Analysis of the contribution of the globin and reductase domains to the ligand-binding properties of bacterial haemoglobins

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Bacterial Hbs (haemoglobins), like VHb (*Vitreoscilla* sp. Hb), and flavoHbs (flavo-haemoglobins), such as FHP (*Ralstonia eutropha* flavoHb), have different autoxidation and ligand-binding rates. To determine the influence of each domain of flavoHbs on ligand binding, we have studied the kinetic ligand-binding properties of oxygen, carbon monoxide and nitric oxide to the chimaeric proteins, FHPg (truncated form of FHP comprising the globin domain alone) and VHb-Red (fusion protein between VHb and the C-terminal reductase domain of FHP) and compared them with those of their natural counterparts, FHP and VHb. Moreover, we also analysed polarity and solvent accessibility to the haem pocket

of these proteins. The rate constants for the engineered proteins, VHb-Red and FHPg, do not differ significantly from those of their natural counterparts, VHb and FHP respectively. Our results suggest that the globin domain structure controls the reactivity towards oxygen, carbon monoxide and nitric oxide. The presence or absence of a reductase domain does not affect the affinity to these ligands.

Key words: bacterial haemoglobin, globin domain, ligand binding, nitric oxide, *Ralstonia eutropha* flavohaemoglobin (FHP), *Vitreoscilla* sp. haemoglobin (VHb).

INTRODUCTION

Hbs (haemoglobins) are proteins capable of reversibly binding oxygen that have been found in all kingdoms of life. Hbs have also been identified in bacteria, where they are classified into three broad groups (structural families). The most abundant group comprises flavoHbs (flavo-haemoglobins), which are single-chain proteins with an N-terminal globin domain and a C-terminal reductase domain structurally related to the ferredoxin: NAD(P)H⁺ reductase family [1]. The second protein group includes small Hbs, which are 20–40 residues shorter than classical Hbs, referred to as trHbs (truncated Hbs) [2]. The third group is composed of bacterial Hbs, characterized by a single globin domain (monomeric or homodimeric). FlavoHbs and bacterial Hbs are closely related, and the tertiary structure of their globin domains consists of seven α -helical regions adopting the classical ‘three-over-three’ α -helical globin fold [1,3,4], while the tertiary structure of trHbs is based on a two-on-two α -helical sandwich and the iron is hexaco-ordinated [2].

Although all these Hbs are studied extensively, there is no conclusive evidence for the roles of these proteins in micro-organisms. HMP (*Escherichia coli* flavoHb) is one of the most extensively studied proteins within the bacterial flavoHb family. At present, the physiological function most often attributed to HMP is to provide protection from NO (nitric oxide; nitrogen monoxide is the name recommended by the International Union of Pure and Applied Chemistry) stress [5–8]. There exists also supporting evidence showing that other flavoHbs behave in a similar manner [9,10]. Extensive biochemical and genetic studies on the Hb of the bacterium *Vitreoscilla*, VHb, have suggested that it is able to facilitate oxygen delivery to the respiratory apparatus

with concomitant enhancement of cell growth or improvement of cellular metabolism [11]. It has also been shown that VHb may act as an alternative terminal oxidase itself [12], but these findings have not been verified by others. On the other hand, VHb is also able to protect growing cells against NO-generating compounds and shield the terminal oxidases from inactivation by NO [13,14]. Several authors point to a common role for bacterial Hbs and flavoHbs. In fact, the non-covalent assembly of bacterial Hbs with a FAD-containing reductase domain could yield a new flavoHb-like protein. Indeed, VHb has been co-purified with an NADH:methaemoglobin reductase [15]. In spite of the similarities between the globin domains of flavoHbs and bacterial Hbs, in our recent report we demonstrated that a clear difference exists between the ligand-binding properties of the two groups of bacterial Hbs [16] suggesting a distinct role for these Hbs in the cell.

Several studies have analysed the contribution of each of the domains of flavoHbs, as well as the addition of a reductase domain to a bacterial Hb, to growth enhancement, oxygen metabolism or protection against nitrosative stress. Frey et al. [17] showed that the expression of FHP (*Ralstonia eutropha* flavoHb) and the chimaeric VHb-Red (fusion protein between VHb and the C-terminal reductase domain of FHP) increased significantly (1.7-fold) the final cell density compared with VHb- and FHPg (truncated form of FHP rendering the globin domain alone)-expressing cells, under hypoxic conditions in bioreactors. A second study showed that the presence or absence of C-terminal reductase domain does not significantly change the protective effect under nitrosative stress conditions *in vivo*. However, the NO consumption activity, *in vitro*, is clearly improved in the presence of a reductase domain [13]. Kaur et al. [14], using a

Abbreviations used: flavoHb, flavohaemoglobin; *fhp*, *Ralstonia eutropha* flavoHb gene; FHP, *Ralstonia eutropha* flavoHb; *fhp*g, truncated form of *fhp* rendering the globin domain alone; FHPg, truncated form of FHP rendering the globin domain alone; Hb, haemoglobin; HMP, *Escherichia coli* flavoHb; Mb, myoglobin; MEK, methyl ethyl ketone; TMB, 3,3',5,5'-tetramethylbenzidine; trHb, truncated Hb; *vhb*, *Vitreoscilla* sp. Hb gene; VHb, *Vitreoscilla* sp. Hb; *vhb-red*, gene fusion between *vhb* and the C-terminal reductase domain of *fhp*; VHb-Red, fusion protein between VHb and the C-terminal reductase domain of FHP.

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similar type of chimaeric protein, reported the same result. Finally, Hernández-Urzuá et al. [18] showed that overexpression of only the haem domain of the flavoHb HMP resulted in an improvement of growth to a similar extent to that observed with VHb, the bacterial Hb. In addition, they showed that although the globin domain alone could give protection to growing cells against NO stress, maximal protection was provided only by holoHMP. In contrast, the globin domain was unable to provide protection against NO-induced inhibition of the respiratory chain.

In the present study, we have analysed the contribution of each of the domains of flavoHbs to their ligand-binding properties. We have studied the kinetic ligand-binding properties to oxygen, CO (carbon monoxide) and NO of the chimaeric proteins, FHPg and VHb-Red, in comparison with their natural counterparts, FHP and VHb.

EXPERIMENTAL

Cloning, expression and purification of Hbs and flavoHbs

Template DNA for the amplification of VHb and VHb-Red was obtained from vectors pRED2 [19] and pAX4 [17] respectively, and for FHP and FHPg from vector pAX5 [17]. Oligonucleotide primers were designed to incorporate EcoRI and BamHI restriction sites at the 5'- and 3'-ends of the gene respectively. The coding sequence for six additional histidine residues at the C-terminus of the Hb was also included at the 3'-ends of the PCR products by using specifically designed primers (Oli2 and Oli5). The DNA primer pairs Oli1 [17] and Oli5 (5'-CGGG-ATCCCGTTATCAGTGATGGTGATGGTGATGCTCAGCAA-ACAGGTCGGGACCAAAC) and Oli1 [17] and Oli2 (5'-CGG-GATCCCGTTATCAGTGATGGTGATGGTGATGTTGTTCCG-CAGAGCGCTCATACAACT) were used to amplify *fhp* (*Ralstonia eutropha* flavoHb gene) and the *fhp*g (truncated form of *fhp* coding for the globin domain alone) gene fragment respectively. The DNA primer pairs Oli4 [17] and Oli3 (5'-CGGGATCCCGTTATCAGTGATGGTGATGGTGATGTTCA-ACCGCTTGAGCGTACAAATCTG) and Oli4 [17] and Oli5 were used to amplify *vhb* (*Vitreoscilla* sp. Hb gene) gene and *vhb-red* (gene fusion between *vhb* and the C-terminal reductase domain of *fhp*) gene construct respectively. All PCR-amplified genes were introduced into pUC19 and transformed into *E. coli* DH5 α [F⁻, *endA*1, *hsdR*17(r_k⁻ m_k⁺), *supE*44, *thi*-1, λ ⁻, *recA*1, *gyrA*96, *relA*1, ϕ 80d lac Δ (lacZ)M15, Δ (lacZYA-argF)U169, Bethesda Research Laboratories] by using standard techniques [20]. PCR products were verified by DNA sequencing as described previously [21,22]. EcoRI and PstI fragments were subcloned into pKQV4 [23] expression vector.

E. coli cells were grown in LB (Luria-Bertani) medium supplemented with ampicillin (100 mg/l) in an Incubator 210 (LH) bioreactor, 3.5 litres working volume, for production of the His-tagged proteins. The other process parameters were: 37°C, 400 rev./min and 1.4 litres/min airflow, and the pH was kept constant at 7 with 2 M NaOH and 2 M H₃PO₄. Growth medium was inoculated at 1% (v/v) using an overnight culture. The cultures were grown to a D₆₀₀ (attenuance) of 1 and induced by adding IPTG (isopropyl β -D-thiogalactoside) to a final concentration of 1 mM. The cells were harvested after 6 h by centrifugation. Purification of the His-tagged proteins was performed as described in [16].

Protein characterization

The protein, haem and FAD contents were assayed as described previously [16]. Haem staining was performed on SDS/PAGE

gels using TMB (3,3',5,5'-tetramethylbenzidine) [24]. FHPg apoprotein was obtained using the MEK (methyl ethyl ketone) method following the procedures described by Asakura [25].

Preparation of ligand-bound Hbs

Solutions of the oxygenated forms of VHb and FHPg were prepared by reducing the proteins with a slight excess of sodium dithionite and by purifying the resulting mixtures over a PD-10 column (Amersham Biosciences). The concentrated protein stock solutions were diluted in sealed cells or in gastight SampleLock Hamilton syringes with 0.1 M sodium phosphate buffer (pH 7.0) containing the required O₂ concentration. Solutions of the oxygenated forms of FHP and VHb-Red were generated in sealed cells or gastight SampleLock Hamilton syringes by adding, immediately before the measurements, NADH (400 μ M, final concentration) to a solution containing the Fe(III) form of the corresponding Hb protein and the required O₂ concentration. Because of the NADH oxidase activity of FHP, these solutions were used within 5 min; older solutions were discarded.

The CO-bound form of the Hb proteins was prepared by flushing concentrated protein stock solutions with argon for 15 min, adding sodium dithionite (~2–5 equiv.), and finally diluting the resulting mixtures with CO-saturated buffer. This stock solution was then diluted in sealed cells or gastight SampleLock Hamilton syringes with phosphate buffer containing different CO concentrations.

Solutions of the Fe(II)-NO form of FHP and VHb-Red were prepared by first degassing protein stock solutions in a sealed cell with argon for approx. 15 min and then diluting them with phosphate buffer containing different concentrations of NO. Finally, the required amount of an anoxic NADH solution was added to obtain a final concentration of 400 μ M. Despite the fact that we tried different procedures, it proved impossible to prepare stable solutions containing the Fe(II)-NO form of VHb. Neither the reductive nitrosylation procedure normally used for obtaining MbFe(II)-NO nor the replacement of the co-ordinated CO with excess NO led to the formation of the Fe(II)-NO form, as confirmed by UV-visible spectroscopy. Alternatively, we prepared the Fe(II)-NO form of VHb by first reducing the corresponding concentrated Fe(III) protein solutions with sodium dithionite (6–15 equiv. were needed, giving a final concentration in the range 55–200 μ M) in sealed degassed cells (flushed for 15 min with argon). The resulting mixtures were then diluted with phosphate buffer containing the required NO concentration. Unexpectedly, despite the strict anaerobic conditions (and the presence of an excess of dithionite), the VHb Fe(II)-NO solutions were stable only for 10–30 min and slowly oxidized to the corresponding Fe(III)-NO form.

Solutions of the Fe(III)-NO form of the proteins were prepared by diluting a thoroughly degassed Fe(III) Hb protein solution in a sealed cell with a buffer containing the required NO concentration.

Kinetic measurements

All kinetic experiments were performed at 20°C, and protein samples were buffered in 0.1 M phosphate buffer at pH 7. O₂ and CO stock solutions were prepared by equilibrating the buffer with the corresponding pure gas at room temperature (20°C). NO-containing solutions were prepared as described in our previous work [26]. Association time courses for O₂, CO and NO [(*k*_{O₂}(Fe²⁺), *k*_{CO}(Fe²⁺) and *k*_{NO}(Fe³⁺)] were measured by laser flash photolysis, following the procedures already described [16]. If required, saturated stock solutions (1300 μ M O₂, 1000 μ M CO and 2000 μ M NO) were diluted with degassed buffer in gas-tight SampleLock Hamilton syringes. All samples

were prepared immediately before use, and their stability was checked spectrophotometrically before and after the kinetic measurements. Final protein concentrations were 2.5–10 μM . Time courses (average for three to ten traces) were monitored at two wavelengths (maximum and minimum of the differential spectra ligand-bound protein minus ligand-free protein) and measured for different ligand concentrations (O_2 , 260–1300 μM ; CO, 200–1000 μM ; and NO, 250–2000 μM). Second-order association rate constants were obtained from the slopes of the linear plots of the observed pseudo-first-order rate constants against ligand concentration.

Dissociation rate constants for O_2 and CO [$k_{\text{O}_2}(\text{Fe}^{2+})$ and $k_{\text{CO}}(\text{Fe}^{2+})$] were measured directly by carrying out replacement reactions as described previously [16]. The ligands of a 10–20 μM solution of the O_2 - and CO-bound forms of the Hb proteins were replaced by a high concentration of displacing ligand (500 μM CO and 1000 μM NO respectively). Measurements were done at two different wavelengths (maximum and minimum of the differential spectra) and at least two different ligand concentrations (65–325 μM O_2 and 50–250 μM CO). The observed rates were obtained from the fits of at least five single traces. $k_{\text{O}_2}(\text{Fe}^{2+})$ was then calculated from the expression $k_{\text{O}_2} = k_{\text{obs}}(1 + k'_{\text{O}_2}[\text{O}_2])/k'_{\text{CO}}[\text{CO}]$ by using $k'_{\text{O}_2}(\text{Fe}^{2+})$ and $k'_{\text{CO}}(\text{Fe}^{2+})$ determined in the present study. For the determination of CO dissociation rates, the equation reduces further and $k_{\text{CO}}(\text{Fe}^{2+}) = k_{\text{obs}}$. Average values and S.D. values obtained from the different measurements are reported.

Solvent accessibility and polarity of the haem pocket

The solvent accessibility to the haem pocket was measured with the software CASTp [27].

To determine the polarity of the haem pocket, we first determined the residues forming the haem pocket with the software CASTp [27]. The residues that interact with the haem molecule were determined with the LPC software [28]. The classification of the amino acids as polar or non-polar was carried out by the method of Timberlake [29].

RESULTS

Protein production and purification

Cultures generating recombinant Hbs were cultivated in bioreactors and bacterial Hb and flavoHb proteins were purified by His-tag-affinity purification. Identical functional behaviour of His-tagged and non-tagged Hbs has been previously reported [30]. All purified proteins showed the expected molecular size and purities ranged from 90 to 95 % as judged by SDS/PAGE gels (results not shown). The molar ratios of haem to protein were approx. 1, except for FHPg for which the determination of the haem content was not possible. Purified flavoHbs were partially lacking FAD. The molar ratios of FAD to haem were 0.05 and 0.8 for VHb-Red and FHP respectively. FAD was supplemented in all reactions to have equimolar amounts of haem to FAD.

Surprisingly, purified FHPg protein solutions displayed an intense green colour. In fact, cell pellets from cultures producing FHPg were already green. Although different growth strategies were tested, e.g. low induction levels and addition of reducing agents to the medium, cell pellets were always green. ApoFHPg was generated by removing the haem cofactor by using the MEK method [25]. Reconstitution of the apoprotein with haemin gave a reddish-brown protein, indicating that the green colour in FHPg is caused by a haem modification.

Haem staining with TMB (stain for peroxidase activity detection) on SDS/PAGE gels showed protein bands corresponding

Figure 1 Haem staining

Purified Hb proteins were separated by SDS/PAGE and stained with TMB for haem detection. Lane 1, molecular-mass markers; lane 2, VHb-Red; lane 3, VHb-Red; lane 4, VHb; lane 5, VHb-Red; lane 6, FHP; and lane 7, FHPg.

to the size of VHb and VHb-Red purified proteins, but none were observed corresponding to purified FHP and FHPg proteins (Figure 1). The loss of haem is a known problem with any method that relies on the measurement of a haem-associated peroxidase activity on SDS/PAGE when haem is non-covalently bound to the protein [24]. These results could indicate that the binding of the haem group to VHb is stronger than the binding to FHP. In fact, the haem group of ligand-free ferric VHb has been reported to be more deeply buried in its protein cavity than in the case of FHP and of Mb (myoglobin) [31].

Spectral and stability characterization

Similar spectra to those previously reported for VHb and FHP [32,33] were obtained. No spectral data have been reported so far for the truncated versions of FHP, FHPg and the fusion protein VHb-Red. The absorbance maxima for the different proteins and complexes studied are summarized in Table 1.

The oxygenated form of FHP was obtained with the addition of NADH to an oxidized protein solution. FHP, like other flavoHbs [34,35], has an NADH oxidase activity that, within minutes, consumes all NADH added, leading finally to the oxidation of the haem iron. Furthermore, it was not possible to obtain FHP in the oxygenated form by means of dithionite reduction followed by desalting. On the other hand, the VHb-Red oxygenated form was also obtained after addition of NADH to an oxidized protein solution, demonstrating that the attached reductase domain was active. In contrast with FHP, no significant NADH oxidase activity was observed. The oxygenated form of VHb-Red could be obtained by dithionite reduction and desalting, showing that VHb-Red is stable in the oxygenated form in contrast with FHP.

FHPg could not be obtained in the oxygenated form. Spectroscopic analysis suggests that the recombinant FHPg is purified in the oxidized form (Figure 2). The green protein derives its colour from the absorbance band at 679 nm. FHPg can only be partially reduced with the addition of an excess of sodium dithionite, as reflected in the spectra shown in Figure 2. The CO and Fe(III)NO spectra of FHPg were similar to those of FHP, except for the additional band at 679 nm. ApoFHPg reconstituted with haemin gave a similar spectra as FHPg, except for the missing band at 679 nm. Kinetic measurements were performed with the green form of FHPg.

Table 1 Spectroscopic data for Fe(II), Fe(III) and complexed forms of the different bacterial globins

Protein form	λ_{max} (nm)		
	Soret	Visible	
VHb(Fe ²⁺)	432	557	
VHb-Red(Fe ²⁺)	431	555	
FHP(Fe ²⁺)	435	560	
FHPg(Fe ²⁺)	—*	—*	
VHb(Fe ³⁺)	402	498	625
VHb-Red(Fe ³⁺)	402	500	
FHP(Fe ³⁺)	400	486	645
FHPg(Fe ³⁺)	402	535	679
VHb(Fe ²⁺)O ₂	411	542	577
VHb-Red(Fe ²⁺)O ₂	413	542	578
FHP(Fe ²⁺)O ₂	413	542	578
FHPg(Fe ²⁺)O ₂	—*	—*	—*
VHb(Fe ²⁺)CO	420	539	565
VHb-Red(Fe ²⁺)CO	420	538	565
FHP(Fe ²⁺)CO	423	540	569
FHPg(Fe ²⁺)CO	422	538	568/679
VHb(Fe ²⁺)NO	418	548	Shoulder
VHb-Red(Fe ²⁺)NO	418	547	Shoulder
FHP(Fe ²⁺)NO	420	546	569
FHPg(Fe ²⁺)NO	—*	—*	—*
VHb(Fe ³⁺)NO	419	533	565
VHb-Red(Fe ³⁺)NO	419	533	565
FHP(Fe ³⁺)NO	419	535	566
FHPg(Fe ³⁺)NO	419	531	566/679

* FHP_g can only be partially reduced.

Figure 2 FHPg spectra

Black line, purified protein, oxidized form; grey line, purified protein in the presence of sodium dithionite, partially reduced form.

O₂, CO and NO kinetic measurements

Ligand association rate constants were measured by flash photolysis. Time courses were monitored at two wavelengths (maximum and minimum of the differential spectra, ligand-bound protein minus ligand-free protein) and measured for different ligand concentrations. Time courses for O₂ rebinding could be fitted well

Figure 3 O₂, CO and NO recombination kinetics after flash photolysis

(A) Time courses for O₂ rebinding to bacterial Hbs and flavoHbs after flash photolysis in the presence of 650 μM O₂. (B) Time courses for CO rebinding to bacterial Hbs and flavoHbs after flash photolysis in the presence of 500 μM CO. (C) Time courses for NO rebinding to Fe(III) form of bacterial Hbs and flavoHbs after flash photolysis in the presence of 400 μM NO. To facilitate comparison of the data for the various proteins, the absorbance changes were normalized to 1 at the maximum absorbance. Line 1, VHb; line 2, VHb-Red; line 3, FHP; line 4, FHPg. Protein concentrations were 2–10 μM in 0.1 M sodium phosphate buffer (pH 7.0). The temperature was 20 °C.

with a single-exponential expression. In contrast, time courses for CO rebinding to the Fe(II) and NO rebinding to Fe(III) form of the Hb proteins were biphasic. Typical time courses for O₂ and CO rebinding to the Fe(II) form of the Hb proteins and NO rebinding to Fe(III) form of the Hb proteins are shown in Figure 3. The absorbance changes were normalized to 1 at the maximum absorbance for comparison. Absorbance changes obtained with FHPg were mostly small, and thus the traces had a smaller signal-to-noise ratio. Second-order association rate constants (*k'*) for each ligand were obtained from the slopes of the linear fits of the observed rate constants (*k*_{obs}) versus ligand concentration. The measured *k'* values are summarized in Table 2 in comparison with previously published values for these bacterial globins. The original value for *k*_{O₂}(Fe²⁺) by Orii and Webster [36] reported in 1986 is not included as it is considered to be wrong. Indeed, those authors used the *y*-axis intercept of the plot of *k*_{obs} against [O₂], which led to a wrong estimate. They also miscalculated the results from CO replacement reactions.

Owing to the impossibility of obtaining VHb and VHb-Red in the Fe(II) form without the presence of a large excess of

Table 2 Rate constants for O₂, CO and NO binding to natural and engineered FHP and VHb

Association (k') and dissociation (k) rate constants were measured at 20 °C with 0.1 M phosphate buffer at pH 7.0 as described in the Experimental section. The errors for k' are the S.D. values from the slopes of plots of the observed pseudo-first-order rate constants against ligand concentration. The errors for k are S.D. values from the mean of at least three independent measurements. Results for the slow phase of the biphasic reactions are given in italics. n.d., Not determined.

Protein	Reference	(Fe ²⁺)O ₂		(Fe ²⁺)CO		(Fe ³⁺)NO
		k' ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	k (s^{-1})	k' ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	k (s^{-1})	k' ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)
FHP	[35]	50	0.2	0.11	0.08*	2.4
	The present study	8 ± 2	0.3 ± 0.02	$2^* \pm 0.4$ <i>0.16 ± 0.05</i>	0.16 ± 0.02	$14^* \pm 5$ <i>3.0 ± 0.7</i>
FHPg	The present study	n.d.	n.d.	$6^* \pm 2$ <i>0.8 ± 0.2</i>	0.11 ± 0.01	$14^* \pm 2$ <i>4.5 ± 0.5</i>
VHb	[36]	78	n.d.	n.d.	n.d.	n.d.
	[41]	200 ± 30	$4.2^{\dagger} \pm 0.2$ <i>0.15 ± 0.04</i>	n.d.	n.d.	n.d.
	[47]	174	0.20	60 <i>2.5</i>	0.037	
	[45]	n.d.	n.d.	7.5*	0.0036	8.4*
	The present study	118 ± 34	0.3 ± 0.05	$29^* \pm 2$ <i>2 ± 0.3</i>	0.22 ± 0.07	$263^* \pm 55$ <i>38 ± 6</i>
VHb + lipid	[45]	8.8 ± 1.6	6.6	0.58‡	0.011	0.35‡
VHb-Red	The present study	83 ± 47	0.3 ± 0.05	$37^* \pm 7$ <i>5 ± 2</i>	0.09 ± 0.04	$178^* \pm 28$ <i>16 ± 5</i>

* The reactions were biphasic; the fast phase accounts for 60 % or more of the reaction.

† The reactions were biphasic; the fast phase accounts for 15 % of the reaction.

‡ Essentially monophasic.

dithionite, NO rebinding to the Fe(II) form of the Hb proteins was not measured. Dithionite and/or the products derived from its decomposition interfered with the laser flash photolysis measurements as described previously for other bacterial globins [16], preventing any reliable measurements. In fact, early studies on VHb show that when reduced by dithionite in the absence of oxygen and then exposed to oxygen, the product obtained is VHb(Fe³⁺) and not VHb(Fe²⁺)O₂ [37].

Since dissociation rate constants (k) were mostly very small, they were determined independently by stopped-flow spectroscopy for better accuracy. $k_{\text{O}_2}(\text{Fe}^{2+})$ and $k_{\text{CO}}(\text{Fe}^{2+})$ were determined by ligand displacement with CO and NO respectively. All kinetic traces could be fitted well with a single-exponential expression. All calculated k values for the different ligands studied are also summarized in Table 2 in comparison with previously published values for these bacterial globins.

Solvent accessibility and polarity of the haem pocket

Some features of the active site of these proteins were analysed with computer tools to gain a better understanding of the effect of the structure on the autoxidation and ligand-binding rates. The three-dimensional structures of only two flavoHbs (FHP, 1CQX and HMP, 1GVH) and one bacterial Hb (VHb, 1VHB, 2VHB and 3VHB) are known today. These were the proteins considered for the analysis and Mb was included for comparison. A large number of three-dimensional structures are available for Mb, six non-liganded forms from different organisms have been used (1WLA, 1BZ6, 1A6N, 1MYG, 1MYT, 1LHS) for *in silico* analysis. In this way, Mb analysis provided a better idea of the possible variation that could be expected for the other Hb proteins analysed if a larger number of structures would be available.

The solvent accessibility to the haem pocket was measured with the software CASTp [27]. The program measures the empty concavities (pockets) on a protein surface into which solvent

Table 3 Haem pocket features: size, mouth opening and solvent accessibility

Analysed with the CASTp software [27]. Three-dimensional structures considered: Mb (1WLA, 1BZ6, 1A6N, 1MYG, 1MYT, 1LHS), VHb (1VHB, chain A and B), FHP (1CQX, chain A and B), FHPg (1CQX, chain A and B, residues 1–147) HMP (1GVH) and HMPg (1GVH, residues 1–146). Mean values and standard deviations are reported.

Protein	Haem pocket		Mouth opening		
	Area* ($\text{\AA}^2$)	Volume* ($\text{\AA}^3$)	Number	Area† ($\text{\AA}^2$)	Length‡ ($\text{\AA}$)
Mb	344 ± 50	189 ± 46	1 ± 0	46 ± 20	59 ± 14
VHb	349 ± 3	166 ± 1	2 ± 0	38 ± 0	51 ± 0
FHP	2427 ± 99	2687 ± 80	8 ± 4	208 ± 68	213 ± 38
FHPg	1095 ± 13	1291 ± 18	3 ± 1	197 ± 16	132 ± 6
HMP	1787	1455	3	267	197
HMPg	393	179	3	16	37

* Solvent-accessible surface.

† Total sum of the solvent-accessible area of the mouth openings; a measure of the pore or mouth openings of the pocket.

‡ Total sum of the solvent-accessible circumference of all the pocket openings.

(probe sphere of 1.4 $\text{\AA}$; 1 $\text{\AA}$ = 0.1 nm) can gain access, i.e., these concavities have mouth openings connecting their interior with the outside bulk solution. The results are summarized in Table 3. The size and connectivity to the outside solution of the haem pocket of VHb are not significantly different from that of Mb. However, the haem pocket of flavoHbs is much bigger and has more openings to the outside compared with the pocket of VHb and Mb. In flavoHbs, the cavities that allocate the haem and FAD molecules are connected and consequently they form a single pocket. FHP has a much bigger haem pocket compared with HMP, probably as a consequence of the additional lipid molecule that accommodates in the haem distal site [1]. When the globin domain of FHP and HMP flavoHbs is analysed alone, the size of the haem

Figure 4 Haem pocket opening of FHP and FHPg

The structure on the left is FHP (1cqx); that on the right is FHPg (1cqx, residues 1–147). The Figure shows the globin domain (magenta), the reductase domain (ice blue), the haem molecule (red), FAD (yellow) and lipid (green).

Table 4 Polarity of the haem pocket

Percentage of non-polar residues in the pocket and in contact with the haem, details of distal and proximal sites. Analysed with the CASTp software [27] and LPC software [28]. Classification of amino acids according to Timberlake [29]. Three-dimensional structures considered: Mb (1WLA, 1A6N, 1BZ6, 1MYT, 1MYG, 1LHS), VHb (1VHB chains A and B, 2VHB chains A and B, 3VHB chains A and B), HMP (1GVH), HMPg (1GVH, residues 1–146), FHP (1CQX chains A and B) and FHPg (1CQX, residues 1–147, chains A and B). Means $\pm$ S.D. are reported.

Protein	Non-polar residues (%)			
	Pocket	Contact haem		
		Total	Distal site*	Proximal site*
Mb	64 $\pm$ 6	59 $\pm$ 3	55 $\pm$ 5	62 $\pm$ 5
VHb	73 $\pm$ 5	71 $\pm$ 2	90 $\pm$ 1	57 $\pm$ 3
HMP	56	61	67	56
HMPg	57	61	64	58
FHP	52 $\pm$ 0.4	55 $\pm$ 0	60 $\pm$ 0	53 $\pm$ 0
FHPg	65 $\pm$ 1.5	58 $\pm$ 0	60 $\pm$ 0	57 $\pm$ 0

* Percentage calculated considering only the residues from this site.

pocket is reduced significantly compared with the native protein. The size of the haem pocket of HMPg is not significantly different from that of VHb or Mb, whereas the haem pocket of FHPg is still significantly bigger. It has been shown that the isolated FHP globin domain keeps the phospholipid in the active site [38], thus the bigger size can be enforced by the bulky lipid that is located inside the haem pocket. From the analysis of the pocket openings, we observe that FHPg has reduced the haem pocket openings to the outside, in comparison with FHP, but the solvent-accessible area of the mouth openings remains almost invariant. As a result, FHPg has a much bigger opening to the outside than FHP. This can be appreciated in the surface molecular representation shown in Figure 4.

The polarity of the haem pocket was another aspect considered in our studies and the results are summarized in Table 4. The haem pocket of VHb shows a clear tendency to apolarity relative to the other Hbs analysed. When comparing the percentage of non-

polar residues interacting with the haem, the differences between VHb and the rest of the Hb proteins analysed become clearly significant: VHb has a much more apolar active site. If the distal and proximal sites of the haem are analysed separately, we observe that basically the differences between VHb and the rest of Hbs analysed are found in the distal site of the haem.

DISCUSSION

In previous studies we showed that bacterial Hbs and flavoHbs have different autoxidation and ligand-binding rates [16]. The main goal of the present study was to identify whether the differences in the kinetics are linked to the structure of the globin domain or to the presence or absence of a reductase domain.

Unexpectedly, purified FHPg had a green colour. In previous studies we have analysed the effect of FHPg expression in *E. coli* to improve microaerobic cell growth [17] and to alleviate nitrosative stress [13]. In none of the cases we could appreciate any colour peculiarity, and the CO differential spectrum of cellular soluble fractions was like the one of FHP. In fact, purified FHPg also gave a correct CO differential spectrum. Either the protein in those cases was also green and, due to the conditions of the assays it was not possible to detect it, or the overproduction procedures preceding the protein purification steps lead to this form of the protein. A green Hb was also obtained with the newly characterized cytoglobin (also named histoglobin) [30]. A correlation between the level of oxygen saturation during the cultivation and the proportion of green-versus red-coloured protein was observed; the higher the oxygen concentration in the culture the more green protein was produced [30]. Even though, in the present study, different growth strategies were applied to FHPg production cultures, in all cases a green Hb was obtained. Vollaard et al. [39] have recently shown that oxidatively modified haem species that do not result in a haem-to-protein cross-link can be produced under milder conditions. One of these non-covalently bound oxidatively modified haems has been characterized as a ‘green haem’ iron chlorine with a distinct optical spectrum [39]. From the computer analysis of solvent accessibility and polarity

of the haem pocket, we observe that the haem pocket of FHP is more polar and has a bigger opening to the outside compared with the one of VHb. It is conceivable that the isolated globin domain of FHP alone is more susceptible to oxidation due to the higher solvent accessibility to the haem site and the lack of an efficient reduction system. Our results are in line with the finding of Kobayashi et al. [40], who reported that the haem domain of *Candida norvegensis* flavoHb, if separated, undergoes extremely rapid autooxidation. The authors proposed that the binding of FAD as well as the presence of the reductase domain can produce extensive changes in the distal haem pocket of *C. norvegensis* flavoHb blocking solvent accessibility into the haem pocket.

The instability of FHP in the oxygenated form is due to the structure of the globin domain. The addition of a reductase domain to VHb does not destabilize the oxygenated form. Removal of the reductase domain from FHP yields an Hb with a modified haem cofactor, which cannot be kept in the oxygenated form. This is probably a common feature with all flavoHbs.

The present study shows that the kinetics of ligand binding to these Hbs is often complex. Kinetics studies with previously characterized globins have also shown that these reactions often proceed with biphasic time courses for ligand association and dissociation. In some cases, the biphasic time courses have been attributed to different conformers, as in the case of VHb [41] or mutant Mbs [42]. In trHbs, plant Hbs [43] and neuroglobin [30], the intramolecular co-ordination to yield the hexaco-ordinate state competes with rebinding of the exogenous ligand, and thus biphasic time courses are observed. In HMP and VHb [44,45], it has been shown that the presence or absence of bound lipid in the haem distal site alters the ligand-binding properties, shifting from fast biphasic time courses in the lipid-free protein to slower and monophasic time courses in the lipid-bound protein. In the present study, the presence or absence of the phospholipid was not determined. Nevertheless, both VHb and VHb-Red have the same spectra and they both have biphasic time courses; therefore they are both in the same form. We are left with the question of the cause of the biphasic time courses for some of the constants measured. The most probable explanation for the observed behaviour of the studied Hbs is the different protein conformations.

Although differences exist between our values and previously published values for VHb and FHP, they follow the tendency already described in our previous paper [16] for other bacterial Hbs and flavoHbs. No big differences were observed in dissociation rate constants. In contrast, association rate constants for O₂ and CO binding to bacterial Hbs were severalfold higher than those for flavoHbs. A similar trend is also observed for NO binding to the Fe(III) form of the proteins. On the other hand, the kinetic constants of the engineered proteins, VHb-Red and FHPg, do not differ significantly from those of their natural counterparts, VHb and FHP respectively. The differences in the kinetic constants for the same Hb protein (Table 2) can be, in part, attributed to the differences in the methodology used for the measurements and as already mentioned [44–46] to the presence or absence of lipid molecules in the binding site, which was not assessed in most of the cases.

In conclusion, the kinetics results presented here clearly indicate that the ligand-binding properties of flavoHbs and bacterial Hbs are linked to the structure of their globin domains and are not influenced by the presence or absence of a reductase domain. Possibly, only when bacterial Hbs and flavoHbs act as an NADH oxidase, does the presence of the reductase domain become important [18].

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