Structure and function of the Gondwanian hemoglobin of *Pseudaphritis urvillii*, a primitive notothenioid fish of temperate latitudes

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Abstract

The suborder Notothenioidei dominates the Antarctic ichthyofauna. The non-Antarctic monotypic family Pseudaphritidae is one of the most primitive families. The characterization of the oxygen-transport system of euryhaline *Pseudaphritis urvillii* is herewith reported. Similar to most Antarctic notothenioids, this temperate species has a single major hemoglobin (Hb 1, over 95% of the total). Hb 1 has strong Bohr and Root effects. It shows two very uncommon features in oxygen binding: At high pH values, the oxygen affinity is exceptionally high compared to other notothenioids, and subunit cooperativity is modulated by pH in an unusual way, namely the curve of the Hill coefficient is bell-shaped, with values approaching 1 at both extremes of pH. Molecular modeling, electronic absorption and resonance Raman spectra have been used to characterize the heme environment of Hb 1 in an attempt to explain these features, particularly in view of some potentially important nonconservative replacements found in the primary structure. Compared to human HbA, no major changes were found in the structure of the proximal cavity of the α -chain of Hb 1, although an altered distal histidyl and heme position was identified in the models of the β -chain, possibly facilitated by a more open heme pocket due to reduced steric constraints on the vinyl substituent groups. This conformation may lead to the hemichrome form identified by spectroscopy in the Met state, which likely fulfils a potentially important physiological role.

Keywords: Antarctica; fish; *Pseudaphritis urvillii*; hemoglobin; Bohr/Root effects; evolution; electronic absorption spectroscopy; Resonance Raman spectroscopy; molecular modeling

Antarctica was once part of the supercontinent Gondwana, which began fragmentation 135 million years ago. The continental drift carried Antarctica close to its present position about 65 million years ago. The physicochemical features of the Antarctic marine environment have been gradually

changing over the past 30–40 million years. The continent became completely isolated 22–25 million years ago due to the opening of the Drake Passage. This event generated the Circum-Antarctic Current and the development of the Antarctic Polar Front. With the reduction of heat exchange from northern latitudes, cooling of the environment proceeded to the current extreme conditions.

Diversification of the teleost perciform suborder Notothenioidei, largely confined within Antarctic and sub-Antarctic waters, has occurred in parallel with the climatic

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changes. Notothenioidei are the dominant component of the Southern Ocean fauna; they account for 96 of the 213 benthic species of the continental shelf and upper continental slope, and 122 (this figure includes non-Antarctic species) of the 313 Southern Ocean species described to date (Gon and Heemstra 1990; Eastman 2000). Information on the place of origin and existence of a transition fauna is lacking. Indirect indications suggest that notothenioids appeared in the early Tertiary (Eastman 1993) and began to diversify in isolation on the Antarctic continental shelf in the middle Tertiary, gradually adapting to progressive cooling. The ancestral notothenioid stock probably arose as a sluggish, bottom-dwelling teleost species that evolved some 40–60 million years ago in the shelf waters (temperate at that time) of the Antarctic continent. Molecular phylogeny has recently begun to provide indications about the time of radiation in the Antarctic.

The initial divergence of some of the families took place during the early fragmentation of Gondwana. At this time stocks appear to have become established in brackish water on isolated continental blocks (e.g., Pseudaphritidae in Australia). With the local extinction of most of the temperate Tertiary fish fauna as the Southern Ocean cooled, the suborder experienced extensive radiation, dating from the mid-Miocene, approximately 7–15 million years ago (Bargelloni et al. 1994), that enabled it to exploit the diverse habitats provided by a now chronically freezing marine environment.

According to a recently revised classification (Balushkin 1992; Pisano et al. 1998), Bovichtidae, Pseudaphritidae, Elegendinopidae, Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae, and Channichthyidae are the families of the suborder. Notothenioids are red-blooded except Channichthyidae (Ruud 1954), the only known adult vertebrates whose blood is devoid of hemoglobin (Hb). Some species of Bovichtidae and Nototheniidae also inhabit waters north of the Antarctic and sub-Antarctic, as well as monotypic Pseudaphritidae and Elegendinopidae.

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

The Hb system of temperate fish is often characterized by multiple, functionally distinct Hbs, probably required to cope with environmental variability. Temperate habitats with constant physicochemical features are inhabited also by species having a single major component (di Prisco and Tamburrini 1992). Notothenioids differ from temperate and tropical species in the reduced Hb concentration and multiplicity; Hb-less Channichthyidae represent the extreme of this trend. Antarctic red-blooded notothenioids are mostly bottom dwellers and have a single major Hb (Hb 1, accounting for 95%–97% of the total), often accompanied by a

minor component Hb 2 (di Prisco 1998), both displaying strong Bohr (Riggs 1988) and Root (Brittain 1987) effects, with few exceptions. Hb 1 and Hb 2 always have one of the chains in common. The minor components Hb 2 often differ from Hb 1 precisely in the residues found only in the Antarctic species. On the basis of these observations, and taking into account the fact that in all Antarctic notothenioids Hb 2 is functionally indistinguishable from the major components, we have hypothesized that in adult fish the minor components are vestigial or larval remnants. Hb 2 is indeed one of the dominant components in the juvenile stage (G. di Prisco, unpubl.), still synthesized in limited amounts by adult fish. Hb 1 and Hb 2 would bear very different evolutionary pressures, on one hand reflecting the needs that larvae and juveniles have to survive, and on the other, the much more refined adjustments developed by adult fish.

This communication reports the structure and function of Hb 1 of the non-Antarctic notothenioid *Pseudaphritis urvillii* (common name, Congolli) of the monotypic family Pseudaphritidae. Comparison between cold-adapted and noncold-adapted species is a useful approach to understanding the fish evolutionary history, as well as the molecular mechanisms of cold adaptation. In fact, *P. urvillii* is a noncold-adapted catadromous species from coastal waters, estuaries, and rivers of Southern Australia. Most likely, the ancestors of modern *P. urvillii* became associated with the Australian component of Gondwana 40–50 million years ago. The fragmentation of Gondwana and separation of Australia from Antarctica is likely to be the vicariant event that led to the speciation of *P. urvillii* (Bargelloni et al. 2000). It is euryhaline and may migrate upstream as far as 120 km from the sea (Andrews 1980). Because of its geographical origin, this Gondwanian species is especially important for calibrating the evolution molecular clock in Antarctica.

Besides the lifestyle, some features differentiate this species from cold-adapted Antarctic notothenioids, for example, it has glomerular kidneys (Eastman 1993) and has neither antifreeze glycoproteins nor the genes encoding antifreeze glycoproteins (Cheng et al. 2003). Other features are shared with cold-adapted species of the suborder. In freshwater, it is almost neutrally buoyant (Eastman 1993). Similar to most notothenioids, *P. urvillii* has a major (Hb 1) and a minor (Hb 2) component, in the typical notothenioid proportions and having the α -chain in common. The primary structure of Hb 1 has high identity with Antarctic globins; however, the values are at the lower end of the range. Hb 1 shows several nonconservative substitutions, some of which may alter the geometry of the invariably hydrophobic heme pocket.

Hb 1 displays strong, effector-enhanced, Bohr and Root effects. (The oxygen-binding properties of the minor component Hb 2 will not be discussed in detail, in view of the physiologically irrelevant levels of this component, and of

the similarity with the functional features of Hb 1, shown by appropriate key experiments.) Some of the functional features of Hb 1, namely the oxygen affinity and subunit cooperativity, appeared exceptional. Molecular modeling, electronic absorption and Raman spectroscopy, powerful techniques that provide information about the overall environment of the heme pocket, were used in an attempt to shed light on possible links between these features and the molecular structure of the heme.

Results

Purification of Hbs and separation of globins

Similar to most Antarctic notothenioids (di Prisco et al. 1991), ion-exchange chromatography of the hemolysate showed the presence of Hb 1 and Hb 2. The major component, Hb 1, accounts for over 95% of the total. Hb 1 and Hb

2 have different β -chains (indicated as β^1 and β^2 , respectively), a feature shared with only one Antarctic notothenioid, the bathydraconid *Cygnodraco mawsoni* (Caruso et al. 1991); Hb 1 and Hb 2 of all the other species differ by the α -chains.

Primary structure

The sequences of the α - and β -chains (142 and 146 residues, respectively) which constitute *P. urvillii* Hb 1 and Hb 2 are reported in Figure 1. The functionally important residues suggested by Perutz and Brunori (1982) to be involved in the molecular mechanism of the Bohr and Root effects in fish Hbs (Ser F9, Glu FG1, Gln HC1, and His HC3) are all conserved in the β -chains. The sequences of the two chains (α and β^1) of Hb 1 have higher similarity with major Hbs of Antarctic notothenioids (73%–82%, Table 1) than with minor Hbs of the same species (64%–68%). In contrast, the β^2

Figure 1. Amino acid sequences of the α - and β -chains of *P. urvillii* Hbs. The α -chain is shared by Hb 1 and Hb 2. β^1 , Hb 1; β^2 , Hb 2. In the β -chains, the similar residues are indicated in gray boxes.

Table 1. Sequence identity (%) between the globins of Hb 1 of a selection of species of Notothenioidei (di Prisco 1998)

	<i>P. urvillii</i>	<i>N. ang.^a</i>	<i>P. ant.</i>	<i>G. acut.</i>	<i>C. maw.</i>	<i>A. mit.</i>	<i>T. bern.</i>	<i>T. newn.</i>
α-Chain								
<i>Notothenia coriiceps</i>	80	99	95	82	83	83	89	87
<i>Trematomus newnesi</i>	78	94	89	92	90	90	97	
<i>Trematomus bernacchii</i>	79	96	92	91	91	90		
<i>Aethotaxis mitopteryx</i>	73	91	88	84	84			
<i>Cygnodraco mawsoni</i>	78	89	87	93				
<i>Gymnodraco acuticeps</i>	76	89	85					
<i>Pleuragramma antarcticum</i>	81	94						
<i>Notothenia angustata^a</i>	78							
β-Chain								
<i>Notothenia coriiceps</i>	77	93	86	80	88	82	90	86
<i>Trematomus newnesi</i>	77	85	84	80	84	83	93	
<i>Trematomus bernacchii</i>	82	91	90	83	87	86		
<i>Aethotaxis mitopteryx</i>	77	84	84	77	80			
<i>Cygnodraco mawsoni</i>	75	87	82	85				
<i>Gymnodraco acuticeps</i>	75	81	80					
<i>Pleuragramma antarcticum</i>	77	88						
<i>Notothenia angustata^a</i>	81	—						

^a Temperate notothenioid.

chain of Hb 2 shows a reverse correlation (70%–77% with major Hbs and 80%–88% with minor Hbs). In comparison with Hb 1 of other notothenioids, the primary structure of Hb 1 reveals several nonconservative substitutions. In the α -chain, Glu and Met replace Leu in F7 and FG3, and Thr replaces Lys in E10 and Ala in E6; in the β^1 chain Cys replaces Leu in B13. It is worth noting that Cys is also present in B13 of *Trematomus bernacchii* Hb.

Phylogenetic positions of *P. urvillii* globins

The trees inferred from the α - and β -globin data sets are shown in Figure 2, A and B, respectively. The same topologies were recovered with the two methods used for phylogenetic analysis (i.e., Local Rearrangement and exhaustive search). In both trees, the globins of major and minor Antarctic fish Hbs cluster in two separate, strongly supported groups, with the globins of temperate fish Hbs forming the most ancestral clade. The α -chain of *P. urvillii* Hb 1 and Hb 2 branches off the clade of the major Antarctic Hbs, and the same applies to the β -chain of Hb 1. The β -chain of Hb 2 places itself in a basal position with respect to the clade of the Antarctic minor Hbs.

Oxygen-binding properties

The oxygen-binding properties were investigated (Fig. 3) in the absence and presence of allosteric effectors such as protons, chloride ions, and organophosphates (ATP and IHP). The effect of IHP, although not physiologically relevant, was investigated because it is known that this heterotropic

ligand, possessing a high number of negative charges, may strongly stabilize the low-affinity T state, within the framework of the two-state allosteric model (Kister et al. 1987; Robert et al. 1988).

As shown in Figure 3C, where oxygen saturation at atmospheric pressure is reported as a function of pH, Hb 1 is characterized by a marked Root effect; at a specific pH value, the oxygen release induced by protons is enhanced by ATP. This is not a novel finding, but once again it highlights the physiological role of anions and in particular of organophosphates which, besides modulating the oxygen affinity, appear to finely control the region of pH in which the Root effect is operative. This is even clearer in Figure 3A, where the oxygen-binding experiments, performed in the absence and presence of allosteric effectors, are illustrated in terms of $\log p_{50}$ as a function of pH. The Bohr effect is strongly enhanced by organophosphates; in particular, going from stripped conditions to 3 mM ATP or IHP, the Bohr coefficient ($\phi = \Delta \log p_{50} / \Delta \text{pH}$), which denotes the average number of protons bound upon heme oxygenation, is increased, respectively, by about 150% and 80%. The effect of chloride is of the same order of magnitude as that observed in human HbA. However, in Hb 1 from *P. urvillii*, the presence of chloride influences the pH dependence of both oxygen affinity (Fig. 3A) and cooperativity (Fig. 3B).

In comparison with Antarctic notothenioids, Hb 1 displayed a much higher oxygen affinity. In the absence of effectors, $\log p_{50}$ remained below zero in the pH range 8.7–7.5. In addition to this remarkable difference, the oxygen affinity and cooperativity in the presence of chloride and ATP at pH 6.3 and pH 8.7 call for special attention. At

Figure 2. (Continued on next page)

alkaline pH the oxygen affinity was very high (0.90 mmHg) and cooperativity almost absent, whereas at pH 6.3 the oxygen affinity was very low (102 mmHg) and cooperativity completely abolished. This unusual modulation of cooperativity by pH is clearly depicted by the bell-shaped curve of the Hill coefficient (Fig. 3B), in which n_{50} reaches 2.0–2.5 at intermediate values, approaching one at both extremes of pH.

The stripped hemolysate had oxygen-binding features reflecting those of the major component Hb 1 (Fig. 3D,E,F). Whole blood and intact erythrocytes were assayed in the absence of effectors, and also showed strong Bohr and Root effects (not shown).

Thermodynamics of oxygen binding

The temperature dependence of oxygen-binding equilibria of stripped hemolysate and Hb 1, was investigated in the range 10°–16°C (Table 2). Under all experimental conditions, that is, in the absence and presence either of chloride alone, or of both chloride and organophosphates, the highest value (in absolute terms) of the enthalpy change of oxygenation was at pH 8.7, where Hb 1 may be considered locked in the high-affinity R state. At lower pH values, the overall enthalpy change progressively decreased as a function of increasing proton concentration, due to the endothermic

Figure 2. Trees resulting from maximum likelihood analysis of α - (A) and β -chains (B) of Antarctic and non-Antarctic fish Hbs. Trees were inferred as described in Materials and Methods. Maximum likelihood scores are $\ln L = -2014.68$ for the α -tree (A) and $\ln L = -2468.27$ for the β -tree (B). Numbers in bold are bootstrap values calculated as the sum of the bootstrap of all the topologies showing the node being analyzed by the RELL method. Constrained nodes are marked by asterisks. Numbers in normal typing refer to the bootstrap values obtained by Local Rearrangement analysis. The numbers near the taxon names are the SWISS-PROT accession numbers of the globin sequences. Additional sequences are from Stam et al. 1997. The species used are *Trematomus newnesi*, *T. bernacchii*, *Notothenia coriiceps*, *N. angustata*, *Pagothenia borchgrevinki*, *Gobionotothen gibberifrons*, *Aethotaxis mitopteryx*, *Pleurogramma antarcticum*, *Cygnodraco mawsoni*, *Gymnodraco acuticeps*, *Electrophorus electricus*, *Cyprinus carpio*, *Carassius auratus*, *Oncorhynchus mykiss*, and *Chrysophrys auratus*.

contribution of the heterotropic effectors, released upon oxygen binding, and of the allosteric transition. At pH 6.3 the overall enthalpy change of the low-affinity T state was measured. Under all conditions, the difference in enthalpy change of oxygenation of the two states was 10–12 kcal · mole⁻¹. The value at pH 6.6 in the simultaneous presence of chloride and ATP was of special interest. In this case, the enthalpy change was positive (i.e., endothermic),

indicating that oxygen release in the tissues is favored by low temperature. On the grounds of the primary structure analysis, a hypothesis on the binding of chloride will be discussed below.

ΔH was also measured in the unstripped hemolysate, containing physiological ATP concentration (not shown). As expected, the values were much less negative than those of mammalian Hbs.

Figure 3. Oxygen-equilibrium isotherms (Bohr effect), subunit cooperativity, and oxygen saturation at atmospheric pressure (Root effect) as a function of pH of Hb 1 (A–C, respectively) and stripped hemolysate (D–F, respectively). (A,B,D,E) 100 mM HEPES at 10°C, in the absence of effectors (filled circles); in the presence of 100 mM NaCl (open circles); 100 mM NaCl, 3 mM ATP (open triangles); and 100 mM NaCl, 3 mM IHP (filled triangles). (C,F) 100 mM HEPES at 10°C, in the absence of effectors (filled circles) and in the presence of 3 mM ATP (open triangles).

Spectroscopic characterization

Spectroscopy was the first approach aimed at finding a structural explanation to the striking oxygen-binding features. The electronic absorption and resonance R (RR) spectra of Hb 1 are shown in Figures 4 and 5, respectively.

The spectra of deoxy Hb 1 at pH 6.2 are similar to those of human deoxy HbA (Perutz et al. 1974b; Spiro and Strekas 1974) and characteristic of a pentacoordinate high-spin (5c HS) heme state. However, unlike in HbA, there is evidence for a second $\nu(\text{C}=\text{C})$ vinyl stretching mode (at 1625 cm^{-1}) in addition to that at 1617 cm^{-1} (Fig. 5). Accordingly, in the low-frequency region, two $\delta(\text{C}_\beta\text{C}_\alpha\text{C}_\beta)$ bending modes of the vinyl substituents at 405 and 412 cm^{-1} are observed (not shown). Deoxy Hb 1 displays an RR

$\nu(\text{Fe-Im})$ stretching mode at essentially the same frequency (215 cm^{-1}) as in deoxy HbA (Nagai et al. 1980), suggesting a largely unchanged proximal cavity in *P. urvillii* Hb 1 compared to human HbA.

In oxy Hb 1, the 2-nm blue-shift of the electronic absorption maxima at pH 7.7 (Fig. 4), compared to those reported for oxy HbA at neutral pH (Antonini and Brunori 1971), and the very weak band at 630 nm indicate that a small amount of Met-Hb 1 is present (see below). The RR bands at 1469 and 1478 cm^{-1} , assigned to 5c HS (deoxy form) and hexacoordinate high spin (6c HS) (Met form), respectively, result from photolysis of the FeO_2 bond induced by the laser irradiation. The formation of the Met form at the expense of the oxy upon laser exposure is confirmed by a slight Soret-band blue-shift after the RR experiment. The $\nu(\text{Fe-O}_2)$

Table 2. Overall oxygenation enthalpy change (ΔH)^a of *P. urvillii* hemolysate and Hb 1

pH	6.29	6.61	7.0	7.46	8.11	8.71	9.4
Stripped hemolysate							
Without effectors	-8.74	-1.72	-4.94	-4.53	-18.28	-14.30	
100 mM NaCl	-7.71	-4.65	-5.77	-3.41	-12.47	-15.49	
100 mM NaCl, 3 mM ATP	-0.66	-0.02	-4.05	-3.86	-10.33	-10.67	-11.90
Hb1							
Without effectors	-8.74	-7.45	-7.38	-26.23	-3.97	-18.74	
100 mM NaCl	-3.09	-7.98	-10.76	-6.46	-12.47	-15.49	
100 mM NaCl, 3 mM ATP	-3.61	+5.88	-0.80	-4.38	-8.38	-14.33	

^a ΔH values are in kcal · mole⁻¹ O₂ (1 kcal = 4.184 kJ)

stretching mode was observed (not shown) at a frequency (568 cm⁻¹) very close to that of HbA (Robert et al. 1988).

As the frequencies of the Fe–CO complex are more sensitive to the properties of the heme cavity than those of the oxy form, carbomonoxy Hb 1 was also studied. The electronic absorption spectrum of the CO complex is characterized by bands at 419, 537, and 567 nm (not shown), very similar to human HbA–CO (Antonini and Brunori 1971). The frequencies of the $\nu(\text{Fe–C})$ and $\nu(\text{C=O})$ stretching modes at 502 and 1952 cm⁻¹, respectively (not shown), differ only slightly from those of the corresponding bands of carbomonoxy HbA (507 cm⁻¹ and 1951 cm⁻¹, respectively; Tsubaki et al. 1982). Hence, again any variation in the properties of the heme cavities of the two proteins must be of limited extent.

The electronic absorption spectrum of human Met-HbA at pH 6.5 is characterized by bands at 405, 500, 540, 580, and 631 nm, indicative of a dominant aquo 6c HS state in

equilibrium with a hydroxy hexacoordinate low-spin (6c LS) state (Perutz et al. 1974a). In contrast, Met-Hb 1 at pH 6.1 displays a mixture of HS and LS heme spin states, in which the LS form constitutes a significant component (Fig. 4). Furthermore, the ~525-nm (β -band) and 560-nm (α -band) wavelengths are consistent with an LS species resulting from a hemichrome, rather than a hydroxide complex (Smulevich et al. 1991). In agreement with the principal features of the electronic absorption spectrum, the high-frequency RR spectrum of Met-Hb 1 (Fig. 4) reveals a mixture of 6c HS (1478 [ν_3], 1512 [ν_{38}], 1560 [ν_2], and 1578 [ν_{37}] cm⁻¹) and 6c LS (1508 [ν_3], 1578 [ν_2], 1604 [ν_{37}], and 1638 [ν_{10}] cm⁻¹) species. As observed for the other *P. urvillii* Hb forms, two vinyl stretching modes are evident at 1620 and 1627 cm⁻¹.

At pH 9.2 the absorption spectrum of Met-Hb 1 red-shifts compared to that at pH 6.1 (the band maxima are at 409, 536, 570, and 630 nm, not shown). This is consistent with an increased proportion of LS species compared to pH 6.1, as confirmed by the corresponding RR spectrum (not shown). Slight differences are evident particularly in the visible region compared to the spectrum of HbA at alkaline pH (George et al. 1964; Feis et al. 1994), consistent with *P. urvillii* Hb 1 at pH 9.2 being a mixture of the hydroxide LS complex characteristic of HbA at alkaline pH, a hemichrome LS species, and a small amount of aquo 6c HS heme. The transition to the hydroxide complex could not be followed to completion as the protein was denatured at pH values higher than 9.2.

The complex formed between Met-Hb 1 and fluoride at pH 5.6 gives rise to electronic absorption and RR spectra (Figs. 4,5) typical of a 6c HS state, indicating that fluoride binds strongly to *P. urvillii* Hb 1. However, the presence of a weak band at ~1510 cm⁻¹ (ν_3 of LS) suggests that fluoride does not fully bind to the protein. The absorption spectrum is similar to that of HbA (Perutz et al. 1974a) except for a 2-nm Soret blue-shift in the fluoride adduct, consistent with the presence of two vinyl stretching modes in the RR spectrum (at 1620 and 1637 cm⁻¹) and, therefore, a lower degree of vinyl conjugation with the porphyrin macrocycle (Neri et

Figure 4. Electronic absorption spectra of Hb 1. The Met-F complex at pH 5.6 in 5 mM citrate-5 mM MES, the Met form at pH 6.1 in 50 mM MES, the oxy form at pH 7.7 in 125 mM Tris-HCl, and the deoxy form at pH 6.2 in 50 mM MES. The visible region is expanded sixfold.

Figure 5. Resonance Raman spectra of Hb 1. Experimental conditions: 5 cm^{-1} resolution; 406.7 nm excitation, (1450–1595 cm^{-1}) 2 sec/0.5 cm^{-1} and (1595–1660 cm^{-1}) 11 sec/0.5 cm^{-1} collection interval, 10 mW laser power at the sample (Met-F); 406.7 nm excitation, 11 sec/0.5 cm^{-1} collection interval, 10 mW laser power at the sample (Met); 413.1 nm excitation, (1440–1590 cm^{-1}) 8 sec/0.5 cm^{-1} and (1590–1660 cm^{-1}) 26 sec/0.5 cm^{-1} collection interval, 10 mW laser power at the sample, a cylindrical lens was used to obtain the spectrum (oxy); 413.1 nm excitation, 10 sec/0.5 cm^{-1} collection interval, 25 mW laser power at the sample (deoxy).

al. 1997). It is worth stressing that only one vinyl band is present in the corresponding spectrum of the HbA-F complex (Choi et al. 1982).

Thus, the spectroscopic analysis of Met-Hb 1 provides evidence for hemichrome formation and supports the presence of two vinyl stretching modes.

Molecular modeling

Another approach aimed at correlating the structure of the heme pocket with the oxygen-binding properties was molecular modeling. Models of α - and β -chains of *P. urvillii* Hb 1 were constructed to allow visualization of the heme environment in deoxy, carbomonoxy, and oxy forms. Deoxy and carbomonoxy models were based on the X-ray coordinates of *T. bernacchii* Hb, whereas the crystallographic structure of human HbA was used as template for the oxy model. Detailed analysis of the final models was performed in comparison to human HbA and *T. bernacchii* Hb crystallographic structures, including those of HbA under low salt conditions (PDB codes 1bbb and 1hbb). *T. bernacchii* Hb displays the highest sequence identity with *P. urvillii* Hb 1 (79% and 82% for the α -chains and β -chains, respectively). Figure 6 reports the sequence alignments.

The α chain of Hb 1 comprises the important replacement of Leu FG3 (strongly conserved in human and fish Hbs) by Met. Modeling indicates that the methyl group (CD1 atom, according to the standard nomenclature for amino acids and heme group used by the Protein Data Bank) of Leu FG3 is at only 3.5 Å from the porphyrin ring of HbA (PDB code 2hhb). However, the α -heme proximal cavity of *P. urvillii* Hb 1 is able to accommodate the Leu FG3 $\rightarrow$ met replacement with no significant effect on the heme and on the proximal (F8)-histidyl conformation. Furthermore, the analysis has shown that even the nonconservative replacement of Leu F7 with Glu does not affect the α -heme proximal side of *P. urvillii* Hb 1, probably due to orientation of the carboxyl oxygen of Glu protruding out of the proximal cavity toward the protein surface.

The distal side of the heme in Hb 1 was compared to that of HbA. The most notable change in the model of the α -heme distal cavity is Thr replacing Gly E6 (Ala in *T. bernacchii* Hb) and Lys E10, which might perturb the hydrogen-bonding interaction pattern of the α -heme distal His. However, a slight conformational modification of His E7, with respect of HbA, was detected only in the carbomonoxy form. The models of the carbomonoxy and oxy forms of Hb 1 suggest that Thr E10 does not interact with ligands, as its hydroxyl group is oriented away from distal His. Consequently, it is also unlikely that the nonconservative replacements Lys E10 $\rightarrow$ Thr and Gly E6 $\rightarrow$ Thr induce a significantly modified electrostatic field in the vicinity of His E7 in the α -chain of *P. urvillii* Hb 1.

It is worth noting that Thr is also present in E10 of sperm-whale myoglobin; the side chain of Thr E10 is always directed toward the solvent in deoxy, carbomonoxy, and oxy forms of myoglobin (PDB codes 1a6n, 1a6g and 1a6m, respectively; Vojtechovski et al. 1999), hydrogen bonded to water crystallographic molecules. The side chains of E10, E7, and E11, by means of feasible movements, have been indicated (Case and Karplus 1979) as a possible pathway for ligand escape in sperm-whale myoglobin.

A different situation is seen in the β -chain. The replacement of the residue interacting with heme vinyl 4 (Leu B13 $\rightarrow$ Cys) reduces the steric hindrance by distal side chains. In particular, the distance of Leu B13 methyl group (CD2 atom) from the CBC atom of heme vinyl 4 is 4.0 Å, 4.8 Å, and 4.5 Å in the three forms of human HbA considered, deoxy (PDB code 2hhb), carbomonoxy (PDB code 2hco), and oxy (PDB code 1hho), respectively. Replacement of Leu B13 with Cys in *P. urvillii* Hb 1 increases the distance of the B13 side chain from the heme and, consequently, induces a different conformation of β -heme vinyl 4. The SG atom of Cys B13 is 6.2 Å, 7.1 Å, and 5.6 Å away from the vinyl CBC atom of deoxy, carbomonoxy, and oxy Hb 1, respectively. Superimposition of β -heme environment of the deoxy structures of *P. urvillii* Hb 1 and human HbA is reported in Figure 7.

Figure 6. Sequence alignment of α - (A) and β^1 -chains (B) of *P. urvillii* Hb 1 with the α - and β -chains of *T. bernacchii* Hb and human HbA. Identical and similar residues are in dark and in white boxes, respectively.

Thus, although the most important replacements leave the α -heme conformation surrounding the proximal and distal histidines essentially unmodified in comparison with HbA, a different conformation in the β -heme vinyl 4 was detected, in agreement with the spectroscopic characterization.

Discussion

Evolution of notothenioid

Multiplicity of Hb components in fish is usually linked to the need to respond to a mutable environment or different

habitats. For this reason, a single Hb in lower amounts is regarded as the consequence of a lesser role for oxygen carriers in Antarctic notothenioids, possibly due to their sluggish mode of life, slower metabolism, and to the peculiarity of the environmental conditions (high stability and constancy of physicochemical features). Hb is a direct link between environment and body requirements, and must fulfill its primary function under a wide variety of conditions; consequently, it has experienced major evolutionary pressures to adapt and modify its functional features, while largely retaining its molecular structure. Similar to a large number of notothenioid species, the hemolysate of *P. urvil-*

Figure 7. The steric constraints on the β -heme vinyl groups. Superimposition of backbone atoms of β -heme environments from the deoxy model of *P. urvillii* Hb 1 (green) and the crystallographic structure of deoxy human HbA (orange) shows the increase of the distance of the B13 side chain of *P. urvillii* Hb 1 from the heme. In each protein, selected residues are labeled in the color of the backbone trace. The distances in Å between Cys B13 (SG atom) and the heme vinyl group (CBC atom) of *P. urvillii* Hb 1 and between Leu B13 (CD2 atom) and the heme vinyl group (CBC atom) of human HbA are shown in white.

lii has a single major Hb (Hb 1) and a minor component (Hb 2). The low amount of Hb 2 can be considered a synapomorphy, connecting *P. urvillii* to the other notothenioids.

Three notothenioid families, that is, Bovichtidae (only one out of 10 species is Antarctic), Pseudaphritidae, and Eleginopidae, presumably diverged and became established in waters around areas corresponding to New Zealand, Australia, and South America before Antarctica became isolated. The absence of any detectable antifreeze glycoprotein coding sequence in *Bovichtus variegatus*, *P. urvillii*, and *Eleginops maclovinus* is consistent with this hypothesis (Cheng et al. 2003). However, the three families carry the trypsinogen-like protease gene, in keeping with occurrence of diversification before the evolution of the antifreeze glycoproteins gene from this ancestral gene.

From the evolutionary standpoint, the analysis of the oxygen-transport system of non-Antarctic notothenioids is of great interest. The characterization of the Hb system of *P. urvillii* (among the most primitive notothenioid species) provides useful insights. Taking advantage of the wealth of information available on a high number of Antarctic notothenioid species (e.g., Hb amino acid sequences), this sub-order has been considered as a suitable source for the study of the evolution of the oxygen carrier in fish, in a molecular phylogenetic approach. In this study, methods affording the highest level of reliability have been used to build phylogenetic trees of α - and β -chain amino acid sequences of Hbs of *P. urvillii*, Antarctic notothenioids, and several non-Antarctic fish species, thus developing and improving previous preliminary results (Stam et al. 1997). The trees are in

agreement with those obtained by morphological analysis and sequence studies on mitochondrial RNA (Ritchie et al. 1996), and give strong support to the monophyly of Antarctic notothenioids, with non-Antarctic *P. urvillii* as their sister taxon.

Although *P. urvillii* has never developed cold adaptation, the amino acid sequence reveals high identity with the globins of Antarctic notothenioids, and most of the residues that differentiate Antarctic notothenioids from temperate fish are found in Hb 1. However, the identity between Hb 1 of *P. urvillii* and other Antarctic fish is close to, or lower than, the low extreme of the ensemble of values of Antarctic notothenioids (82%–99% and 77%–93% for the α - and β -chains, respectively). This argues in favor of a common origin within notothenioids, but also suggests that the primary structure of the major Hb has undergone modifications only to a limited extent. If sequence mutations in Antarctic fish are indeed related to the development of cold adaptation, this may imply divergence during the first stages of the cooling process, and in any case before the event that gave rise to antifreeze glycoproteins.

The relevance of studying Hbs of non-Antarctic species in the evolution of Notothenioidae is highlighted by the analysis of the features of the Hb system of another temperate notothenioid. Recent studies indicate that the genome of the more recent *Notothenia angustata* (family Nototheniidae), common near the coast of southern New Zealand, does have the antifreeze glycoproteins genes (Cheng et al. 2003), supporting the hypothesis that this species had developed cold adaptation before migration from the Antarctic

continental shelf in a relatively recent geological time. The amino acid sequence identity between Hbs of noncold-adapted *N. angustata* and cold-adapted *Notothenia coriiceps* (a species of the same family) is the highest ever found among notothenioids (Fago et al. 1992). The presence of antifreeze genes suggests that, unlike *P. urvillii*, this fish may have migrated from Antarctic to temperate waters in a relatively recent time and was cold adapted prior to radiation. The very high similarity of Hb sequence of *N. angustata* and the lower one of *P. urvillii* with Antarctic notothenioids may indeed bear a proportional relationship to cold adaptation, and at the same time appear to reflect the differences in divergence and migration times. On the other hand, both species have high hematocrit, erythrocyte number, Hb content, and cellular concentration. Unlike primary structure, these features might be considered a short-term response to environmental changes. In evolutionary processes, in the same time span changes in amino acid sequence would occur at a much slower overall rate, thus producing fewer variations. We suggest that the common phylogenetic origin remains the prevailing factor in primary structure in the course of readaptation of a notothenioid to a temperate environment. Changes in factors other than sequence, such as (for instance) regulatory processes or enhanced protein synthesis, may well be additional mechanisms of temperature compensation.

Oxygen affinity and subunit cooperativity

P. urvillii Hb 1 has a strong Bohr effect in the presence and absence of the physiological effector ATP. The Root effect is largely ATP-induced. In the temperature dependence of oxygen binding, the positive (endothermic) enthalpy change observed around pH 6.6 may have an important physiological significance. At pH values where the T state becomes preferentially populated, but still in the presence of a significant degree of heterotropic interactions, the contribution of the effectors and of the conformational transition may even overcome that of the T state, thereby producing a change of sign in the overall enthalpy change, which, in fact, becomes endothermic, favoring oxygen release when the organism is challenged by lower temperatures.

The β -chain of *P. urvillii* Hb 1 shows two remarkable replacements with respect to HbA: Lys A5 $\rightarrow$ Arg and Ala E20 $\rightarrow$ Lys. In *P. urvillii* Hb 1, these residues may represent an "additional" oxygen-linked chloride binding site, as observed in a number of Hbs characterized by a low effect of temperature. It has been shown (De Rosa et al. 2004) that the endothermic contribution by the oxygen-induced release of the "additional" chloride ions (one for each β -chain) may significantly contribute to lower the overall heat of oxygenation, thus providing a molecular basis to explain the thermodynamics of the oxygenation–deoxygenation cycle of several mammal Hbs. Similar to human fetal, horse, and

bear Hbs, *P. urvillii* Hb 1 also displays a small oxygenation enthalpy only in the simultaneous presence of both chloride and organophosphate. The β -chain of these Hbs has basic residues in A5 and E20; in the mammal Hbs, the interaction with 2,3-DPG in its pocket induces a distortion in helix A, thereby influencing the structure so that Lys A5 (Arg in *P. urvillii* Hb 1) is able to switch on the additional binding site. Such conclusion, supported by crystallographic data, may apply to *P. urvillii* Hb 1.

The affinity and cooperativity are modulated by pH and allosteric effectors in a remarkable way. The oxygen affinity of Hb 1 is exceptionally high compared to other notothenioids. Although the Hb multiplicity closely resembles that of sedentary Antarctic bottom dwellers, this high affinity (possibly related to the peculiar habitat constraints) clearly differentiates this species from other notothenioids. A high oxygen affinity may result, at least in part, from an altered intrinsic affinity in the α - and β -subunits. In the α -chain of *P. urvillii* Hb 1 several substitutions may alter the geometry of the invariably hydrophobic heme pocket. For instance, Glu and Met replace Leu in F7 and FG3; the substitution in $\alpha 87$ (F7) is uncommon, and Glu is indeed found only in this species. Interestingly, all Antarctic Notothenioidei have Gln, whose codon differs from that of Glu of *P. urvillii* Hb 1 by a single base change in the first position. Moreover, Thr replaces Gly E6 (in HbA) and Ala E6 (in *T. bernacchii* Hb) and Lys E10 (in both Hbs). As discussed in detail below, the spectroscopic and modeling studies indicate, however, that each of these replacements leave the conformation and electrostatic field surrounding His E7 essentially unmodified with respect to both HbA and *T. bernacchii* Hb. In fact, although *T. bernacchii* Hb has lower oxygen affinity, the affinity of *P. urvillii* Hb 1 and HbA are comparable. In the β -chain, Leu B13 is replaced by Cys. This nonconservative substitution, although uncommon in fish Hbs, is found also in *T. bernacchii* Hb. Although this replacement, therefore, cannot directly explain the high affinity of *P. urvillii* Hb 1, it does induce a conformational change with significant implications (see below).

The subunit cooperativity, mostly induced by allosteric effectors, is regulated by pH in a very unusual way, in that it not only disappears at low physiological pH (as in all Root-effect Hbs), but is absent also at high pH. Thus, the curve of the Hill coefficient as a function of pH is bell-shaped, and its maximum identifies the pH value where the ligand-induced conformational transition is fully active. Moreover *P. urvillii* Hb 1 appears to be a rare type-case of Hb, in which the molecule is fully locked in the low-affinity conformation at acidic pH and tends to be locked in the high-affinity state at alkaline pH. In the common sting ray, *Dasyatis sabina* (Mumm et al. 1978), Hb also exhibits a bell-shaped curve of the Hill coefficient, with maximal value of 2.0 near pH 9.0 but approaching one below 7.0 and above 10, in the absence and the presence of effectors.

Heme pocket properties

The spectroscopic measurements reveal two characteristics which distinguish Hb 1 from HbA, namely the presence of a hemichrome species in the Met form and reduced steric constraints on vinyl group 4. It is noteworthy that the proportion and nature of the LS species observed in *P. urvillii* Met-Hb 1 are in contrast with those found in HbA. At both pH 6.1 and 9.5 *P. urvillii* Hb 1 displays a significant proportion of LS heme (compared to HbA) due to a hemichrome conformation, rather than a hydroxy complex as in HbA. Hemichromes have been generally observed in association with Hb-denaturation processes but, unlike hemichromes of other heme proteins that are reduced to hemochrome, the oxidized form of *P. urvillii* Hb 1 is reduced by dithionite to the deoxy form which can then bind oxygen or CO. Although the formation of hemichrome in a native Hb structure is rare, it has recently been reported in the monomeric Hb of the sea cucumber *Caudina arenicola* (Mitchell et al. 1995), the homodimeric Hb of rice (Hargrove et al. 2000), the tetrameric Hb of the horse (at pH 5.4) (Robinson et al. 2003), and the major Hb 1 of the Antarctic notothenioid *Trematomus newnesi* (Riccio et al. 2002). Moreover, it has recently been found that in five Hbs from four Antarctic notothenioids the oxidation process, promoted by air or ferricyanide, leads to the reversible formation of hemichrome (Vitagliano et al. 2004), in marked contrast with human HbA, and suggesting that this behavior is common in fish. These findings provide new information on tetrameric Hbs in the hemichrome state, and suggest a physiological role of in vivo hemichrome formation in fish, which appears to occur regardless of whether a species is cold adapted or not.

It is worth noting that fish are highly sensitive to a number of environmental stress factors. Although fish erythrocytes may contain low levels of Met-Hb, stress often leads to rapid increase of this form (Graham and Fletcher 1986). It is feasible that fish might use hemichrome formation as a protective response to otherwise irreversible stress-induced Hb oxidation. In other words, spontaneous autoxidation, occurring at higher rates than in mammalian Hbs, might be balanced by hemichrome formation. This in vivo mechanism has been suggested also in other organisms (Robinson et al. 2003).

The formation of a hemichrome (bisHis) in *P. urvillii* Met-Hb 1 indicates a change in conformation compared to HbA that allows distal His to bind to the iron atom. A slightly modified distal cavity, compared to HbA, is also suggested by retention of an aquo HS species in the Met form at alkaline pH, which indicates that the pK_a of the alkaline transition in *P. urvillii* Hb 1 is higher. Although the plant, invertebrate, and vertebrate Hbs mentioned above differ significantly in their primary structures, all exhibit two hemichrome-induced structural characteristics, namely a

shift of the heme group to a more exposed position and a narrowing of the heme pocket. These differences found in *P. urvillii* Met-Hb 1 compared to HbA do not appear to have significant consequences on the ligand-bound form (oxy, carbomonoxy). In fact, in accordance with the modeling studies, which suggest a slightly modified distal His disposition in the ligand-bound forms but a largely unchanged electrostatic field, the Fe–CO and CO frequencies of the CO complex are not significantly modified. These frequencies are sensitive indicators of modified heme interactions (H-bonding, polarity and steric hindrance) and hence confirm the similarity of the heme cavities of *P. urvillii* Hb 1 and HbA. The absence of any variation in the Raman frequency of the Fe–O₂ stretching mode of *P. urvillii* Hb 1 compared to HbA is consistent with this conclusion, although it should be noted that the frequency of the Fe–O₂ Raman mode is not particularly sensitive to perturbation of the distal cavity (Hirota et al. 1996).

The presence of two vinyl modes in the RR spectra of *P. urvillii* Hb 1, compared to the single mode observed in HbA, is particularly interesting. A direct relationship between the $\nu(C=C)$ stretching frequency and the orientation of the vinyl groups, as induced by specific protein interactions, has recently been found in heme-containing peroxidases (Smulevich 2001; Marzocchi and Smulevich 2003). In particular, it was found that, when the protein matrix exerts no constraints on the vinyl groups, the latter take the torsional conformation usually found in model compounds in solution (Kalsbeck et al. 1995), and two $\nu(C=C)$ stretching modes should be observed in the RR spectrum. Hence, it is suggested that the presence of two distinct vinyl stretches in the RR spectra of *P. urvillii* Hb 1 is related to a reduced steric hindrance of vinyl 4 caused by replacement of Leu B13 with Cys in the β -chain. This spectroscopic finding is consistent with the reduced steric constraints of the vinyl 4 substituent group of *P. urvillii* Hb 1 highlighted by the modeling studies. It can be anticipated that the reduced steric hindrance upon replacement of the residue interacting with heme vinyl 4 contributes to the increased mobility of the heme, allowing it to shift to a more exposed position, as required for hemichrome formation.

In conclusion, spectroscopy and molecular modeling were used to investigate correlations between the peculiar oxygen-binding features of *P. urvillii* Hb 1 and the overall environment of the heme pocket, and showed the unexpected lack of a direct linkage between the nonconservative replacements found in the amino acid sequence and the oxygen affinity of these Hbs on one hand. On the other hand, two closely related and unique features distinguish *P. urvillii* Hb 1 from HbA, namely (1) the hemichrome species in the Met form, whose formation appears to be mediated by (2) the reduced steric constraints on vinyl group 4. Although their correlation with the oxygen-binding features of *P. urvillii* Hb 1 remains an open question, the occurrence of a

significant amount of Hb in the hemichrome state under physiological conditions in notothenioids (regardless of their temperature adaptation) suggests an important functional role of this form in fish life style.

Materials and methods

Materials

DEAE-cellulose (DE52) was from Whatman. All other reagents were of the highest purity commercially available.

Hb purification

Specimens of *P. urvillii* were captured in North West Bay River, Margate, Tasmania, Australia. Blood samples were drawn from the caudal vein of anesthetized fish with heparinized syringes. Hemolysates were prepared as described (D'Avino and di Prisco 1988). Separation of Hbs was achieved by fast protein liquid chromatography (FPLC) on a DE52 ion-exchange column (1.8 × 30 cm), equilibrated with 10 mM Tris-HCl (pH 7.6), and by stepwise elution with the same buffer, at a flow rate of 1.0 mL/min. The Hb 1-containing fractions were pooled and dialyzed against 10 mM HEPES (pH 7.7). All steps were carried out at 0°–5°C. For oxygen-binding, aliquots of a solution of carbon monoxide Hb 1 were stored at –80°C prior to use within a maximum of 7 days. For each experiment, one aliquot was thawed, converted to the oxy form by exposure to light and oxygen, and immediately used; no oxidation was spectrophotometrically detectable, indicating that final Met-Hb formation was negligible (<2%).

Amino acid sequencing

Purification of globins, fractionation of tryptic and cyanogen-bromide-cleaved peptides, and subsequent amino acid sequencing were carried out as previously described (Verde et al. 2002).

Phylogenetic analysis

Globin amino acid sequences of selected species of temperate and Antarctic fish were aligned using the program CLUSTAL X. Maximum likelihood phylogenetic trees of both α - and β -globin sequences were inferred by the method implemented in the software package MOLPHY (Adachi and Hasegawa 1996), using the model of amino acid substitution (Jones et al. 1992), adjusted with the empirical amino acid frequencies. The following strategy was applied for inferring the trees. First, a distance matrix and the corresponding neighbor-joining (NJ) tree were estimated. Second, the NJ tree was subjected to a local rearrangement search using the program PROTML contained in the MOLPHY package. An exhaustive topology search of 1000 top-ranking trees was performed on a partially constrained starting tree where the taxa of some undisputed clades were clustered together. The constrained topology contained the major and minor Antarctic globin sequences as fixed groups because their topology was well supported by bootstrap analysis and was congruent with previous assessments (Verde et al. 2003). Finally, the user tree option was used to pick up the best tree among the 1000 trees inferred by exhaustive search. The relative bootstrap probabilities were computed with the ReEstimation of Log Likelihood (RELL) method. Bootstrap values for internal tree branches were calculated as the sum of the

bootstrap probabilities of all the trees showing the node being analyzed among the alternatives.

Oxygen binding

Hemolysate stripping was carried out as described (Tamburrini et al. 1994). Oxygen equilibria were measured in 100 mM HEPES/MES in the pH range 6.0–9.4, at 10°C and 16°C (keeping the pH variation as a function of temperature in due account) at a final Hb concentration of 0.5–1.0 mM on a heme basis. An average standard deviation of $\pm 3\%$ for values of p_{50} was calculated; experiments were performed in duplicate. To obtain stepwise oxygen saturation, a modified gas diffusion chamber was used, coupled to cascaded Wösthoff pumps for mixing pure nitrogen with air (Weber et al. 1987). In the diffusion-chamber experiments, the oxygenation range was between 10% and 90%. pH was measured with a Radiometer BMS Mk2 thermostatted electrode. Sensitivity to chloride was assessed by adding NaCl to a final concentration of 100 mM. ATP or inositol hexakisphosphate (IHP) were added to a final concentration of 3 mM; hence, a large excess over tetrameric Hb. Oxygen affinity (p_{50}) and cooperativity (n_{50}) were calculated from the linearized Hill plot of $\log S/(1-S)$ versus $\log pO_2$ at half saturation (S denotes fractional oxygen saturation).

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

Molecular modeling

As the crystal structure of *P. urvillii* Hb 1 has not yet been established, molecular modeling studies were carried out on a Silicon Graphics O2 R12000 workstation using Insight II (Accelrys Inc.).

Sequence similarity searches within the Protein Data Bank were performed using PSI-BLAST running on the server at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). The sequence alignments were obtained by CLUSTAL W (Thompson et al. 1994). The PostScript files from CLUSTAL W alignment results were generated with the ESPript program (Gouet et al. 1999).

Crystallographic structures of deoxy and carbomonoxy *T. bernacchii* Hb (PDB codes 1hbb and 1pbx, respectively; Camardella et al. 1992; Ito et al. 1995) were used as templates for building corresponding models of Hb 1 of *P. urvillii*. As the PDB coordinates of oxy *T. bernacchii* Hb were not available, a model of oxy Hb 1 of *P. urvillii* was built based on human HbA (PDB code 1hho; Shaanan 1983), which displayed 51% and 47% sequence identity with the α - and β -chains of *P. urvillii* Hb 1, respectively.

Based on CLUSTAL W alignments, three-dimensional models of α - and β -chains of deoxy, carbomonoxy and oxy Hb 1 of *P. urvillii* were then generated by the MODELER program (Sali and Blundell 1993) implemented in Insight II. This program is based on a distance-restraint algorithm using spatial restraints extracted from a multiple alignment of the target sequences with the template structures and from the CHARMM-22 force field (Brooks et al. 1983). Three models, optimized by a short simulated annealing refinement protocol available in MODELER, were generated for each alignment. The model with the lowest value of MODELER objective function (F , molecular probability density function violation) was selected as the representative model for each chain in each structure. To further validate the modeling procedure, models obtained by MODELER were analyzed by the program PROCHECK (Laskowski et al. 1993) to confirm their stereochemical quality.

Spectroscopy

As *P. urvillii* Hb 1 was isolated in the oxy form, the oxy samples were used without further manipulation. The Met-Hb derivatives were prepared by oxidation of the oxy form using excess potassium ferricyanide followed by gel filtration on a Biogel P-6DG column equilibrated with the appropriate buffer to remove the oxidant. The deoxy samples were prepared by adding 2–3 μ L of sodium dithionite (20 mg/mL) to 50 μ L of deoxygenated buffered solution of Met-Hb. The carbomonoxy complex was prepared by flushing Met-Hb with nitrogen, then with CO and reducing the protein as described above. Fluoride (F^-) adducts were prepared by adding 2 μ L of a saturated solution of sodium fluoride, dissolved in 100 mM sodium citrate (pH 5.0), to 40 μ L of solution of Met-Hb in 5 mM MES at pH 6.0. The protein concentration of all samples, for both resonance Raman (RR) and electronic absorption spectroscopy, was 30 μ M on a heme basis.

Electronic absorption spectra were measured with a Cary 5 spectrophotometer. Infrared spectra were recorded with a Bruker IF120HF FTIR spectrophotometer. Carbomonoxy Hb samples (250 μ M) were transferred by a gas-tight syringe flushed with CO into a CaF_2 IR cell (path length, 0.1 mm), previously flushed with CO. Tris-HCl at pH 7.6 (10 mM) was used as reference. RR spectra were obtained at room temperature with excitation from the 406.7- and 413.1-nm lines of a Kr^+ laser (Coherent, Innova 302C). The back-scattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HG2S) equipped with a cooled photomultiplier (RCA C31034A) and photon-counting electronics. To minimize local heating by the laser beam, all samples except deoxy Hb were cooled by a gentle flow of nitrogen run through liquid nitrogen. RR spectra were calibrated to an accuracy of 1 cm^{-1} for intense isolated bands, using indene as standard for the high-frequency region, and indene and CCl_4 for the low-frequency region.

The protein sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL knowledgebase under the accession numbers P83623 (α), P83624 (β^1), P83625 (β^2).

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