb, $\Delta H = 14$, $\Delta H^\ddagger = 21$; *Physeter catodon*, $\Delta H = 15$; rat, $\Delta H^\ddagger = 21$) [29]. Studies on Hbs of fishes from low-temperature habitats did not report a lower activation energy [31]. The observed lower energy barrier of oxygen dissociation for the Antarctic *Y. eightsi* Hb might be due to many structural changes in the haem pocket such as a weaker hydrogen bond between a distal residue and the Fe–O₂ bond, if any, or due to the haem geometry. This is in contrast with the very high affinity of *Ascaris* Hb [32] or trematode Hbs [33], where there is an additional hydrogen bond.

The binding of CO is similar to that of mammalian Mbs. The CO equilibrium constant was calculated from the kinetic data at 20 °C; this value is in the range of CO affinity of other Mbs. As a result, the partition coefficient K_{CO}/K_{O_2} of 250 is very high compared with other Mbs, but similar to human Hb (Table 1).

In the kinetic experiments on CO binding two phases were observed. The amount of slow phase increases with protein concentration (Figure 2). Moreover, there was a dependence

Figure 4 Oxygen equilibrium curve at 25 °C for Hb *Yoldia*, presented as a Hill plot where Y is the fraction saturation and PO_2 is the partial pressure in oxygen (mmHg)

The data can be simulated as a single affinity (straight line) of 15 mmHg; the deviations of a few percent at the endpoints are within the limits of error of spectral normalization.

Figure 5 Correlation of oxidation rate and oxygen affinity

Plotted is the reciprocal of the oxidation rate at 37 °C versus the oxygen affinity at 25 °C. Natural proteins human Hb (Hb), sperm whale Mb (SW Mb), Hb *Yoldia* and FixL are shown. Various mutations of sperm whale Mb are also shown.

Sequence is reconstructed from relevant peptides obtained by CNBr (cnbr) and trypsin (trp) digestion and N-terminal deblocking (debloking) and is aligned with reference to Mbs with known tertiary structure. Positions of helices A–H in each species are shown above the sequence. Phys, *Physeter catodon* Mb, accession number P02185; Aplim, *Aplysia limacina* Mb, accession number GGGAA; Luc I, *Lucina pectinata* Hb I, accession number A61437; Sca I, *Scapharca inaequalivalvis* Hb I, accession number GGNKID [35].

At 37 °C, the autoxidation rate of *Y. eightsi* Hb (0.27/h) is four times higher than for other Mbs, in agreement with the current idea that low-affinity Hbs oxidizes more rapidly [34]. Hbs may also display an enhanced oxidation rate for reasons of protein stability or access of the pocket to water molecules [34]. Hbs from poikilotherms living at low temperature are not stable at 37 °C, the body temperature for homeotherms; other Hbs from Antarctic fishes, even having quite high oxygen affinity, display higher autoxidation rates [31]. *Y. eightsi* Hb did not display any stability problems (Figure 5).

Using existing templates, the *Y. eightsi* Hb sequence can be aligned with globins with known tertiary structure, including those from three molluscan species (*Aplysia limacina*, *Lucina pectinata* Hb I and *Scapharca inequivalvis* I; Figure 6), as well as with all other mollusc globin sequences available in our database

Figure 8 Schematic representation of known intron-insertion positions of different vertebrate and invertebrate globin genes

Helix notation is used to define the intron-insertion positions, e.g. B12.2 is the intron inserted after the second base of the codon of amino acid 12 on the B-helix and G7.0 is the intron inserted between the codons of amino acids 6 and 7 on the G-helix. NA means the amino acid sequence preceding the A-helix whereas HC means the amino acid sequence following the H-helix. Pc, precoding sequence.

Figure 9 Phylogenetic tree of molluscan globins

[11,42]. In *Y. eightsi* the presence of dimeric and/or polymeric globin genes is unknown. However, this does not exclude the possibility that the NA1.2 intron in *Y. eightsi* Hb might be a potential point for dimerization.

As in *Biomphalaria* Mb, the *Y. eightsi* Mb has no leader sequence. The NA1.2 intron therefore cannot be considered as a

‘displaced’ precoding intron separating the leader and coding sequence. Consequently it must be considered as a newly inserted intron, supporting the intron late hypothesis [38,39]. We therefore suggest that the globin gene family is a dynamic system wherein intron insertion and loss occur in addition to the conservation of evolutionarily ancient introns.

Phylogeny

A neighbour-joining tree constructed on the basis of 34 molluscan globin sequences is shown in Figure 9. Sperm whale Mb is used as an outgroup. Two major monophyletic groups, the Gastropoda and Bivalvia, can be seen. *Y. eightsi* however does not cluster with either of them. There are two things to consider when looking at these clusters. (i) The molluscan monophyly is not advocated by both small-subunit rRNA and globin-based trees [42,43]. (ii) Globin-based trees are a mixture of gene and species trees and include information on the evolution of the species as well as the gene. As such, Figure 9 displays a gene tree rather than a species tree, underlining the adaptation of *Y. eightsi* Hb to the extreme environment of Antarctica by means of divergent evolution.

Conclusions

The Hb from *Y. eightsi* displays an adaptive mechanism for its local environment near 0 °C. While the oxygen affinities of haemoproteins are highly sensitive to temperature, the actual affinity at any given temperature can be regulated by several orders of magnitude [33]. For *Y. eightsi* Hb a decrease in the oxygen-dissociation rate compensates the temperature effect, in order to maintain a physiological affinity. The large increase in oxygen off-rate, accompanied by a lower binding energy, suggests a significant change in binding interactions. Yet the usual distal and proximal histidines are conserved, suggesting only tertiary effects, as occur for human Hb in the transition from the high- to the low-affinity (deoxy) conformation. The presence of an unprecedented intron (NA1.2) and a comparison with other molluscan Hb sequences underlines the peculiarity of this Antarctic Hb.

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