Structure and Ligand Selection of Hemoglobin II from *Lucina pectinata*^{*[S]}

Received for publication, June 19, 2007, and in revised form, January 10, 2008 Published, JBC Papers in Press, January 18, 2008, DOI 10.1074/jbc.M705026200

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Lucina pectinata ctenidia harbor three heme proteins: sulfide-reactive hemoglobin I (HbI_{Lp}) and the oxygen transporting hemoglobins II and III (HbII_{Lp} and HbIII_{Lp}) that remain unaffected by the presence of H₂S. The mechanisms used by these three proteins for their function, including ligand control, remain unknown. The crystal structure of oxygen-bound HbII_{Lp} shows a dimeric oxyHbII_{Lp} where oxygen is tightly anchored to the heme through hydrogen bonds with Tyr³⁰(B10) and Gln⁶⁵(E7). The heme group is buried farther within HbII_{Lp} than in HbI_{Lp}. The proximal His⁹⁷(F8) is hydrogen bonded to a water molecule, which interacts electrostatically with a propionate group, resulting in a Fe-His vibration at 211 cm⁻¹. The combined effects of the HbII_{Lp} small heme pocket, the hydrogen bonding network, the His⁹⁷ trans-effect, and the orientation of the oxygen molecule confer stability to the oxy-HbII_{Lp} complex. Oxidation of HbI_{Lp} Phe(B10) → Tyr and HbII_{Lp} only occurs when the pH is decreased from pH 7.5 to 5.0. Structural and resonance Raman spectroscopy studies suggest that HbII_{Lp} oxygen binding and transport to the host bacteria may be regulated by the dynamic displacements of the Gln⁶⁵(E7) and Tyr³⁰(B10) pair toward the heme to protect it from changes in the heme oxidation state from Fe^{II} to Fe^{III}.

Hemoglobins are key proteins in symbiotic relationships between invertebrates and chemoautotrophic bacteria. The

clam *Lucina pectinata* inhabits the sulfide-rich tropical mud and produces three different types of hemoglobin in its gills, first described by Read (1) and characterized by Kraus and Wittenberg (2, 3). Different functionalities have been described for the three hemoglobin variants: hemoglobin I (HbI_{Lp})² is a sulfide-reactive monomeric protein, whereas hemoglobin II and III (HbII_{Lp} and HbIII_{Lp}) are oxygen transporters. HbII_{Lp} and HbIII_{Lp} self-associate in a concentration-dependent manner forming a tetramer that remains unaffected in the presence of H₂S (2, 3). The mechanisms underlying ligand selection control between HbI_{Lp} and HbII_{Lp}/HbIII_{Lp} remain unknown. The structure of HbI_{Lp} has been solved with different ligands bound at the distal position: water (Protein Data Bank code 1FLP) (4), cyanide (PDB code 1BOB) (5), and hydrosulfuric acid (PDB code 1MOH) (6). The primary structure of HbII_{Lp} was determined by Edman degradation (7) and from its cDNA sequence (8). HbI_{Lp} only shares 32% of its amino acid sequence with HbII_{Lp}, but both have a conserved glutamine in the E7 position, and a phenylalanine and a tyrosine in the B10 position for HbI_{Lp} and HbII_{Lp}, respectively. The Tyr(B10) and Gln(E7) distal heme pocket residues of HbII_{Lp} are also found in other hemoglobins (8, 9). Hemoglobins from *Ascaris suum* (Hb_{Asc}) (Tyr(B10) and Gln(E7)) (10) and *Mycobacterium tuberculosis* hemoglobin N (Tyr(B10) and Leu(E7)) (11, 12) show high oxygen affinity and a very slow release of the bound oxygen. The three different hemoglobins from the clam *Scapharca inaequalis* (Hb_{Si}) show a very different heme pocket (Met(B10) and His(E7)) (13). Hb_{IIA}, _{Si} and Hb_{IIB}, _{Si} assemble to form a heterotetramer (like HbII_{Lp} and HbIII_{Lp}), whereas Hb_I, _{Si} forms homodimers (like HbI_{Lp}). The different types of hemoglobin from this clam bind oxygen cooperatively. Hb_{IIB}, _{Si} exhibits a Hill coefficient for oxygen binding of 2.1 when in a heterotetramer, whereas the homodimer has a Hill coefficient of 1.5 (14). Interestingly, even though the *S. inaequalis* hemoglobins and *L. pectinata* HbII_{Lp} and HbIII_{Lp} share ≥40% amino acid similarity, the *Lucina* hemoglobins do not behave cooperatively (2).

^{*} This work was supported by National Science Foundation Grant NSF-MCB-0544250 (to J. L. G.), National Institutes of Health NIGMS MBRS-SCORE 2 Grant S06GM08103-34 (to J. L. G.), Grants P20RR016470 and G12RR03051 from the National Center for Research Resources, National Institutes of Health, Grant BIO2006-15517-C02-02 from the Spanish Ministry of Education and Sciences, Project RMN-1344, the Junta de Andalucía (Spain) (J. A. G.), Factoría de Cristalización funded by the program Consolider-Ingenio 2010, and National Institutes of Health Grant HL65465 (to S. R. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 2OLP) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

[S] The on-line version of this article (available at <http://www.jbc.org/>) contains supplemental Figs. S1 and S2.

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² The abbreviations used are: Hb_{Lp}, *L. pectinata* hemoglobin; Hb_{Asc}, *A. suum* hemoglobin; Hb_{Si}, *S. inaequalis* hemoglobin; HbI_r, *L. pectinata* recombinant HbI; trHb, truncated hemoglobins; FPLC, fast performance liquid chromatography; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

Early biophysical studies (15) compared the structure and functionality of HbII_{Lp} and hemoglobin from *A. suum* because both heme proteins have heme pockets with Tyr(B10) and Gln(E7). The low oxygen off rates were attributed to differences in the hydrogen bonding network of both oxy-protein complexes. Additional studies using UV-visible spectroscopy and pH titration of HbII_{Lp} and a HbI_{Lp} Phe(B10) → Tyr mutant have revealed bands at 486, 541, 577, and 603 nm for both proteins at neutral conditions (3, 9). At basic pH values, the barrier for the reaction increases as the tyrosine adopts a closed conformation and the heme (Fe^{III}) hydroxyl complex replaces the met-aquo species, which suggested the existence of an open and closed conformation due to the interactions between Tyr(B10) and the heme iron. The presence of these conformers were confirmed by resonance Raman spectroscopy showing that, in a neutral environment, met-aquo HbII_{Lp} was present as a mixture of coordination and spin states with values for the ν_2 mode at 1558 cm⁻¹ (6C HS) and 1580 cm⁻¹ (6C LS) and for the ν_3 mode at 1479 cm⁻¹ (6C HS), 1492 cm⁻¹ (5C), and 1503 cm⁻¹ (6C LS) (9). The infrared spectra of the complex HbII_{Lp}CO also showed the presence of the A₃ (closed) and A₀ (open) conformers at 1924 and 1964 cm⁻¹, respectively (9). We proposed that in the open conformation Tyr(B10) swings away from the heme iron, whereas in the closed conformation Tyr(B10) is closer to and may interact with the ligand. Similarly, the reactions between hydrogen peroxide and both HbII_{Lp} and the HbI_{Lp} Phe(B10) → Tyr mutant showed that Tyr(B10) tailors, in two very distinct ways, the reactivity of compounds I and II ferryl species (9). First, increasing the reaction pH from 4.9 to 7.5, and then to 11.2, the second order rate constant for HbII_{Lp} decreases from 141.60 to 77.78 M⁻¹ s⁻¹, and to 2.96 M⁻¹ s⁻¹, respectively. This pH dependence is associated with the disruption of the heme-tyrosine (603 nm) protein moiety, which controls access of H₂O₂ to the heme protein active center, thus regulating the formation of the ferryl species. Second, the existence of a hydrogen bonding network between the heme pocket amino acids (*i.e.* Tyr(B10)) and ferryl compound I created a much faster path than 3.0×10^{-2} s⁻¹ for the decay of compound I to compound II. Moreover, the presence of Tyr(B10) in HbII_{Lp} and the HbI_{Lp} Phe(B10) → Tyr mutant appears to afford a more stable O₂ adduct in the oxygenated HbI_{Lp}. The contribution of Tyr(B10) to the stability of the HbII_{Lp} and HbI_{Lp} Phe(B10) → Tyr heme pocket against peroxide attack has recently been shown to be partially due to the presence of hydrogen bonding between the ferryl moiety and the heme pocket amino acids, including Tyr(B10), which ultimately enhances the removal of peroxide by the peroxidative cycle (16, 17). In addition, the close proximity of Tyr(B10) with Gln(E7) to the heme iron contributes largely to the distal control of NO binding, thus providing a model for the design of future oxidative stable oxygen hemoglobins with little or no vasoactivity (16).

Here, we report detailed structural data of HbII_{Lp} obtained by x-ray crystallography, providing information about the heme pocket, distal amino acids, and their interaction with the dioxygen molecule. Our results, supported by resonance Raman measurements, suggest a mechanism where HbII_{Lp} selects oxygen by tailoring the hydrogen bonding environment

between the Tyr(B10) and Gln(E7) and heme-O₂ moiety, stabilizing the heme (Fe^{II}) oxidation state.

EXPERIMENTAL PROCEDURES

Wild-type HbI_{Lp} and HbII_{Lp} Sample Preparations—Proteins were isolated and purified as described with minor modifications (9). HbI_{Lp} was separated from HbII_{Lp}/HbIII_{Lp} using a Hi Load 26/60 Superdex 200 gel filtration column (AKTA FPLC, Amersham Biosciences). HbI_{Lp} was further purified by cation exchange chromatography using DEAE Sephadex Fast Flow equilibrated with 25 mM ammonium bicarbonate buffer, pH 8.3. HbII_{Lp} was purified from the HbII_{Lp}/HbIII_{Lp} fraction by ion exchange chromatography with a HiPrep 16/10 Q FF column equilibrated with 10 mM triethanolamine/acetate buffer at pH 8.3 and eluted with a gradient of sodium chloride concentration from 0 to 180 mM. The purity of the proteins was confirmed by SDS-PAGE.

Recombinant HbI_{Lp} and Site-directed Mutant Preparation—The mutants from *L. pectinata* HbI (HbI_{Lp}Phe(B10) → Tyr, HbI_{Lp}Gln(E7)Val, HbI_{Lp}Gln(E7) → Asn, and HbI_{Lp}Gln(E7) → His) were obtained by introducing single amino acid substitution using the QuikChange Mutagenesis kit (Stratagene, La Jolla) into the HbI_{Lp} coding region cloned into the pET28(a+) vector (18). The HbI_{Lp} mutants were expressed in *Escherichia coli* Bli5 cells as described (18). Dark red cell pellets were lysed and centrifuged to separate the soluble from the insoluble fractions. The soluble fraction was equilibrated with CO and purified in Co²⁺ affinity columns (Talon, Invitrogen) followed by size exclusion chromatography (AKTA FPLC, Amersham Biosciences) (9). The purified HbI_{Lp}Phe(B10) → Tyr showed UV-visible spectra typical of an oxyHbII_{Lp}. Met-aquo and CO HbI_{Lp}Phe(B10) → Tyr derivatives were obtained in a similar manner as the HbII_{Lp} complexes (18).

HbII_{Lp} Sequence Analysis—Amino acid sequences were obtained from the NCBI protein sequence data base. The accession numbers for *L. pectinata* hemoglobins are HbI AAG01380, HbII AAO89499, and HbIII AAB28352. Data base searches for sequences with high sequence similarity was performed using the HbII_{Lp} sequence and PSI-BLAST (19). Multiple sequence alignments were performed with ClustalW (20). Sequence alignments were used to assess the variation at specific positions and the resulting differences in the overall structure and distal heme cavity.

Crystallization and Data Collection—HbII_{Lp} was crystallized as previously reported (21). In brief, lyophilized HbII_{Lp} was dissolved in 50 mM BisTris propane pH 7.0 buffer, 0.5 mM EDTA in a final concentration of 30 mg/ml. The crystals were grown by the counter-diffusion technique with a three-chamber configuration (22) using ammonium sulfate as precipitant. X-ray diffraction intensity data were collected at the BM-16 station of the European Synchrotron Radiation Facility (ESRF) using a 0.97-Å wavelength in a Mar CCD-165 detector. Data were indexed, integrated, and scaled with HKL2000 suite (23) at a resolution limit of 1.93 Å.

Structure Solution and Refinement—The structure of HbII_{Lp} was solved by molecular replacement methods as reported in Ref. 21. In brief, coordinates from HbI_{Lp} (PDB code 1EBT) without the water molecule present in the distal position of the

TABLE 1

Data collection and refinement statistics of HbII_{LP}-O₂ structure

Statistical values for the highest resolution shell, 1.96–1.93 Å for data collection and 1.98–1.93 Å for refinement, are given in parentheses.

Data collection	
Wavelength (Å)	0.977
Temperature (K)	100
Space group	P4 ₂ 2 ₁ 2
<i>a</i> = <i>b</i> , <i>c</i> (Å)	73.92, 152.35
α, β, γ (°)	90
Monomers per asymmetric unit	2
Resolution (Å)	20.0–1.93
No. of observed reflections	338,327
Redundancy	10.5 (10.6)
Completeness (%)	99.6 (100.0)
<i>R</i> _{merge} ^a (%)	5.0 (33.6)
Average <i>I</i> /σ(<i>I</i>)	40.0 (7.0)
Refinement	
<i>R</i> value (%)	16.5 (17.5)
<i>R</i> _{free} value (%)	19.3 (22.5)
No. of reflections in working set	30,675 (2158)
No. of reflections in test set	1,643 (137)
No. of solvent molecules	283
Average <i>B</i> factor (Å ²)	30.42
Root mean square deviations	0.013
bond length (Å)	
bond angles (°)	1.841
Ramachandran plot	
Most favored regions (%)	94.8
Allowed regions (%)	5.2
General allowed regions (%)	0.0
Disallowed regions (%)	0.0

$$^a R_{\text{merge}} = \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_{hkl} \sum_i I_i$$

heme iron were used as a search model and the molecular replacement solution was found using the CNS suite (24). Refinement was conducted using CNS and REFMAC5 software from CCP4 (25). After several cycles of restrained refinement, manual model building against the electron density maps was conducted with the program COOT (26). Once all the residues were replaced by the HbII_{LP} sequence, the *R*-factor and *R*-free decreased to 0.30 and 0.36, respectively. Additional refinement was carried out with REFMAC5 using the TLS parameters (27). The inclusion of TLS parameters in the refinement process improved the *R*-factor and *R*-free to a final value of 0.17 and 0.19, respectively. Water molecules were placed in electron density difference maps using the ARP/wARP version 5.0 program from the CCP4 suite. All the structures were checked using the refinement with MolProbity (28), and before deposition using PROCHECK (29). Details on data collection and structure refinement are summarized in Table 1. The secondary structure was tested with iMolTalk (30), whereas β-turn geometry was calculated using the program PROMOTIF version 2.0 (31). Superposition and root mean square deviations of the structures were performed using the CCP4 program LSQKAB (32). Protein interfaces in the crystal were characterized using the PISA server (33). Distances between amino acids were calculated using the program CONTACT from CCP4 (25). The accessible surface areas values were computed with NACCESS (34) with a probe radius of 1.4 Å and a slice width of 0.05 Å. Protein cavities were computed using the CASTp server (35). The coordinates and structure factors of *L. pectinata* HbII were deposited at the RCSB PDB with entry code 2OLP.

Potassium Ferricyanide Titration and Resonance Raman Spectroscopy—Deoxyhemoglobin was obtained by adding sodium dithionite to the purified protein samples, followed by a

FIGURE 1. Structure of the HbII dimer from *L. pectinata*. Each helix, A to H, is labeled and identified by the colors: red, blue, yellow, magenta, orange, pink, light blue, and salmon, respectively.

purification step in a Hi-Trap desalting column (FPLC, GE Healthcare). Oxyhemoglobin complexes were obtained by flushing the deoxy derivatives with oxygen. The initial concentration for the oxyHb_{LP} species was 3.40 μM, at pH 7.5. The titration of oxyHb_{LP} was initiated with the addition of 15-μl aliquots of a 10% potassium ferricyanide solution (2) up to a total volume of 150 μl to obtain the met-aquo HbI_{LP}. Full oxidation of HbII_{LP} and the HbI_{LP}Phe(B10) → Tyr mutant proteins required a decrease of pH from 7.5 to 5.0, because the above conditions were not adequate. The resonance Raman measurements were made by focusing the output of a krypton ion laser at 413.1 nm (Spectra Physics) to a ~30-μm spot on a rotating cell to prevent photodamage. The laser power was set at 10 milliwatts using a CCD back-illuminated detector (800 × 2000 pixels) coupled to a modified Spex 1401 centered at 410 nm. Three spectra were collected, each composed of 60 accumulations of 10 s. Hemoglobin concentration was ~100 μM.

RESULTS

HbII_{LP} Structure and Sequence Analysis—The HbII_{LP} crystallizes in space group P4₂2₁2, and diffracts x-rays with a resolution better than 2.0 Å, having unit cell parameters *a* = *b* = 73.92 Å and *c* = 152.35 Å, and two molecules forming a dimer in the asymmetric unit and a solvent content of 61% per volume. All the residues are placed in the most favorable region of the Ramachandran plot. HbII_{LP} shows the characteristic globin fold with six α-helices surrounding the central heme pocket, and two minor helical segments between B and E helices (Fig. 1). The distance between the iron atoms in the dimer is 17.8 Å, and the plane orientation of the porphyrin ring is almost perpendicular. This short distance between the heme groups is a characteristic feature of the EF-dimers in other types of molluscan hemoglobin (36). The dimer interface includes 25 residues of each monomer and covers a surface of 845 Å². At the interface, four hydrogen bonds, namely, Lys^{95B}–Ser^{46A} (2.77 Å),

	A-helix	B-helix	C-helix	
HbII <i>L. pectinata</i>	-----TT LT--NPKAA IRSSWSKRM	NEVSNQGGF MDLFKAFET		40
HbIII <i>L. pectinata</i>	-----SSG LT--GPQKAA LKSSWSRFRD	NAVNTGNTNF MDLFKAYPDT		41
HbI <i>L. pectinata</i>	-----SLEAAQKSN VTSSWAKASA	AWGTAGPEEF MALFDAHDDV		39
HbI <i>S. inaequivalvis</i>	MPSVYDAAQ LT--ADVKKD LRDSWKVIGS	DKKNGVALV TFLFADNQET		48
HbIIA <i>S. inaequivalvis</i>	--XVDAAVAK VCGSEAIKAN LRSSWGVLSA	DIEATGLML SNLFLTRPDT		48
HbIIB <i>S. inaequivalvis</i>	--SKVAELANA VVSNADQKDL LRMSWGVLSV	DMEGTGLML ANLFKTSPPA		49
Hb domainI <i>A. suum</i>	-----A NKTRELCKMS LEHAKVDTSN	EARQDGLIDL KHMFFENYPL		41
	C-helix	D-helix	E-helix	F-helix
HbII <i>L. pectinata</i>	LTPEKSLFGE ITLALQDNE KMKRSLVFC	NGMSSFVHL	DNDMLVYL	90
HbIII <i>L. pectinata</i>	LTPFKSLFED VSFNQMTDHP TMKRALVFC	DGMSSFVDNL	DDHEVLVVL	91
HbI <i>L. pectinata</i>	FAKESGLFSG AAKGTVKNTE EMARQSEK	GLVSNVVDNL	DNAGALEGC	89
HbI <i>S. inaequivalvis</i>	IGYFKRLG-- -DVSQGMAND KLRGHSITLM	YALQNFIDQL	DNPDDLVCVV	95
HbIIA <i>S. inaequivalvis</i>	KTYFTRLG-- -DVQGMKANS KLRGHATITL	YALNNFVDSL	DDPSRLKCVV	95
HbIIB <i>S. inaequivalvis</i>	KGKFARLG-- -DVSAGKDNS KLRGHSITLM	YALQNFVDAL	DDVERLKCVV	96
Hb domainI <i>A. suum</i>	RKYTSREYY -TAEDVQNDP FFAMGSKIL	LACHVLCATY	DDRETFNAT	90
	F-helix	G-helix	H-helix	
HbII <i>L. pectinata</i>	QAMAKLHNE GIFA--SDLR TAYDILIHVM	EDHN--HMG	GAKDAWEVY	136
HbIII <i>L. pectinata</i>	QAMAKLHNE GIRI--KEIR DGYGVLLAYL	EDHC--HVEG	STKNWEDFI	137
HbI <i>L. pectinata</i>	RTFAANHEK GISA--GQLE AAFKVLGSEM	KSYG-----	GDEGAVFA	131
HbI <i>S. inaequivalvis</i>	EXFAVNHIT KISA--AEFG KINGENKVL	ASKN--FGD	KYANAWAKLV	140
HbIIA <i>S. inaequivalvis</i>	EXFAVNHIN KISG--DAFG AIVEEMKETL	KARMGNVYSD	DVAGAWAALV	143
HbIIB <i>S. inaequivalvis</i>	EXFAVNHIN QISA--DEFG EIVGPLRQTL	KARMGNVYSD	DTVAWASLV	144
Hb domainI <i>A. suum</i>	RELLDRHAR HVHMPFEVMT DFWKLFEEYL	GKKT--TLDE	PTKQAWHEIG	138
	G-helix			
HbII <i>L. pectinata</i>	GFICKTLGDY MKELS	151		
HbIII <i>L. pectinata</i>	AYICRVQGDY MKERL	152		
HbI <i>L. pectinata</i>	GALMGEIEPD M----	142		
HbI <i>S. inaequivalvis</i>	AVVQAAL----	147		
HbIIA <i>S. inaequivalvis</i>	GVVQAAL----	150		
HbIIB <i>S. inaequivalvis</i>	AVVQASL----	151		
Hb domainI <i>A. suum</i>	REFAKEINKH GR---	150		

FIGURE 2. Multiple sequence alignment of the three types of hemoglobin from *L. pectinata*, the bivalve mollusk *S. inaequivalvis*, and domain one of the nematode *A. suum* hemoglobin. The corresponding helices as predicted by the program suite iMoltalk applying the STRIDE method are labeled (red squares). Conserved residues are shaded in cyan and residues at position B10 (Tyr³⁰ in *L. pectinata*), E7 (Gln⁶⁵ in *L. pectinata*), Lys⁹² and Arg¹⁰⁰ (*L. pectinata*) are shaded in yellow. The gaps are labeled in green.

FIGURE 3. Stereo view of the hydrogen bond network in the oxygen bound HbII_{LP} structure. Residues at a 5-Å distance cut-off are shown. The omit map (contoured at 3 σ) and bond interactions are shown in blue and green lines, respectively. Distances between residues are shown in Table 2.

Lys^{95A}–Ser^{46B} (2.98 Å), Lys^{92B}–Gln^{65A} (2.73 Å), and Lys^{92A}–Gln^{65B} (2.66 Å), and one salt bridge Asp^{82B}–Lys^{63A} (3.05 Å) are found. It is noteworthy that the HbII_{LP} structure confirms the existence of one aspartate in position 84 and an acetyl group in the N terminus as previously suggested by comparison of matrix-assisted laser desorption/ionization-mass spectrometry results and the cDNA-derived amino acid sequences (8).

The sequence alignments of the hemoglobins of *L. pectinata*, *S. inaequivalvis*, and *A. suum* are shown in Fig. 2. The multiple alignment has three gaps: one between the C and D helices, a second one within the G helix, and the third gap after the G helix. The B10 residue varies from a Phe in HbI_{LP}, Tyr in HbII_{LP}, HbIII_{LP}, and Hb_{Asc} to a Met in the three types of hemoglobins from *S. inaequivalvis*, whereas the E7 position contains a His

residue in all the *S. inaequivalvis* hemoglobins and a Gln in the rest of the aligned polypeptides. In the HbII_{LP} structure, the Arg¹⁰⁰(F11) and Lys⁹²(F3) form H-bonds with the propionates of the heme group. The Lys⁹²(F3) and Arg¹⁰⁰(F11) are conserved in all the hemoglobin sequences, except in Hb_{Asc} that are replaced by Glu⁹² and Asp¹⁰⁰, respectively. The replacement of Lys⁹² by a Phe⁹³ in the HbI_{LP}, which cannot form the salt bridge or hydrogen bonds, suggests that Lys⁹² could be responsible for the HbII_{LP} dimer stability.

HbII_{LP} is known to form large aggregates at high concentrations (2). Analysis for protein complex assembly using the PISA Server (31) predicted two additional assemblies: a dimer of dimers to form a tetramer, and a dimer of the tetramer to form an octamer, both of low predicted stability. The superposition of the HbII_{LP} dimer with HbI_{Si} has an overall average root mean square deviation of 2.2 Å (supplemental Fig. 1). The tetrameric HbII_{AB, Si} in the form of two heterodimers is composed by chains HbII_{A, Si} and HbII_{B, Si} (37). The hypothetical HbII_{LP} tetramer in the unit cell is different from that of the *S. inaequivalvis* hemoglobin.

A channel of water molecules is buried in the HbII_{LP} dimer interface, 40 of them located at a distance <10 Å of the heme groups. They form H-bonds with several residues located between the heme groups in the dimer. This interfacial water has been previously reported in the homodimeric HbI_{Si} contained in

the coelomic fluid erythrocytes from the bivalve mollusk *S. inaequivalvis* (38) and they have been proposed to play an important role in the allosteric behavior of hemoglobin HbI_{Si} (39).

The major differences between chains A and B in the *L. pectinata* HbII dimer are found in the β -turn formed by residues Gly¹²²–Gly¹²³–Leu¹²⁴–Thr¹²⁵ with a maximum root mean square deviation of 3.4 Å. The β -turn is a type I in chain A, and a type IV in chain B. This difference in conformation arises from a difference in the environment, the β -turn has several contacts with the symmetry related molecule in chain A, whereas in chain B, it is exposed to the solvent. This β -turn is also one of the major differences between the HbII_{LP} and HbI_{LP} structures.

TABLE 2

Heme contacts in HbII_{LP}-O₂ structure

Residue 1	Atom	Residue 2	Atom	Distance (Å)	
				Chain A	Chain B
HEM	O1A	Arg ¹⁰⁰	NH ₂	2.71	2.52
		W6/W7	O	2.62	2.57
	O2A	Arg ¹⁰⁰	NH1	2.98	2.88
		Arg ¹⁰⁰	NH ₂	3.47	3.34
	O1D	W9/W2	O	2.59	2.63
		Lys ^{92a}	NZ	2.63	2.72
	O2D	W43/W5	O	2.64	2.67
		W8/W3	O	2.62	2.66
		W34/W5	O	2.59	3.41
		W43/33	O	3.42	2.62
Fe	Fe	W57/139	O	2.96	2.98
		His ⁹⁷	NE2	2.08	2.04
		Oxy	O1	2.94	2.85
		Oxy	O2	2.11	1.69
		Gln ⁶⁵	NE2	3.00	3.07
		Oxy	O1	1.94	1.78
Tyr ³⁰	OH	Oxy	O2	2.99	2.95
		Met ⁹³	O	2.98	2.79
His ⁹⁷	ND1	W9/W2	O	3.45	3.41
		Lys ^{92a}	NZ	2.73	2.66
Q65A	NE2	Tyr ³⁰	OH	3.00	3.07
		Oxy	O1	3.02	3.04
		Oxy	O2	3.43	3.51

^a Marked contacts are inter-polypeptides chains.

HbII_{LP} Distal and Proximal Heme Pocket—Fig. 3 shows the structure and the hydrogen bonding network of the oxyHbII_{LP} proximal and distal sites. The porphyrin ring adopts a planar configuration with the iron atom in the plane, and the average distance of the pyrrolic nitrogens to the iron is 2.06 Å. The dioxygen forms an angle of 123° with the iron atom in chain A, and 162° in chain B. The heme pocket in chain B is slightly smaller than in chain A and the distance between the OH of the distal tyrosine and the iron atom is shorter (4.84 Å in chain A and 4.53 Å in chain B, but the differences in the volume size of the heme cavity are 912.6 Å³ in chain A and 831.9 Å³ in chain B) might arise from small differences in the position of the atoms as a consequence of the average coordinates position. In the HbII_{LP} heme cavity most of the aromatic residues are conserved when compared with HbI_{LP} (for instance, Trp(A11), Phe(B9), Phe(C7), Phe(CD5), Phe(E11), and Trp(H8)), except Tyr(B10), Tyr(G8), and Tyr(G14), which are replaced by phenylalanine in the HbI_{LP} sequence, and Phe(E18) that is replaced by tryptophan. Although the HbII_{LP} proximal heme pocket is mainly hydrophobic, it contains one buried water molecule (W⁹). This water molecule forms a hydrogen bond with the heme propionate group (O2A) within a distance of 2.59 Å and with His⁹⁷(ND1) within a distance of 3.45 Å (Table 2). It is also part of the water network in the interface of the HbII_{LP} dimer. A water molecule is also present in the heme pocket of the HbI_{LP} as part of an identical hydrogen network.

When HbI_{LP} and HbII_{LP} are superimposed using the heme group as a reference, the HbI_{LP} as well as the proximal histidine show a displacement of ~1 Å. The orientation of the proximal His⁹⁷(F15) is roughly perpendicular to the heme plane and the average distance between the His⁹⁷(NE2) atom and the iron atom is 2.06 Å, which is a slightly shorter distance when compared with the sulfide (PDB code 1MOH), cyanide (PDB codes 1ETB and 1B0B), and water-bound HbI_{LP}, where the distances range from 2.13 to 2.31 Å. It has been suggested that within the various kinds of hemoglobin and myoglobin a hydrogen bond

FIGURE 4. Resonance Raman spectra of deoxy samples of (a) HbI_{LP}, (b) HbI_{LP}Phe(B10) → Tyr, and (c) HbII_{LP} showing the Fe-His stretching mode and trans-effect of the proximal His.

between the proximal histidine His(F8) and the side chain of a residue next to it modulates the strength of the His-Fe bond, regulating the ligand affinity (37, 40). In HbII_{LP}, His(F8) has two possible hydrogen bond interactions, the first one with the buried water molecule and the second one from the interaction of the carbonyl group of Met⁹³ and the ND1 from histidine (Table 2). A similar hydrogen bond is formed between the carbonyl of Met⁹³ and the histidine ND1 atom in HbI_{LP}.

Heme Oxidation and Resonance Raman Spectra of Oxy Species—HbI titration with potassium ferricyanide required an additional oxidizing agent to complete oxidation to the met-aquo HbI_{LP} form, shifting the Soret band from 416 nm (oxyHbI_{LP}) to a maximum at 407 nm (supplemental Fig. 2). A pH-dependent titration for oxyHbII_{LP} and the oxyHbI_{LP}Phe(B10) → Tyr mutant required a decrease in pH from 7.5 to 5.5 to completely change the Soret band from 414 to 403 nm, a characteristic of the met-aquo HbII_{LP} species. This result suggests that Tyr in the B10 position is responsible for the pH oxidation control of oxyHbII_{LP} and the oxyHbI_{LP}Phe(B10) → Tyr mutant. Similarly, Fig. 4 compares the low frequency resonance Raman spectra of ferrous deoxygenated recombinant HbI_{LP}, HbI_{LP}Phe(B10) → Tyr, and HbII_{LP}, which provides information on the Fe-His stretching mode (ν_{FeN}) and the trans-effect of the proximal histidine on the Fe-O₂ mode. The vibrational frequencies (Fig. 4, a and b) for HbI_{LP} and the HbI_{LP}Phe(B10) → Tyr mutant are located at 219 cm⁻¹, similar to the myoglobin spectrum. However, for HbII_{LP} the ν_{FeN} is present at a lower frequency, 211 cm⁻¹ (Fig. 4c), suggesting that the factors controlling the strength of the Fe-N bond are not the same for both sets of hemoproteins. Normal mode assignments were based on the presence or absence of a particular frequency on the spectra for the HbI_{LP}-ligand complexes and compared with the values reported in the literature (17, 41). The ν_{FeN} for hemoglobin and myoglobin has been found in the resonance Raman spectra between the 217 and 244 cm⁻¹ regions (17, 41, 42). The spectra also show a band at 370 cm⁻¹, which has pre-

FIGURE 5. A, resonance Raman spectra of oxy species HbII_{LP}, HbI_{LP}, and HbI_{LP} mutants. B, oxygen isotopic shift for the Fe-O₂ normal mode vibration of the oxyHbII_{LP}, oxyHbI_{LP}, and oxyHbI_{LP} mutants.

viously been attributed to the C_β-C_c-C_d bending motion of the peripheral propionate heme substituents in HbI_{LP}. The spectra for HbI_{LP} and HbI_{LP}Phe(B10) → Tyr also show the ν₈ band at 347 cm⁻¹. This is characteristic of the combination of the metal-pyrrole stretch and the in-plane substituent bend, and a shoulder at 331 cm⁻¹ that has been assigned to the out-of-plane pyrrole-tilting mode. In contrast, in HbII_{LP} the spectral bands have been displaced to 350 and 310 cm⁻¹, respectively.

Fig. 5A shows the vinyl region and the Fe-O₂ vibration for HbII_{LP}, recombinant HbI_{LP} and a series of HbI_{LP} mutants. Some differences in their vibrational modes are observed as function of the polarity of the heme pocket and oxygen isotopic substitution. For example, the recombinant HbI_{LP} (Fig. 5A, a) shows a relatively strong band at 412 cm⁻¹ with a small shoulder at 421 cm⁻¹. The former corresponds to an in-plane vinyl-bending mode (δC_βC_αC_β-vinyl), whereas the latter is attributed to either a contribution from both vinyls or the same vinyl reflecting heterogeneity among heme subunits. Only small intensity changes are observed as a function of amino acid substitution. Similarly, the ν₈ band is found at 345 cm⁻¹, which is invariant among the different hemeprotein derivatives. The shoulder of the out-of-plane pyrrole-tilting mode at 333 cm⁻¹ is almost absent in the samples. Fig. 5B shows the ¹⁶O₂-¹⁸O₂ isotope difference spectra, where the Fe-O₂ stretching mode of the heme O₂ moiety presents a significant frequency change as a function of the heme pocket amino acids. Thus, the spectrum of recombinant HbI_{LP} (Fig. 5B, a) shows a band at 567 cm⁻¹, which was previously attributed to the Fe-O₂ stretching mode, and in this work, has been confirmed by the oxygen isotopic substitution. Changing the residue at the E7 position in the HbI_{LP}Gln(E7) → Val and HbI_{LP}Gln(E7) → Asn mutants (Fig. 5B, b and c) produced a decrease in the stretching mode to 563 cm⁻¹, whereas changing the residue at the E7 position with the HbI_{LP}Gln(E7) → His or at the B10 position in HbI_{LP}Phe(B10) → Tyr mutants (Fig. 5B, d and e) increased the frequency of the Fe-O normal mode to 569 and 572 cm⁻¹, respectively. For the wild-type HbII_{LP} this frequency is present at 573 cm⁻¹ (Fig. 5B, f), suggesting that the oxygen in the heme O₂ moiety is strongly hydrogen bonded to the

distal pocket Tyr³⁰(B10) and Gln⁶⁵(E7), in good agreement with the proposed x-ray structure.

DISCUSSION

The crystal structure of oxyHbII from *L. pectinata* has been solved and it shows relevant differences when compared with the ferric HbI_{LP} sequence and structure (the oxyHbI_{LP} structure has not been resolved yet). The overall hemoglobin fold is conserved in these clam proteins. Some important differences between them are found in the additional residues in the sequence of HbII_{LP} and the flexible β-turn formed by the GGLT residues. The heme cavity is conserved between the HbI_{LP} and HbII_{LP} forms, with the exception of the B10 position, which is occupied by Phe and Tyr, respectively. A significant displacement of the heme group is observed in the least square superimposition of both types of hemoglobins. The heme group is buried farther in HbII_{LP} than in HbI_{LP} because of the minor steric hindrance of the Met⁹³ and Phe⁷⁶ residues that are replaced by Phe⁹² and Trp⁷⁵, respectively. Oxygen is tightly bound to the HbII_{LP} heme distal site through simultaneous hydrogen bonds with Tyr(B10) and Gln(E7) (Fig. 3). This last pair of amino acids has been proposed to stabilize the oxygenated form of HbII_{LP} by means of strong and weak hydrogen bonds, respectively (37, 40). Hb_{Asc} also has a Tyr(B10) and a Gln(E7), and a high oxygen affinity and unusually slow off rate for oxygen dissociation (15, 41). The Hb_{Asc} crystal structure (PDB 1ASH) shows that the distance between the iron to oxygen O1 is 1.90 Å, and between Tyr(B10)(OH) to O₂ is 2.73 Å (40). Table 2 presents several distances between the oxygen heme moiety and the amino acids in the distal pocket of HbII_{LP}. For example, the iron to oxygen O1 is 2.94 Å, and from Tyr(B10)(OH) to O₂ is 2.99 Å. These longer distances are rather similar to those found in the structure of *M. tuberculosis* hemoglobin N (12). The orientation of the dioxygen bound to the heme plane is 110°, similar to the value found in the HbII_{LP} structure, but in this case, the oxygen molecule is pointing toward the Tyr(B10)(OH) group. A CASTp calculation resulted in an average volume of 1000 Å³ for the heme cavity in *M. tuberculosis* hemoglobin, which is slightly larger than the average size of 872 Å³ for HbII_{LP}. Similarly, a large cavity volume was observed in the ferric derivative of the Mb triple mutant (L29F/H64Q/V68F), Mb, and HbI_{LP}. The crystal structure reveals the existence of a much greater heme freedom and larger distal cavity volume in HbI_{LP} than sperm whale Mb, in both the H₂S bound and unbound to the heme group, because of the lack of hydrogen bonding between the heme propionate groups and nearby amino acid residues (43). This dynamic behavior is absent in HbII_{LP}, where the heme group is firmly anchored in place.

Cooperativity and Allosteric Effects—In contrast to a remarkably conserved tetrameric arrangement in vertebrate hemoglobins, invertebrate hemoglobins show a large diversity in quaternary structures (36). The aggregation state of the hemoglobin plays a central role in their cooperative behavior. Hemoglobins from *L. pectinata* show that HbI_{LP} is a monomer in all of the conditions assayed, whereas HbII_{LP} and HbIII_{LP} behave as apparent monomers at low concentrations and aggregates at higher concentrations (2). Lys⁹² of HbII_{LP} may play an impor-

tant role in dimer stability, because a Thr that disrupts the possibility to form a bond between chains replaces this residue in the HbI_{Lp} sequence. Lys⁹² is conserved in HbIII_{Lp} opening up the possibility to form a heterodimer as occurs in *S. inaequalis* hemoglobins. The oligomeric hemoglobins from *S. inaequalis* show cooperativity in oxygen binding. However, a Hill coefficient of 1.1 was obtained for the oxygen binding measurement of HbII_{Lp}, regardless of the aggregation state (2, 3). Several mutant forms of the HbI_{Si} dimer have structurally characterized, suggesting that two residues play an important allosteric role. First, Phe⁹⁷(F3) is packed tightly in the heme pocket in the deoxy HbI_{Si} state, and exposed to the dimer interface upon ligand binding (44). The displacement of the bulky Phe⁹⁷ side chain to the dimer interface displaces several water molecules leaving the interface with less water and a poorer ordered water network. A similar behavior has been described for the heterotetramer of HbII_{IA, Si} and HbII_{IB, Si} in which mutation of the Phe⁹⁷ to leucine provokes a loss of cooperativity (44). Another residue playing a determinant role is Thr⁷², and both Phe⁹⁷ and Thr⁷² are replaced in HbII_{Lp} by methionine and valine, respectively.

The superpositions of the HbII_{Lp} structure to the oxygen bound (PDB code 3SDH) and unbound (PDB code 4SDH) hemoglobin states of the *S. inaequalis* show that Asn¹⁰⁰ in these hemoglobins is replaced by Leu⁹⁶ in HbII_{Lp}, leading to the loss of a strong hydrogen bond between Asn¹⁰⁰ and the heme propionate group. Moreover this propionate moiety of the heme group in *S. inaequalis* shows an important displacement between the oxygen-bound and unbound forms. In particular, the orientation of the unbound form is equivalent to the orientation of the propionate in the oxyHbII_{Lp} structure. The interaction with the nearest residues is the same in Hb_{Sp}, even a water molecule equivalent to the water molecule (W⁹) bound to His⁹⁷ (Fig. 3) is found at a distance of 2.52 Å. Interestingly, this water molecule disappears in the oxygen-bound structure of *S. inaequalis*. This observation supports the hypothesis that this water molecule plays an important role in the regulation of the heme group affinity to ligands.

Geometry of the Heme-Oxygen Moiety and Hydrogen Bonding Network—The hydrogen bonding network between the heme bound oxygen and the Gln(E7) and Tyr(B10) of HbII_{Lp} is almost identical to the active center of Hb_{Asc}. In both cases, it has been proposed that the hydrogen bonding network is responsible for their extremely low oxygen dissociation rates (15, 41). Despite this analogy, the oxygen dissociation rate constant for HbII_{Lp} and Hb_{Asc} shows a difference of 2 orders of magnitude (0.11 and 0.004 s⁻¹, respectively). This suggests that other factors, like the His⁹⁷(F8) trans-effect and its orientation, could play an important role in determining this rate difference. The resonance Raman measurements allows a comparison between the Fe-His stretching mode of Mb, HbII_{Lp}, and Hb_{Asc}, at 220, 211, and 202 cm⁻¹, respectively, whereas for recombinant HbI_{Lp} and the HbI_{Lp}Phe(B10) → Tyr mutant, the Fe-N stretching mode is characterized by a band at 219 cm⁻¹ (Fig. 4). The Fe-His normal mode falls into the group of heme proteins that have a neutral proximal histidine, characteristic of those responsible for transporting oxygen, where the proximal histidine environment differs for these three hemoproteins. The strength of the

FIGURE 6. Oxygen heme moiety. Least square superposition of HbII_{Lp} (yellow) and Hb_{Asc} (cyan, PDB code 1ASH). The superposition was carried out taking into account all the residues in the structure (amino acids and prosthetic group). The water molecule forming a hydrogen bond between the propionate group and His⁹⁷(F8) in HbII_{Lp} is also shown. This water molecule is not present in the oxygen bound structure of Hb_{Asc}. The oxygen molecule shows opposite orientation in both structures (the oxygen molecule in Hb_{Asc} is shown in clear cyan).

hydrogen bond formed by a nitrogen proton in the proximal histidine and the polarity of the environment are powerful factors for determining the Fe-His stretching frequency in the heme-histidine moiety. This hydrogen bonding network in hemoglobins and myoglobins has also been related to ligand affinity (37, 40). The significant differences observed in the heme-ligand dissociation rate constants between Mb and Hb_{Asc} have mainly been attributed to the tilted orientation of the His(F8) with regard to the pyrrole rings of the heme (45). Fig. 6 shows an overlay of the oxy heme moiety of Hb_{Asc} and HbII_{Lp} indicating several structural differences in the orientation of the proximal histidine, the bound oxygen, propionates, and vinyl groups. In particular, the presence of a water molecule between the propionate group of heme and His(F8) in HbII_{Lp} suggests that the Fe-His strength and the His-Fe trans-effect may be modulated by this hydrogen bond. Furthermore, the orientation of His(F8), as suggested for Hb_{Asc}, can also contribute to the observed Fe-His frequency. The combination of these factors facilitates the strength of the Fe-O₂ complex in the distal heme pocket. Moreover, the small distal cavity volume of HbII_{Lp} and its hydrogen bonding network with the heme-O₂ moiety is consistent with the relationship between the Fe-O₂ stretching mode and its oxygen dissociation rate constant (*k*_{off}) (46). This is supported by data in Table 3, which shows a rough inverse relationship between these two properties for HbII_{Lp}, HbI_{Lp}, and several HbI_{Lp} mutants. For example, oxyHbII_{Lp} and oxyHbI_{Lp} show Fe-O frequencies at 572 and 567 cm⁻¹ correlated with an oxygen dissociation rate constant (2) of 0.11 and

TABLE 3
vFe-O₂ and dissociation rate constants (*k*_{off}) for HbII_{Lp}, HbI_{Lp}, and HbI_{Lp} mutants

Heme protein	<i>v</i> _{FeO₂} cm ⁻¹	<i>k</i> _{off} s ⁻¹
HbI (Gln(E7), Phe(B10), Phe(E11))	567	61.0 ^a
HbI _r (Gln(E7), Phe(B10), Phe(E11))	567	140.0
HbIGln(E7) → Val	563	500.0
HbIGln(E7) → Asp	563	375.0
HbIGln(E7) → His	569	3.0
HbIPhe(B10) → Tyr	571	0.60
HbII (Gln(E7), Tyr(B10), Phe(E11))	572	0.11 ^a

^a From Ref. 2.

61 s⁻¹, respectively. Similarly, HbI_{Lp}Gln(E7) → Asp and HbI_{Lp}Gln(E7) → Val mutants show an increase in the dissociation rate constant from 140 s⁻¹ (for HbIr specie) to 375 and 500 s⁻¹, whereas the Fe-O normal mode frequency decreases to 563 cm⁻¹ (46). The faster oxygen dissociation rates suggest that the interactions of the E7 residue in these two mutants are much weaker than in HbI_{Lp}, thus corroborating that the absence of a hydrogen bond induces a lower Fe-O vibrational frequency. Although no dipolar interaction exists between the residues in the E7 position in these two mutants, the observed frequencies in the HbI_{Lp} mutants are higher than expected probably due to multipole interactions of the three phenylalanine residues (B10, E11, and CD1) in the distal side of the heme (47). The HbI_{Lp}Gln(E7) → His mutant induces a much stronger polar interaction between this residue and the dioxygen heme complex with a dissociation rate constant (*k*_{off}) of 3 s⁻¹, suggesting the presence of dipolar interactions with the His(E7) center. This is consistent with the increase in the Fe-O energy and the kinetics studies on Mb, which indicated that the hydrogen bond between His(E7) and the oxygen coordinated to the iron control the *k*_{off} rate. The Tyr(B10) in the HbI_{Lp}Phe(B10) → Tyr mutant and in HbII_{Lp} is also stabilizing the coordinated dioxygen molecule as evidenced by the increase in the frequency from 567 to 571 cm⁻¹, and to 572 cm⁻¹, respectively, and the decrease in their dissociation rate constants from 61 to 0.6 s⁻¹, and to 0.11 s⁻¹, respectively. As mentioned above, despite similarities in their hydrogen bonding network, Hb_{Asc} and HbII_{Lp} show significantly different oxygen dissociation rate constants (0.0041 and 0.11 s⁻¹, respectively) (2, 48). Moreover, the Hb_{Asc} show a Fe-His normal mode at 202 cm⁻¹, whereas for HbII_{Lp} this frequency is present at 211 cm⁻¹. Thus, differences in the His(F8) trans-effect and the orientation of the oxygen molecule in the oxyHbII_{Lp} and oxyHb_{Asc} complexes (Fig. 6) must have an important role to explain the observed experimental data. Similarly, this is also supported by studies of CO complexes showing three different conformers at 1912, 1956, and 1965 cm⁻¹ for Hb_{Asc}-CO, whereas the HbII_{Lp}-CO complex show only the presence of the A₃ and A₀ conformers at 1924 and 1964 cm⁻¹ in the infrared spectra (15, 46). Therefore, the interplay between the small HbII_{Lp} heme pocket structure, the hydrogen bonding network to the proximal and distal heme environments, the His(F8) trans-effect, and the orientation of the oxygen molecule in the oxy complex are responsible for the stability of the oxyHbII_{Lp} complex.

In truncated hemoglobins (trHbs), the oxy trHb complex shows, in general, a much lower energy for the Fe-O vibrational

mode (542–560 cm⁻¹) than Mb (49), HbI_{Lp}, and HbII_{Lp}. Moreover, oxy trHbs tend to present an inverse correlation between the Fe-O frequency and the oxygen dissociation rate, similarly for oxyHbI_{Lp} and oxyHbII_{Lp} there is apparently also a rough inverse relationship with the dissociation constants but their Fe-O frequency is present at higher energy (567–572 cm⁻¹). The fact that trHbs have a hydrogen bonding network interacting with the proximal and distal oxygens of the heme-O₂ moiety, whereas HbI_{Lp} and HbII_{Lp} present a hydrogen bonding network interacting only with the distal oxygen of the oxyheme complex may account for the observed trend. Particularly, the Fe^{δ+}-O-O^{δ-} center is highly polar (50) and the formation of hydrogen bonding between heme pocket amino acids and one or both oxygens of the oxyheme complex can generate different moiety resonance structures. Furthermore, because the low frequency Fe-O Raman active vibrational mode arises from the interactions between the stretch and bend motion of Fe^{δ+}-O-O^{δ-}, at equilibrium, an approximate potential leading to the Fe-O vibrational frequency can be expressed as Equation 1,

$$V = f_1 \Delta r_1^2 + f_2 \Phi \Delta r_2^2 \pm f_1 f_2 \Delta r_1 \Delta r_2 + f_\Phi \Delta \Phi^2 \pm f_1 f_\Phi \Delta r_1 \Delta \Phi \pm f_2 f_\Phi \Delta r_2 \Delta \Phi \quad (\text{Eq. 1})$$

where *f*₁, *f*₂, *f*_Φ, *r*₁, *r*₂, and $\hat{O}$ are the force constant and geometries for the Fe-O, O-O, and Fe-O-O atoms, respectively. Similarly, *f*₁ *f*₂, *f*₁ *f*_Φ, and *f*₂ *f*_Φ are the associated interaction force constants, and their signs can be positive or negative (51, 52) depending on the Fe^{δ+}-O-O^{δ-} resonance structures, geometry, and central atom hybridization, which in turn are affected by the hydrogen bonding network present in the oxyhemoglobin species. It is plausible that the combination and magnitude of the sign in these coupling constants may be responsible for the lack of a single correlation between the Fe-O frequency and the hemeproteins oxygen dissociation constant.

Factors Influencing HbII_{Lp} Oxygen Selection—Different roles have been suggested for the hemoglobin variants of *L. pectinata* (1, 3). HbI_{Lp} is a sulfide-reactive monomeric protein, whereas HbII_{Lp} and HbIII_{Lp} are responsible for the oxygen transport and remain unaffected by the presence of H₂S. The factors underlying this unique behavior and ligand selection between HbI_{Lp} and HbII_{Lp}/HbIII_{Lp} clearly depend on the heme iron oxidation state; however, the mechanism underlying the deoxy heme stability in HbII_{Lp} is still unknown. *In vitro* HbI_{Lp} and HbII_{Lp} bind oxygen with an association rate constant of 100–200 × 10⁶ and 0.39 × 10⁶ M⁻¹ s⁻¹, and hydrogen sulfide with association rate constant values of 226 × 10³ and 11.3 × 10³ M⁻¹ s⁻¹, respectively (2, 3). This indicates that HbI_{Lp} binds both ligands much faster than HbII_{Lp}. Similar to HbII_{Lp}, kinetic analysis of the HbI_{Lp}Phe(B10) → Tyr mutant with O₂ and H₂S indicates that in this variant the association constant of both ligands decreases to 6.8 × 10⁶ and 3.37 × 10³ M⁻¹ s⁻¹, respectively, when compared with HbI_{Lp}. Furthermore, the H₂S dissociation constant for HbII_{Lp}, rHbI_{Lp}, and the HbI_{Lp}Phe(B10) → Tyr mutants are 17 × 10⁻³, 0.04 × 10⁻³, and 0.06 × 10⁻³ s⁻¹, respectively, whereas for the oxygen species these values are 0.11, 140, and 0.6 s⁻¹, respectively. Thus, the data indicate that in HbI_{Lp} the Phe(B10) → Tyr mutation cannot account for the

properties of HbII_{LP}-SH₂ complex reactivity but it can explain the smaller dissociation constant of oxyHbII_{LP}. Replacing Phe(B10) by Tyr in the HbI_{LP}Phe(B10) → Tyr mutant may decrease the size of the HbI_{LP} heme cavity, thus causing a reduction in ligand association. Independently of the heme iron oxidation state driving force for the selection of O₂ or H₂S, the heme cavity of HbII_{LP} is smaller (872 Å³) than the HbI_{LP}. Dynamic features in HbI_{LP}, Mb, and triple Mb mutant (L29F/H64Q/V68F) revealed a large cavity, suggesting that the larger cavities favor the binding of H₂S by HbI_{LP} (43). The reduction of the heme cavity of both HbII_{LP} and HbI_{LP}Phe(B10) → Tyr may help to stabilize the Fe^{II}-O₂ moiety once O₂ binds the heme iron center by forming hydrogen bonding interactions with Gln(E7) and Tyr(B10) as evidenced by the resonance Raman and oxygen off rate analyses presented above. Moreover, the reduction of the heme cavities in both proteins, caused in part by Tyr(B10), may help prevent oxidation of the ferrous iron center by impeding access of external water molecules into the distal environment. Indeed, a direct role of Tyr(B10) in preventing oxidation of Fe^{II} as well as ligand selection can be observed in the pH titration analysis of HbI_{LP}, HbI_{LP}Phe(B10) → Tyr, and HbII_{LP}. The data showed that the high affinity of HbII_{LP} and the HbI_{LP}Phe(B10) → Tyr mutant for oxygen is pH-dependent. Similarly, a decrease from pH 7.5 to 5.5 was necessary to fully oxidize both HbII_{LP} and the HbI_{LP}Phe(B10) → Tyr mutant, suggesting that the tyrosine plays an important role in regulating the oxidation of the heme group. Previous experiments has been suggested a relationship between the pK_a values of the ionizable groups associated with the heme and the role of the hydrogen bonding interactions on the heme oxygen dissociation rate (53). Furthermore, for met-aquo HbII_{LP} and HbI_{LP}Phe(B10) → Tyr, the UV-visible pH data shows, at neutral conditions, bands at 486, 541, 577, and 603 nm for both proteins. This suggests the existence of an open and closed conformation due to the interactions in the coordination of the Tyr(B10)(OH) and the ligand, to the heme iron. This means that, in the open conformation the Tyr³⁰(B10) swings away from the iron, whereas in the closed conformation remains at very close distance and may interact with the ligand (47).

Overall, the data suggest a model for the *in vivo* mechanism of the clam *L. pectinata* where the function of HbII_{LP} to bind and possibly transport oxygen to the host bacteria is regulated by the dynamic displacements of the Gln⁶⁵(E7) and Tyr³⁰(B10) pair toward the heme to protect it from the change in the heme oxidation state from Fe^{II} to Fe^{III}. This suggested mechanism avoids the binding of H₂S to HbII_{LP} that disrupts its function of oxygen transport in an environment rich in hydrogen sulfide. In summary, the results from the crystallographic data show that a small heme pocket cavity for HbII_{LP} induces the formation of strong hydrogen bonds between the iron and oxygen molecule. Resonance Raman data supports the existence of a hydrogen bonding network between Gln(E7) and Tyr(B10) that stabilizes the binding of the oxygen to HbII_{LP} complex shown in Fig. 3. This, together with the proximal histidine trans-effect, and the pH dependence of the oxidation state substantiates the role of HbII_{LP} in controlling the oxygen dissociation rate.

REFERENCES

- Read, K. R. (1965) *Comp. Biochem. Physiol.* **15**, 137–157
- Kraus, D. W., and Wittenberg, J. B. (1990) *J. Biol. Chem.* **265**, 16043–16053
- Kraus, D. W., Wittenberg, J. B., Lu, J. F., and Peisach, J. (1990) *J. Biol. Chem.* **265**, 16054–16059
- Rizzi, M., Wittenberg, J. B., Coda, A., Fasano, M., Ascenzi, P., and Bolognesi, M. (1994) *J. Mol. Biol.* **244**, 86–99
- Bolognesi, M., Rosano, C., Losso, R., Borassi, A., Rizzi, M., Wittenberg, J. B., Boffi, A., and Ascenzi, P. (1999) *Biophys. J.* **77**, 1093–1099
- Rizzi, M., Wittenberg, J. B., Coda, A., Ascenzi, P., and Bolognesi, M. (1996) *J. Mol. Biol.* **258**, 1–5
- Hockenhull-Johnson, J. D., Stern, M. S., Martin, P., Dass, C., Desidero, D. M., Wittenberg, J. B., Vinogradov, S. N., and Walz, D. A. (1991) *J. Protein Chem.* **10**, 609–622
- Torres, E., Renta, J. Y., Rodríguez, Y., López-Garriga, J., and Cadilla, C. L. (2003) *J. Protein Chem.* **22**, 683–690
- Pietri, R., Granell, L., Cruz, A., De Jesús, W., Lewis, A., León, R., Cadilla, C. L., and López Garriga, J. (2005) *Biochim. Biophys. Acta* **1747**, 195–203
- Yang, J., Kloek, A. P., Goldberg, D. E., and Mathews, F. S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 4224–4228
- Couture, M., Yeh, S., Wittenberg, B. A., Wittenberg, J. B., Ouellet, Y., Rousseau, D. L., and Guertin, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 11223–11228
- Milani, M., Pesce, A., Ouellet, Y., Ascenzi, P., Guertin, M., and Bolognesi, M. (2001) *EMBO J.* **20**, 3902–3909
- Condon, P. J., and Royer, W. E., Jr. (1994) *J. Biol. Chem.* **269**, 25259–25267
- Royer, W. E., Jr., Heard, K. S., Harrington, D. J., and Chiancone, E. (1995) *J. Mol. Biol.* **253**, 168–186
- Peterson, E. S., Huang, S., Wang, J., Miller, L. M., Vidugiris, G., Kloek, A. P., Goldberg, D. E., Chance, M. R., Wittenberg, J. B., and Friedman, J. M. (1997) *Biochemistry* **36**, 13110–13121
- De Jesús-Bonilla, W., Jia, Y., Alayash, A. I., and López Garriga, J. (2007) *Biochemistry* **46**, 10451–10460
- De Jesús-Bonilla, W., Cruz, A., Lewis, A., Cerda, J., Bacelo, D. E., Cadilla, C. L., and López-Garriga, J. (2006) *J. Biol. Inorg. Chem.* **11**, 334–342
- León, R. G., Munier-Lehmann, H., Barzu, O., Baudin-Creuz, V., Pietri, R., López-Garriga, J., and Cadilla, C. L. (2004) *Protein Expr. Purif.* **38**, 184–195
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25**, 3389–3402
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22**, 4673–4680
- Gavira, J. A., De Jesús, W., Camara-Artigas, A., Lopez-Garriga, J., and Garcia-Ruiz, J. M. (2006) *Acta Crystallogr. Sect. F Struct. Biol. Crystallogr. Commun.* **62**, 196–199
- García-Ruiz, J. M. (2003) *Methods Enzymol.* **368**, 130–154
- Otwinowski, Z., and Minor, W. (1997) *Methods Enzymol.* **276**, 307–326
- Brunger, A. T., Adams, P. D., Clore, G. M., Delano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **54**, 905–921
- Bailey, S. (1994) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **50**, 760–763
- Emsley, P., and Cowtan, K. (2004) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **60**, 2126–2132
- Winn, M. D., Isupov, M. N., and Murshudov, G. N. (2001) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **57**, 122–133
- Lovell, S. C., Davis, I. W., Adrendall, W. B., III, de Bakker, P. I., Word, J. M., Prisant, M. G., Richardson, J. S., and Richardson, D. C. (2003) *Proteins* **50**, 437–450
- Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Crystallogr.* **26**, 283–291
- Frishman, D., and Argos, P. (1995) *Proteins* **23**, 566–579
- Hutchinson, E. G., and Thornton, J. M. (1996) *Protein Sci.* **5**, 212–220
- Kabsch, W. (1976) *Acta Crystallogr. Sect. A* **32**, 922–923
- Krissinel, E., and Henrick, K. (2005) *Comput. Life Sci. Proc.* **3695**, 163–174
- Hubbard, S. J., and Thornton, J. M. (1993) *NACCESS, Computer Program*, Department of Biochemistry and Molecular Biology, University College, London

35. Binkowski, T. A., Naghibzadeh, S., and Liang, J. (2003) *Nucleic Acids Res.* **31**, 3352–3355
36. Royer, W. E., Knapp, J. E., Strand, K., and Heaslet, H. A. (2001) *Trends Biochem. Sci.* **26**, 297–304
37. Smerdon, S. J., Krzywda, S., Wilkinson, A. J., Brantley, R. E., Carver, T. E., Hargrove, M. S., and Olson, J. S. (1993) *Biochemistry* **32**, 5132–5138
38. Royer, W. E., Jr., Pardanani, A., Gibson, Q. H., Peterson, E. S., and Friedman, J. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 14526–14531
39. Pardanani, A., Gambacurta, A., Ascoli, F., and Royer, W. E. (1998) *J. Mol. Biol.* **284**, 729–739
40. Carver, T. E., Brantley, R. E., Jr., Singleton, E. W., Arduini, R. M., Quillin, M. L., Phillips, G. N., Jr., and Olson, J. S. (1992) *J. Biol. Chem.* **267**, 14443–14450
41. Cerda, J., Echevarría, Y., Morales, E., and López-Garriga, J. (1999) *Biospectroscopy* **5**, 289–301
42. Mukai, M., Mills, C. E., Poole, R. K., and Yeh, S. R. (2001) *J. Biol. Chem.* **276**, 7272–7277
43. Fernández-Alberti, S., Bacelo, D. E., Binning, R. C., Echave, J., Chergui, M., and Lopez-Garriga, J., (2006) *Biophys. J.* **91**, 1698–1709
44. Zhou, Y. Q., Zhou, H. Y., and Karplus, M. (2003) *J. Mol. Biol.* **326**, 593–606
45. Das, K. T., Boffi, A., Chiancone, E., and Rousseau, A. D. (1999) *J. Biol. Chem.* **274**, 2916–2919
46. Pietri, R., Granell, L. B., León, R., Cadilla, C. L., and López-Garriga, J. (2006) *Biochim. Biophys. Acta* **1764**, 758–765
47. Navarro, A., Maldonado, M., González-Lagoa, J., López, R., López-Garriga, J., and Colón, J. (1996) *Inorg. Chim. Acta* **243**, 161–166
48. Blaxter, M. L., Vanfleteren, J. R., Xia, J., and Moens, L. (1994) *J. Biol. Chem.* **269**, 30181–30186
49. Lu, C., Egawa, T., Wainwright, L. M., Poole, R. K., and Yeh, S.-R. (2007) *J. Biol. Chem.* **282**, 13627–13636
50. Hirota, S., Li, T., Phillips, G. H., Olson, J. S., Mukai, M., and Kitagawa, T. (1996) *J. Am. Chem. Soc.* **118**, 7845–7846
51. Coulson, C. A., Duchesne, J., and Manneback, C. (1947) *Nature* **159**, 794–795
52. Califano, S. (1976) *Vibrational States*, pp. 95–97, John Wiley & Sons, New York
53. Saroff, H. A. (2004) *J. Theor. Biol.* **229**, 113–118

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J. Biol. Chem. 2008, 283:9414-9423.

doi: 10.1074/jbc.M705026200 originally published online January 18, 2008

Access the most updated version of this article at doi: [10.1074/jbc.M705026200](https://doi.org/10.1074/jbc.M705026200)

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