The X-ray Structure of Ferric Escherichia coli Flavohemoglobin Reveals an Unexpected Geometry of the Distal Heme Pocket*

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Flavohemoglobins are oxygen-binding proteins composed of a typical globin domain containing a B-type heme fused with a ferredoxin reductase-like FAD- and NAD-binding domain. Together with single domain bacterial hemoglobins and six-helices containing “truncated hemoglobins,” flavohemoglobins are part of a vast family of “hemoglobin-like” proteins whose functional properties are still elusive. Because of the identification of flavohemoglobin (39% amino acid residue identity), and the N-terminal globin domain of HMP shares 51% amino acid residue identity with VHB (146 amino acid residues). Amino acid residues in the topological positions that harbor the heme group within the CD corner and the B, E, and F helices are strongly conserved in the whole bacterial hemoglobin family (1, 6). In particular, Tyr-B10, Phe-CD1, Leu-E11, and His-F8 (proximal histidine) are invariably conserved, whereas the position of E7 typically occupied by a histidine residue in vertebrate hemoglobins and myoglobins is most often replaced by a Gln residue. Based on the topology of vertebrate Hbs, the distal pocket residues Tyr-B10 and Gln-E7 are located in a position that dominates the steric interactions with the iron-bound ligand, thus suggesting a common role of these amino acids in determining the functional properties of most bacterial hemo- globins. Nevertheless, the three-dimensional structures of the ferrous lipid-bound FHP (6) and of the ferric ligand-free and azide, thiocyanide, and imidazole-bound VHB (7, 8) derivatives have revealed that the stereochemistry of the heme pocket in these proteins is hardly superimposable to that typical of vertebrate hemoglobins and myoglobins, indicating that predictions based on topological considerations must be used with caution.

The structural details of HMP active site have been inferred by an analogy with the existing tridimensional structures of Alcaligenes eutrophus flavohemoglobin (FHP) (6) and Vitreoscilla sp. (VHB) (7, 8) hemoglobin as well as from spectroscopic data on HMP itself (9, 10). The entire amino acid sequence of HMP is closely related to that of the A. eutrophus flavohemoglobin (39% amino acid residue identity), and the N-terminal globin domain of HMP shares 51% amino acid residue identity with VHB (146 amino acid residues). Amino acid residues in the topological positions that harbor the heme group within the CD corner and the B, E, and F helices are strongly conserved in the whole bacterial hemoglobin family (1, 6). In particular, Tyr-B10, Phe-CD1, Leu-E11, and His-F8 (proximal histidine) are invariably conserved, whereas the position of E7 typically occupied by a histidine residue in vertebrate hemoglobins and myoglobins is most often replaced by a Gln residue. Based on the topology of vertebrate Hbs, the distal pocket residues Tyr-B10 and Gln-E7 are located in a position that dominates the steric interactions with the iron-bound ligand, thus suggesting a common role of these amino acids in determining the functional properties of most bacterial hemoglobins. Nevertheless, the three-dimensional structures of the ferrous lipid-bound FHP (6) and of the ferric ligand-free and azide, thiocyanide, and imidazole-bound VHB (7, 8) derivatives have revealed that the stereochemistry of the heme pocket in these proteins is hardly superimposable to that typical of vertebrate hemoglobins and myoglobins, indicating that predictions based on topological considerations must be used with caution.

Direct structural information on HMP recently obtained by resonance Raman and FTIR studies in solution clearly demonstrated that the protein is endowed with high conformational flexibility and displays an equilibrium between an open and a closed heme pocket conformation (9, 10). In the closed confor-
mation, Tyr-B10 has been suggested to establish an interaction with diatomic ligands (9). This conclusion is in agreement with site-directed mutagenesis studies, which indicate that ligand binding properties of ferrous HMP and, in particular, oxygen release kinetics are strongly affected by the Tyr-B10 → Phe substitution (5).

Altogether, the body of spectroscopic information available on HMP is strongly suggestive of a peroxidase-like active site in which the proximal histidine endows with a significant imidazole character favors electron donation to the iron atom, whereas distal residues Tyr-B10 and Glu-E7 exert a steric control, and contributes to the eventual processing of the iron-bound oxygen (9). In this study, the solution of the x-ray crystal structure of ferric unliganded HMP together with an investigation of the ligand binding properties of the ferric derivative not only disclose the tridimensional details of the active site of HMP but also provides clues for a structural and functional link with the closely related *A. eutrophus* and *Vitreoscilla* sp. proteins.

**EXPERIMENTAL PROCEDURES**

HMP has been expressed and purified as reported previously (10). Particular care was taken to remove bound lipids (11) by means of hydroxypatite chromatography (10). The lipid removal was checked by electrospray mass spectrometry. Protein concentration was measured spectrophotometrically using the extinction coefficient of 150,000 M⁻¹ cm⁻¹ for the ferric cyanide adduct.

Titrations experiments were carried out by adding small volumes of imidazole, sodium cyanide, or sodium azide (Sigma) solutions to the protein solution (8 μM) in 0.1 M sodium phosphate buffer, pH 7.0, at 20 °C in a 1-cm quartz cuvette. A 10-cm path length cuvette was used in the titration with imidazole and cyanide at ligand concentrations below 5 × 10⁻⁴ M and protein concentration of 0.5–1 μM. The absorption spectra were recorded after each ligand addition (2-min equilibration time for imidazole and cyanide and 1-h equilibration time for cyanide) on a Jasco 570 spectrophotometer (Jasco).

Kinetic measurements were carried out by mixing the protein solution with ligand solutions at different concentrations in an Applied Photophysics rapid mixing apparatus (Leatherhead, United Kingdom). The observation wavelengths were 420 nm for cyanide, 415 nm for imidazole, and 413 nm for azide adducts. All experiments were carried out in 0.1 M phosphate buffer, pH 7.0, at 20 °C.

Sedimentation velocity experiments were performed at 10 °C and 35,000 rpm on a Beckman Optima XL-A analytical ultracentrifuge equipped with absorption optics. The protein concentrations were 5–10 × 10⁻⁵ M in 0.1 M sodium phosphate buffer at pH 7.0. Data were collected at 550 and 403 nm for the ferric unliganded protein at 558 and 419 nm for the ferric cyanide-bound derivative.

Crystallographic Data Collection—Crystallization experiments were carried out at 25 °C using the sitting drop vapor diffusion method. A volume of 4 μl of protein sample (22 mg/ml) in water was mixed with an equal amount of the reservoir solution containing 0.1 M sodium acetate buffer, pH 5.1–5.3, 21–26% polyethylene glycol 3350, and 0.2 M NaCl. Crystals grew in 1 week to ∼0.05 × 0.05 × 0.1 mm.

Data were collected as 0.5 oscillation frames on the ELETTRA beamline at Basovizza (Trieste, Italy) at a wavelength of 1.0 Å and at 100 K using 26% polyethylene glycol 200 as cryoprotectant. Data analysis performed with DENZO (12) indicates that the crystals are hexagonal P622 with cell dimensions of \(a = b = 164.86 \text{ Å}, c = 53.46 \text{ Å}, \alpha = \beta = 90°, \gamma = 120°\). The data scaling performed with SCALEPACK (12) gave a \(R_{int}\) of 0.05% for 22,448 unique reflections with a completeness of 99.5% at 2.19-Å resolution. Heavy atom derivatives were generated by soaking crystals in reservoir solution plus 5 mM Hg(CH₃COO)₂ for 36 h or 5 mM K₃PtCl₆ for 48 h. Statistics for all sets of data analyzed are given in Table I.

**Structure Determination and Refinement**—The structure was solved by multiple isomorphous replacement. Coordinates of Pt and Hg were located using the programs CNS (13) and PHASER (14). The data set of the two different derivatives were combined, atom parameters were refined, and the starting phases were calculated using isomorphous and anomalous differences with the maximum likelihood method incorporated in MLPHARE (15). The phases were further improved by solvent flattening using DM (density modification) (16). The model was built using the program XTALVIEW (17). The refinement was carried out with the maximum likelihood method incorporated in REFMAC (18) to an R-factor of 19.5% and a R_free of 27.2% at 2.19-Å resolution.

The model was then complete with the exception of residues 333–336 and 347–354. The residues within these regions corresponding to two external loops were not well defined. To solve the problem, the refinement of the atomic coordinates and displacement parameters was carried out by simulated annealing and by least-squared minimization using the program X-PLOR (19). Water molecules and ions were added manually. The final model includes all 396 residues and 180 water molecules. The final R-factor for 2.19-Å resolution is 18.8% with final R_free of 24.7% and bond lengths r.m.s. deviation of 0.008 and bond angles r.m.s. deviation of 1.8°. The quality of the model was assessed using the program PROCHECK (20). The most favored regions of the Ramachandran plot contain 90.9% of non-glycine residues. The overall G-factor is −0.22, which is better than the expected values. The structural and refinement statistics are listed in Table I.

**RESULTS AND DISCUSSION**

**The Overall Fold**—The overall fold of ferric unliganded lipid-free HMP (Fig. 1A) consists of heart-shaped structure in which three different domains, namely the C-terminal NAD-binding, the FAD-binding, and the N-terminal globin domains, are clearly distinguished.

Despite the high degree of sequence similarity between HMP and FHP (Fig. 2), the superposition of the two structures obtained with the program SEQUOIA (21) results in a high r.m.s. deviation (6.83 Å). Such a deviation is mainly because of a rotation of the NAD binding module (see Fig. 1B) and because of a substantial rearrangement of the E helix positioning within the globin domain in HMP with respect to FHP (see below).

**The Globin Domain**—The architecture of the globin domain corresponds to a classic globin fold with an unusually long H helix and in which the D helix is substituted by a large loop region within the segment CD2-E6 (Asn⁴¹–Asp⁵²) (see Fig. 3). Unexpectedly, the inspection of the distal pocket architecture reveals that the distal position closest to the iron atom is occupied by the Leu⁵²–E11 isopropyl side chain with the CG atom at 3.6-Å distance from the iron atom along the normal to the heme plane (Fig. 4). Leu⁵²–E11 side chain together with the Phe⁶³–CD1 ring, the Ile⁶⁴–E15 and Val⁶⁴–G8 side chains, and the Gln⁵³–E7 backbone segment fill completely the first distal shell (Fig. 4). In contrast, Tyr⁴⁹–B10 and Gln⁵³–E7 side chains previously proposed as the major candidates for iron-ligand distal stabilization (9, 10) are shifted from the heme at a distance of 5.0 Å. The Tyr-B10 phenol ring is confined to the second layer of distal pocket residues with the phenol hydroxyl pointing toward the isopropyl chain of Leu⁵²–E11. This arrangement is strongly suggestive of a ligand-linked conformational change that entails the rotation of the Leu⁵²–E11 isopropyl side chain toward the edge of the heme to allow the Tyr-B10 phenol hydroxyl interaction with the iron-bound ligand. This
interpretation might explain the experimental evidence obtained by resonance Raman and FTIR spectroscopy for the participation of a Tyr hydroxyl, either direct or most probably mediated by a water molecule, to the distal coordination of bound CO in the ferrous protein (9, 10).

The architecture of the heme pocket in the proximal region is dominated by a hydrogen-bonding network between His85-F8, Tyr95-G5, and Glu135-H23 (see Fig. 4) that imparts a rigid orientation to the proximal histidine ring with respect to the heme plane. In particular, the OE2 atom of Glu135 is involved in a hydrogen-bonding interaction at 2.9 Å from the ND1 nitrogen of the proximal histidine. In turn, Glu135 is anchored to the phenol hydroxyl of Tyr95 by hydrogen bonding (OE2 at 2.7 Å from the Tyr oxygen atom). This structural arrangement is in line with the proposed imidazolate character of the proximal histidine resulting from ND1 proton withdrawal toward Glu135 OE2 atom and with a high value of the iron-proximal histidine-stretching frequency of the HMP ferrous unliganded derivative measured by Mukai et al. (9) in resonance Raman experiments.

Another peculiar structural feature of the HMP heme domain consists in the arrangement of the CE loop region. In fact, as shown in Fig. 5, the cavity formed by the residues Asp44-Asn52 is filled with a strong electron density whose electronic shell most probably pertains to a large anion such as chloride or phosphate. Possible electrostatic interactions to the bound anion have also been identified and include a positively charged ammonium ion belonging to residues Arg49, amide nitrogens of Asp52 and Arg54, and/or the O2D oxygen atom of a heme propionate. This finding is of particular interest because of the location of the loop on the heme edge toward the propionate region and in contact with the flavin binding module. A possible functional implication of the anion binding site in modulating the heme ligand binding properties is discussed in the last paragraph.

Comparison between E. coli and A. eutrophus Globin Domains—The structural overlay of the Cα skeleton of HMP and FHP globin domains indicates that a major difference between the two proteins pertains to the positioning of the E helix that is shifted far from the heme plane in the latter protein. It is now clear that in FHP heme domain, the E helix shift is determined by the accommodation of a bulky phospholipid molecule that alters the positioning of the relevant distal pocket residues (6). Particularly, in FHP, the cyclopropane ring of a 9,10-methylene-hexadecanoic chain of a dyacylglycerol-phosphatidic acid, a common fatty acid component within the bacterial membranes, sits on the top of the iron atom apparently displacing the relevant distal site residues (11). In fact, in FHP, Leu-E11 accompanies the lipid-induced E helix movement and is displaced by 4.6 Å away from the heme pocket with respect to its positioning in HMP. Interestingly, HMP is also capable of binding membrane lipids, although with lower affinity with respect to FHP (11). VHB has also been recently reported to be able to interact with the bacterial inner membrane (23). Taken together, these observations suggest that lipid binding is not a
distinctive feature of FHP but may represent a common property of the bacterial hemoglobin family, the functional role of which is still to be defined. In this framework, it is of interest to compare the residues that are in Van der Waals contacts with the lipid in the A. eutrophus protein with the analogous residues in HMP and VHB. Sequence alignments indicate that most residues involved in the formation of the lipid-harboring cavity within the heme domain (residues within 4.0 Å from the phospholipid atoms) are conserved in the three proteins (see Fig. 2). As a last comment, it is worth considering that the CE loop region in FHP is apparently devoid of a preformed cavity that allows the harboring of an anion.

Comparison between HMP Heme Domain and Vitreoscilla Hb—The striking similarity of the overall fold of the heme domain in HMP and Vitreoscilla Hb (C-α structure superposition r.m.s. deviation = 1.58) shown in Fig. 3 is not reflected in a similar architecture of the distal heme pocket in that notable differences concern the nature and/or the geometry of the E8 and E11 topological positions. The proline ring in position E8 in VHB lies close to the normal heme plane at 3.8 Å from the metal in the distal pocket (7) but is substituted by an arginine residue in HMP whose side chain is oriented outside the distal pocket. Leu-E11 in HMP occupies a genuine distal site position along the normal to the heme plane, whereas the same residue in VHB is in a staggered conformation and appears to line the edge of the heme (7, 8). In both HMP and VHB, the Gln-E7 side chain does not contribute to the formation of the first distal pocket shell in the unliganded derivatives.

The FAD-binding Domain—The structures of the FAD- and NAD binding modules in HMP are superimposable to those observed in FHP, and a discussion on the distinctive topological features of these domains in comparison with similar flavin-binding proteins is given in detail by Ermler et al. (6). Nevertheless, two major differences between HMP and FHB need to be considered further. First, as shown in Fig. 1B, the relative orientations of the FAD- and NAD-binding domains in the two proteins differ. In particular, the entire NAD-binding domain in HMP is rotated clockwise with respect to the relative orientation observed in FHP, thus leading to a considerably larger interdomain cleft in HMP. The second point concerns the structural arrangement of the isalloxazine binding motif. The side geometry is strikingly similar in the two proteins in that the segment 205–207 (207–209 in FHP) and the Tyr168 (190 in FHP) exhibit identical orientations with respect to the isalloxazine plane (see Fig. 6). Other conserved contacts (data not shown) to the flavin moiety involves Val269 in HMP (276 in}

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**Fig. 3.** Structural overlay of the globin domains of *E. coli* and *A. eutrophus* flavohemoglobin and *Vitreoscilla* sp. hemoglobin. The overlay among globin domains was obtained by minimizing the C-α distances for each protein using the program SEQUOIA (21) and was depicted with the program MOLSCRIPT (28). The stereo diagram of the globin domain of HMP (red ribbons, yellow heme) is overlaid with the corresponding domain of *A. eutrophus* flavohemoglobin (green ribbons, green heme) in A and with *Vitreoscilla* Hb (cyan ribbons, blue heme) in B.
FHP) and Thr<sup>272</sup> (279 in FHP). In contrast, the re-side, which faces the C-terminal loop region of the NAD-binding domain, is quite different in the two proteins. In fact, in HMP, the aromatic ring of the conserved Phe<sup>390</sup> (396 in A. eutrophus) is packed against the isoalloxazine central ring, whereas in FHP, it protrudes toward the interdomain cleft. Considering the high sequence similarity within the FAD- and NAD-binding domains of the two proteins (see alignments of Fig. 2), both the rotation of the NAD domain and the differences in the re-side of the isoalloxazine binding motif may be suggestive of a conformational change linked to the binding of a phospholipid molecule. Thus, it may be envisaged that the lipid-free species is stabilized by a stacking interaction of the isoalloxazine ring with Phe<sup>390</sup> in HMP, whereas upon lipid binding (i.e. in FHP), the whole C-terminal loop is shifted and rotated toward the ribitol moiety with concomitant loss of the stacking interaction. Despite these significant structural rearrangements, the relative positioning of the isoalloxazine moiety and the heme plane is superimposable in the two proteins, and only a modest decrease in the closest distance between the two cofactors is observed (the distance between the C8AF atom of the isoalloxazine and OA1 of the heme propionate is 6.3 Å in FHP and 5.9 Å in HMP).

**Fig. 4.** Structural details of the heme pocket in *E. coli* ferric unliganded flavohemoglobin. The heme molecule is shown in stereo together with a selection of amino acid residues within the pocket at 5 Å from the macrocycle. The phenolic ring of Tyr<sup>29</sup>-B10 located at >5 Å from the heme is also shown, whereas the Phe<sup>14</sup>-CD1 is hidden for clarity. The picture was obtained with the program MOLSCRIPT (28).

**Fig. 5.** Electron density map of the CE region of *E. coli* heme domain. Electron densities were calculated with the program X-PLOR (19) and contoured at 1σ from the residues Ala<sup>95</sup>-Ala<sup>56</sup>. The anion residing at the center of the map was tentatively assigned to a chloride ion (yellow sphere).

**Fig. 6.** Structural details of the FAD binding site in *E. coli* ferric unliganded (right) and *A. eutrophus* ferrous lipid-bound (left) flavohemoglobins. The isoalloxazine three-membered ring (embedded in a transparent Van der Waals surface) is shown together with a selection of amino acid residues located within 5-Å distance from the macrocycle atoms in the re-side (top) and in the si-side (bottom). Pictures were obtained with the program ViewerLite.

**X-ray Structure of *E. coli* Flavohemoglobin**
ferric heme ligands, namely fluoride, nitrite, nicotinate, and hydrogen peroxide, were also tested but exhibited no detectable binding up to 0.1 M concentration (data not shown). Fig. 7 shows a typical titration of HMP with imidazole. An inspection of the absorption spectrum of the unliganded derivative is consistent with the presence of a pentacoordinated heme adduct. It is worth noting that despite the contribution attributed to the flavin absorption (shoulders at 470 and 490 nm with a broad tail toward the visible region), the peak at 645 nm and the broad Soret band characterized by a low molar absorptivity and centered at ~403 nm are clearly identified as typical pentacoordinate high spin marker bands in which the proximal histidine is the fifth ligand (22). The spectrum of the fully liganded species is typical of hexacoordinate low spin adducts observed in all other hemoglobins and myoglobins. The spectral profiles obtained upon the addition of imidazole are strikingly similar (apart from the flavin contribution) to those observed in the analogous titration carried out in FHP (24). The ligand binding isotherms thus obtained (Fig. 8) are strongly biphasic similar to those obtained in VHB under similar experimental conditions although with significant differences in the values of the apparent binding constant, especially for imidazole (see legend of Fig. 8) (for review see Ref. 9). Such a finding is most intriguing on the basis of the following considerations. (i) There is no spectrally distinct intermediate from the spectra of unliganded and fully liganded species (in other words, all spectra recorded as a function of imidazole concentration can be accounted for by a linear combination of the unliganded and fully liganded spectra). (ii) The amplitudes of the two phases are nearly equal. (iii) As demonstrated by analytical centrifugation, HMP is monomeric in solution in both the liganded and unliganded derivatives (data not shown) and hence no heme-heme interaction may be envisaged to explain the apparently “anti-cooperative” behavior. As a consequence, the present data on HMP dismiss the previously reported anti-cooperative hypothesis formulated to explain the analogous biphasic ligand binding behavior in VHB (7). To find a rationale for the complex binding isotherms in HMP, ligand binding kinetics has been investigated, and a complete data set has been obtained for imidazole and cyanide binding (Fig. 9). Azide binding was too fast, and the change in absorbance was too small to yield reliable data. Imidazole binding is very fast (4 × 10^5 M^-1 s^-1) when compared with vertebrate hemoglobins and myoglobins (25) as well as with VHB (2.3 × 10^4 M^-1 s^-1 for the faster phase), consistent with the sixth iron coordination position being free and accessible for ligand approach to the iron atom.

At variance with VHB, the binding of imidazole and cyanide appears to be a simple second order monophasic process, thus indicating that the biphasic thermodynamic behavior must be entirely accounted for by the ligand-release process. The differences in the observed imidazole binding rate in HMP with respect to VHB may be attributed at first glance to the presence of the rigid Pro-E8 ring on the top of the heme iron in the latter protein. In fact, as demonstrated by Bolognesi and co-workers (7), the ligand binding to ferric VHB entails the movement of the entire E7–E12 segment. Ligand accommodation in HMP distal pocket is most probably achieved by the rotation of the isopropyl side chain of Leu-E11 from an extended to a staggered position.

At present, no simple mechanism can be envisaged to explain the complex kinetic and thermodynamic behavior of HMP and VHB. It should be pointed out that biphasic kinetics has been demonstrated in both proteins also in the ferrous state. In
particular, two phases are observed in the kinetics of CO combination and oxygen release in both VHB (24) and HMP (5). These results, taken together with the presence of two conformations (at equilibrium) in the ferrous CO-bound adduct of HMP (9, 10), indicate that the liganded species are endowed with a significant structural heterogeneity pertaining to the heme pocket itself. Nevertheless, an intriguing speculation concerns the possible involvement of the anion binding site located in the CE loop region above the heme distal pocket (see Fig. 5) in the modulation of heme iron-ligand binding. It may be envisaged that negatively charged ligand molecules (i.e. cyanide and azide) or possibly other molecules capable of making H-bond interactions with nearby residues (i.e. imidazole) are partitioned between the heme iron and the anionic binding loop, thus giving rise to a complex kinetic and thermodynamic behavior. Further studies will be necessary to clarify this aspect and to assess a functional relevance to the observed phenomenon.

CONCLUSIONS

The three-dimensional structure of E. coli flavohemoglobin in the ferric unliganded state reveals a novel design of the heme pocket distal site. The presence of the Leu-E11 isopropyl side chain on the top of the heme iron in a genuine distal site position is unexpected on the basis of structural models proposed in previous spectroscopic investigations on HMP-liganded derivatives (9, 10). In fact, previous resonance Raman and FTIR measurements showed that ferrous CO-bound HMP is characterized by the presence of two conformers in which the diatomic ligand is either stabilized by a hydrogen-bonding interaction to the Tyr-B10 (closed conformation) or free of distal constraints (open conformation) (9). The present structure pertaining to a ligand-free derivative offers a different view in which the Tyr-B10 residue lies far from the diatomic ligand contact region and is shielded by the isopropyl side chain of Leu-E11. Thus, the positioning of Leu-E11 indicates that this residue not only gates the accommodation of the incoming ligand but also hinders the access of the Tyr-B10 phenol hydroxyl to the distal ligand contact area. Such a structural arrangement is highly suggestive of a ligand-linked motion that entails the displacement of the Leu-E11 side chain (open conformation) followed by the approaching of the Tyr-B10 hydroxyl to the iron-bound ligand to form a hydrogen-bonding interaction (closed conformation). At present, the functional relevance of this ligand-linked conformational rearrangement is unknown but still offers several points for further comments and speculations. First of all, although Leu-E11 must necessarily be displaced to accommodate the iron ligand, the steric barrier imposed by the isopropyl moiety does not appear to limit the ligand binding kinetics, even in the case of the bulky imidazole molecule (see the relevant paragraph on ligand binding properties under “Experimental Procedures”). Thus, it is difficult to single out a specific role for Leu-E11 in discriminating among different ligands (e.g. polar versus non-polar), although it is worth mentioning that no ferric iron adducts with nitrite, hydrogen peroxide, and fluoride (typically formed in both ferric hemoglobins and peroxidases) have been detected. In turn, a more complex interplay between Leu-E11 and Tyr-B10 might be envisaged in the control of ligand release kinetics. The presence of two discrete conformations determined by different positions of the Leu-E11 and Tyr-B10 pair in the liganded species might be at the basis of the biphasic ligand binding curves observed in equilibrium titration experiments (Fig. 8). Nevertheless, as an alternative hypothesis, the anion binding site in the CE loop region above the heme distal pocket might participate in modulating the heme iron ligand binding properties.

A second point that requires further investigation concerns the possible interaction of HMP with membrane phospholipids (11). The whole reasoning concerning the interplay between Leu-E11 and Tyr-B10 might result with little relevance whether HMP could be demonstrated to accommodate a long hydrocarbon chain within the distal heme pocket as observed in the A. eutrophus protein. At present, it is not clear whether HMP is involved in any sort of interaction with the bacterial inner membrane in the living cell. Nevertheless, a recent report on the interaction of Vitreoscilla Hb with bacterial membrane (11) as well as the high conservation of the residues involved in phospholipid binding among HMP, VHB, and FHP (see Fig. 2) can be suggestive of such an interaction. Moreover, the structural overlap of the isoaflxazine binding motif in lipid-free HMP versus lipid-bound FHB reveals a major structural rearrangement of the C-terminal segment that face the NAD binding side of the flavin rings (Figs. 1B and 6). This structural change has been interpreted as a specific signature of a phospholipid-induced conformational transition in FHP that also entails a movement of the whole NAD binding module with respect to the FAD-binding domain. The correctness of this hypothesis and its functional implications should await a deeper understanding of the lipid-protein interaction in HMP.

As a final comment, it should be pointed out that the high similarity between the structural details and ligand binding properties of HMP heme domain and VHB strongly suggests a similar function for these proteins, a proposal that clashes with the radically different functional hypotheses formulated to date (i.e. oxygen transport for VHB and nitric-oxide dioxygenase activity for HMP). At present, in the search of a unifying functional hypothesis, it can be argued that HMP is certainly not an oxygen transporter because it is capable in reducing oxygen to peroxide and/or superoxide (26), whereas VHB might function well as a nitric-oxide dioxygenase provided that electrons for the catalytic cycle are supplied by a donor protein (27).

Protein Data Bank Submission—Atomic coordinates and structure factors of ferric unliganded lipid-free E. coli flavohemoglobin have been deposited to the Protein Data Bank (accession code 1gvh).

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