Ligand-Linked Changes at the Subunit Interfaces in Scapharca Hemoglobins Probed through the Sulfhydryl Infrared Absorption†

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ABSTRACT: FTIR spectra of native *Scapharca* homodimeric hemoglobin (HbI) and of the Phe97→Ile mutant have been measured in the region 2400−2700 cm^{-1} where the absorption of the sulfhydryl groups can be observed. In native HbI, the two Cys92 residues give rise to a relatively intense band centered at 2559 cm^{-1} that is shifted to 2568 cm^{-1} and strongly quenched upon ligand binding. In the Phe97→Leu mutant, such ligand-linked changes are not observed and the strong peak at around 2560 cm^{-1} persists in the liganded derivatives. In native HbI, the observed changes have been attributed to the decrease in polarity of the interface due to the ligand-induced extrusion of the Phe97 phenyl ring from the heme pocket to the interface and the subsequent release of several water molecules that are clustered in the vicinity of Cys92. In contrast, in the Phe97→Leu mutant, the Leu residue does not leave the heme pocket upon ligand binding and the interface is unaltered. The Cys92/S−H infrared band, therefore, represents a sensitive probe of the structural rearrangements that take place in the intersubunit interface upon ligand binding to HbI. The heterotetrameric *Scapharca* hemoglobin HbII contains, in addition to the Cys92 residues in the interfaces, two extra sulfhydryl groups per tetramer (Cys9 in the B chain) that are exposed to solvent in the A helix. The frequency of the Cys9/S−H stretching vibration occurs at 2582 cm^{-1} in the unliganded and at 2586 cm^{-1} in the liganded derivative, pointing to the involvement of the A helix in the ligand-linked polymerization characteristic of HbII.

The mollusk *Scapharca inaequivalvis* contains two cooperative hemoglobins, a homodimeric (HbI)[1] and a heterotetrameric component (HbII) that are characterized by unique structure−function relationships. The individual subunits share the same globin fold as vertebrate hemoglobins but have a completely different quaternary assemblage. In HbI, the subunit interface is formed by the heme carrying E and F helices, in which vertebrate hemoglobins are exposed to solvent. This mode of assembly results in the juxtaposition of the two heme groups that are almost in contact through their propionate residues. The HbII tetramer (A_2B_2) is a dimer of heterodimers that are assembled like HbI (*1*). The AB dimer within the HbII tetramer displays structural properties that resemble those of the homodimer since the sequence identity in the relevant interface region is 92% (*2*).

The structural changes that accompany ligand binding in HbI have been unveiled in crystallography studies (*3, 4, 5*) and are thought to be very similar to those occurring in the AB dimer of the HbII heterotetramer (*1*). At variance with vertebrate hemoglobins, in HbI, the ligand-linked quaternary changes are extremely small and most structural rearrangements concern uniquely the heme pocket and its vicinity. A central role in cooperative ligand binding is played by the movement of the Phe97 residue that is packed tightly against the heme in close contact with the proximal histidine in the deoxy derivative but is displaced from the heme pocket to the subunit interface upon ligand binding. This movement is accompanied by a slight shift in the relative positions of the two heme groups and is coupled to the disruption of a well-ordered interfacial water cluster. The importance of this water cluster and of Phe97 has been confirmed by the behavior of the Phe97→Leu HbI mutant (*6*). Substitution of Phe97 with Leu results in a decrease in cooperativity and in an increase in oxygen affinity. These functional effects find their structural counterpart in the absence of the ligand-induced displacement of Leu97 to the interface and in the persistence of the network of interfacial water molecule characteristic of the deoxygenated structure also in the CO derivative. As a whole, these data suggest that the ordered water molecular network in the interface stabilizes the deoxy structure, thus representing a functionally active constituent of HbI.

The understanding of the finer structural details that govern the ligand-linked structural changes in the HbI interface would greatly benefit from the availability of direct spectroscopic probes. In previous reports, attention was focused on the two Cys92 residues (Cys92 is the only cysteine in HbI) that are symmetrically related in the intersubunit interface at one helical turn from Phe97 (*7*). Cys92 displays a different reactivity with a set of organomercurial reagents,

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[1] Abbreviations: HbI, *Scapharca inaequivalvis* homodimeric hemoglobin; HbII, *Scapharca inaequivalvis* heterotetrameric hemoglobin; HbA, human hemoglobin A; Phe97→Ile HbI, dimeric *S. inaequivalvis* hemoglobin mutant in position Phe97→Ile; FTIR, Fourier transform infrared spectroscopy.

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depending on the ligation state of native HbI, thereby demonstrating that the overall accessibility of the subunit interface is ligand-linked. Moreover, the ionization of charged organomercurials bound to liganded and unliganded HbI differs, suggesting the occurrence of ligand-linked changes in the electrostatic potential in the vicinity of Cys92. The protein-bound organomercurials, due to their charge and bulkiness, stabilize the protein in a nonnative cooperative structure and, hence, may not be fully reliable reporter groups of the native state of the protein.

In the present paper, we have monitored directly the two Cys92 sulfhydryl groups at the interface by measuring the infrared absorption of the S−H group as a function of the ligation state of the protein in native HbI, in the Phe97→Leu mutant, and in the heterotetrameric HbII. HbII, in addition to the Cys92 residues in both the A and B subunits, has another Cys residue in position A9 of the B subunit. In a study on HbA, Alben et al. (*8*) were the first to report spectroscopic observations of absorption bands due to fundamental vibrational transition of cysteine sulfhydryl groups in proteins. Further investigations allowed the assignment of the observed signals to the S−H stretching modes relative to the different cysteine residues, namely, the α-104, *β*-112, and *β*-93. These assignments provided the basis for the use of the frequency and intensity of the S−H absorption band to probe the quaternary rearrangements that take place in HbA upon ligand binding (*9, 10, 11*).

The obtained results provide the first heme-independent spectroscopic probe of conformational changes of HbI, which accompany ligand binding. The observed changes in frequency and intensity of the SH absorption band have been attributed to ligand-linked changes in the electrostatic potential in the subunit interface.

MATERIALS AND METHODS

The hemoglobins (HbI and HbII) from the mollusk *Scapharca inaequivalvis* were purified as described previously (*12*). The single mutation Phe97→Leu was obtained as described by Pardanani et al. (*6*). Expression of the mutant in *E. coli* and protein purification were carried out as described for recombinant wild-type HbI (*13*).

The proteins were concentrated on Centricon PM 10 (Amicon Inc. Beverly, MA) concentrators to 6−20 mM heme, in 0.2 M phosphate buffer at pH 7.0.

FTIR spectra were measured on a Nicolet Magna 760 spectrometer equipped with an MCT/A detector at 2 cm^{-1} resolution. The instrument was purged with dry air generated by a Domnik Hunter CoRP 142 system (Domnik Hunter Lim., Dukesway, UK).

The protein solutions were placed in a 0.2 mm CaF_2 cell; for each sample, between 256 and 1000 scans were collected and averaged. From each spectrum, the spectrum of the PMB-reacted protein measured at the same protein concentration was subtracted as baseline. Protein concentrations were determined on each sample by recording the visible absorption spectrum in the same CaF_2 cell used for infrared measurements, with a 0.05 mm spacer. A molar absorptivity on a heme basis of 14,200 M^{-1} cm^{-1} at 578 nm was used for the oxygenated derivative (*12*). Deoxygenated hemoglobins were prepared by addition of a 2−4-fold molar excess of sodium dithionite, buffered at pH 7.0. *p*-Chloro-mercuribenzoate (PMB) (Sigma Chemical Co, St. Louis, U.S.A.) was dissolved in 0.1 M NaOH, buffered at pH 7.0 and centrifuged before use. The concentration of PMB solution was determined spectrophotometrically by using an extinction coefficient of 16900 M^{-1} cm^{-1} at 232 nm (*14*). PMB was added in slight molar excess to the proteins in the oxygenated state. In the case of PMB reacted deoxy HbI, the sample was prepared under a N_2 atmosphere since addition of dithionite causes the cleavage of the mercaptide bond. The infrared absorption of both free and sulfhydryl-bound PMB in the region of interest was negligible under the conditions used.

The integrated intensity of the relevant bands (B_{mM}) were calculated by a simple quadrature routine with the Matlab program (The Math Work Inc., South Natick, MA).

The sulfhydryl spectra in HbII are more complex to interpret due to the overlap of the Cys92 and Cys9 absorption bands. To single out the contribution of each Cys residue from the observed spectral envelope, the overall line shape (V_{obs}) was fitted to the sum of two voigtian curves (eqs 1 and 2). The Voigt profile (eq 1) yielded better fitting results with respect to the simple Lorentzian (*L*) or Gaussian (*G*) curve (15). Four fitting parameters are needed to minimize each single voigtian curve, namely, intensity (*I*), peak frequency (υ_0), Lorentzian width (Γ), and Gaussian width (σ).

$$V(\upsilon,\upsilon_0) = G(\upsilon,\upsilon_0) \times L(\upsilon,\upsilon_i) = I \int \exp[-(\upsilon-\upsilon_0)^2/2\sigma^2] \times \Gamma/[(\upsilon-\upsilon_i)^2 + \Gamma^2]\, d\upsilon \quad (1)$$

$$V_{obs} = V_1(I_1,\upsilon_1,\sigma_1,\Gamma_1) + V_2(I_2,\upsilon_2,\sigma_2,\Gamma_2) \quad (2)$$

Thus, in the fitting procedure for each HbII absorption profile, eight parameters were minimized. Despite the relatively large number of parameters, the fit converged rapidly by assigning to V_1, as initial guesses, the values of the intensity, frequency, and line width characteristic of the Cys92 absorption band in HbI. The integrated intensities, overall line widths, and molar absorptivities of the relevant bands were then calculated for V_1 (Cys92) and V_2 (Cys9), respectively.

FTIR absorption spectra have also been measured on the CO derivatives of native and Phe97→Leu in the CO stretching region. Measurements were carried out on the same solutions used for the SH absorption measurements, using 0.05 mm optical path length cells.

RESULTS

The sulfhydryl groups display an absorption band in the 2500−2700 cm^{-1} region in which water transmits maximally (*9*). In both HbI and HbII, a reliable assignment of the band relative to the S−H stretching mode was obtained after subtraction of the spectra of the PMB-reacted proteins. The assignment was also confirmed by the disappearance of the relevant absorption band at pH values >9.8 (data not shown). In HbI, the Cys92 S−H stretching mode gives rise to a peak at 2559 cm^{-1} in the deoxy derivative. The peak is shifted to 2568 cm^{-1}, is strongly quenched, and also broadened in both the CO and O_2 adducts with respect to deoxy HbI (Figure 1 A and Table 1). In the Phe97→Leu mutant, the S−H stretching band is centered at about 2560 cm^{-1} in the

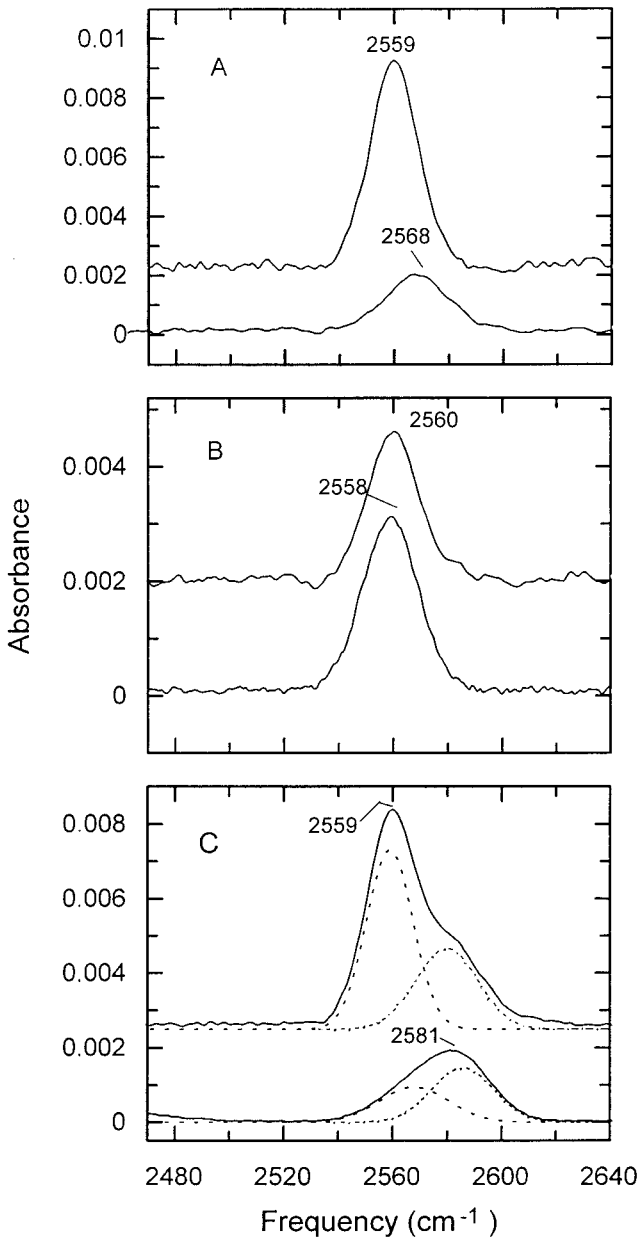

FIGURE 1: Infrared absorption spectra of deoxy- (top) and oxy- (bottom) *Scapharca inaequivalvis* native HbI (A) Phe97→Leu HbI (B), and native HbII (C) derivatives. Spectra have been measured in a 0.2 mm optical path CaF_2 windows in 0.2 M phosphate buffer at pH 7.0 and 20 °C. Protein concentration was 20.1, 6.8, and *10.2* mM (heme) for (A), (B), and (C), respectively. The spectrum of the deoxy derivatives was measured in the presence of 25 mM sodium dithionite. The dashed lines of panel (C) represent the absorption bands of Cys92 (- -) and of Cys9 (-·-), respectively, as obtained from the deconvolution procedure outlined under Materials and Methods. Fitting parameters are for deoxy HbII: $I_1 = 5.2 \pm 0.4 \times 10^{-3}$; $\upsilon_1 = 2559 \pm 0.4$; $\sigma_1 = 15.2 \pm 0.4$; $\Gamma_1 = 2.3 \pm 0.3$; $I_2 = 2.2 \pm 0.2 \times 10^{-3}$; $\upsilon_2 = 2580.4 \pm 0.3$; $\sigma_2 = 13.4 \pm 0.4$; $\Gamma_2 = 2.6 \pm 0.2$; for oxy HbII: $I_1 = 0.9 \pm 0.2 \times 10^{-3}$; $\upsilon_1 = 2568.2 \pm 0.3$; $\sigma_1 = 16.2 \pm 0.5$; $\Gamma_1 = 2.3 \pm 0.3$; $I_2 = 1.6 \pm 0.3 \times 10^{-3}$; $\upsilon_2 = 2588.1 \pm 0.3$; $\sigma_2 = 13 \pm 0.3$; $\Gamma_2 = 2.5 \pm 0.1$.

unliganded protein and is blue shifted by 2 cm^{-1} upon ligand binding. At variance with the behavior of the native protein, the band intensity is slightly increased in the oxy derivative with respect to the deoxy one (Figure 1B).

The situation is more complex in tetrameric HbII due to the additional cysteine residue (Cys9) present on the B chains of the A_2B_2 tetramer. In the deoxy-HbII derivative, the strong peak at 2559 cm^{-1} can be assigned to Cys92 on the basis of the data obtained on HbI. Accordingly, the shoulder at about 2580 cm^{-1} is assigned to the Cys9/S−H stretching mode. In the liganded derivatives, only a broad band centered at ~2583 cm^{-1} is observed that comprises the weak Cys92 signal and the contribution from the Cys9 residue (Figure 1C). The data on HbII were fitted to a sum of two voigtian curves by fixing the frequencies and line widths relative to the Cys92/SH absorption band to the values obtained for the HbI homodimer. Thus, five parameters were minimized in the fitting procedure, namely, the intensities of the two bands (i.e., those relative to both Cys92 and Cys9), the Gaussian and Lorentzian widths, and the peak frequency of the Cys9 band. The result of the fitting procedure, shown in Figure 1C, indicates that in the liganded derivatives the peak position due to the Cys9 absorption band is shifted by 8 cm^{-1} toward higher frequencies and that the integrated intensity is decreased by 18%.

Table 1: Infrared Absorption Bands of Sulfhydryl Groups in Native HbI and HbII Hemoglobins from *Scapharca inaequivalvis* and in the Phe97→Leu HbI Mutant

	υ_{SH} (cm^{-1})	$\Delta\upsilon_{1/2}$ (cm^{-1})	ϵ_{mM} ($mM^{-1}\,cm^{-1}$)	B_{mM} ($mM^{-1}\,cm^{-2}$)
native HbI				
deoxy	2559	12.1	0.023	0.48
oxy	2568.2	16.0	0.005	0.21
CO	2567.8	15.6	0.006	0.22
Phe97→Leu HbI				
deoxy	2560.4	13.3	0.022	0.57
oxy	2558.2	15.3	0.023	0.65
CO	2558.8	15.2	0.022	0.63
native HbII (Cys 92)[a]				
deoxy	2559	12.1	0.023	0.51
oxy	2568.2	16.0	0.007	0.17
CO	2567.8	15.6	0.008	0.18
native HbII (Cys 9)[a]				
deoxy	2580.4	13.5	0.020	0.43
oxy	2588.1	13.1	0.015	0.33
CO	2587.2	13.2	0.016	0.34

[a] Data obtained from the deconvolution procedure outlined under Materials and Methods using the parameters given in Figure 1.

The CO stretching absorption bands have also been measured in native and in Phe97→Leu HbI and in the HbII tetramer. Native HbI and HbII (data not shown) display identical absorption peaks centered at 1945 cm^{-1}, in full agreement with previous resonance Raman and FTIR measurements (*16, 17*). The frequency of the CO stretching band in the Phe97→Leu mutant is very similar (1946 cm^{-1}), and its line width is identical to that observed in the native protein (Figure 2).

DISCUSSION

The dramatic effect of the heme ligation state on the frequency and intensity of the infrared absorption of the Cys92/S−H group in HbI provides the first heme independent spectroscopic marker of the ligand-linked conformational transitions in this unusual hemoglobin. This finding is of special significance given the very small quaternary changes that accompany ligand binding in HbI.

Sulfhydryl infrared absorption bands have successfully been used as probes for the relatively large conformational changes accompanying the R−T transition in HbA due to the strategic positions of the cysteine residues in the two

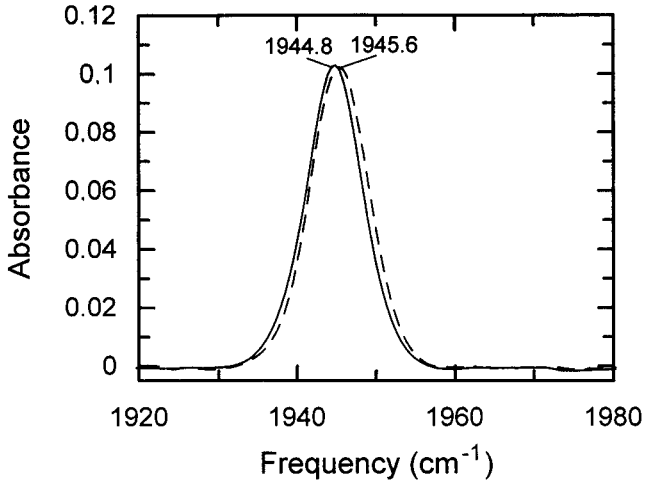

FIGURE 2: Infrared absorption spectra of native and Phe97→Leu carbonmonoxy HbI in the CO stretching region. The samples are those used in Figures 1 and 2; the optical path length was reduced to 0.05 mm. Spectra are normalized to the same protein concentration.

relevant $\alpha_1\beta_1/\alpha_2\beta_2$ and $\alpha_1\beta_2/\alpha_2\beta_1$ interfaces of the tetramer (*8, 11, 18*). In particular, Cysβ93 (F9) is located at the $\alpha_1\beta_2/\alpha_2\beta_1$ interface, while Cysβ112 (G14) and Cysα104 (G11) are buried at the $\alpha_1\beta_1/\alpha_2\beta_2$ interface. The quaternary rearrangements of the $\alpha_1\beta_2/\alpha_2\beta_1$ interface and the tertiary changes occurring at the $\alpha_1\beta_1/\alpha_2\beta_2$ interface that accompany ligand binding are reflected in shifts or intensity changes in the infrared absorption bands of the relevant cysteines. Thus, the Cys sulfhydryl are effective reporter groups of all the ligand-linked changes that take place in HbA.

In deoxy-HbI, the integrated intensity of the Cys92 infrared band is about one-half that corresponding to Cysβ112 or Cysβ93 in the HbA-CO derivative (*8, 18*). In ligand-bound HbI, the Cys92 band undergoes a 3-fold decrease in the integrated intensity, a sizable broadening, and a 9 cm^{-1} shift toward high frequency with respect to the deoxy derivative (Figure 1C and Table 1). Such a large effect on the band frequency and intensity was unexpected given the striking similarity of the crystallographic structures of deoxy- and CO-bound HbI. The interpretation in structural terms is challenging and requires a detailed analysis of the ligand-linked changes occurring at the subunit interface.

The observed effects on the Cys92 infrared spectrum in HbI cannot be interpreted in terms of changes in the strength of hydrogen bonding to another residue in the interface, as in the case of cysteines α104 or β112 in HbA (*8, 18*). In fact, inspection of the crystallographic structures of both ligand-free and CO-bound HbI does not reveal nearest neighbor interactions that can be ascribed to hydrogen bonding of the Cys92 sulfhydryl group. The nearest possible hydrogen bond acceptor is the carboxyl oxygen of Asp89 that, however, is located at 3.36 from the Cys92 sulfur atom in the deoxy derivative and remains essentially in the same position (within 0.05) in the CO adduct (Brookhaven Protein Data Bank). Zooming out the structural frame in the vicinity of Cys92 unveils the interfacial water architecture comprised between residues 94−102. The water network consists of 17 well-ordered molecules in the deoxy derivative, which is reduced to 11 water molecules in HbI−CO due to the extrusion of Phe97 from the heme pocket into the interface (Royer, 1994). The partial dehydration of the interface and the corresponding changes in the overall polarity of the local environment are sensed by the Cys92 sulfhydryl group, located at one helical turn from Phe97. In this framework, the observed quenching of the absorption band intensity upon ligand binding can be ascribed to a change of the bulk dielectric properties in the vicinity of the cysteine residue rather than to a specific interaction of this group with a selected residue. In fact, the increase in the integrated absorption intensity of sulfhydryls in a polar environment with respect to an apolar one is a well-known phenomenon in organic thiols (*8, 18*). The red shift of the absorption peak, coupled to a marked line broadening effect upon lowering the solvent polarity in organic thiols is also consistent with the behavior of Cys92 in HbI (*8*).

The interpretation of the data on native HbI is strengthened by the lack of the above-mentioned effects on the infrared absorption band in the Phe97→Leu mutant. Accurate crystallographic studies have shown that in the absence of the Phe97 residue, the interfacial water molecules network does not undergo ligand-linked changes since the Leu97 side chain is always in the heme pocket (*6*). As a consequence of the conservation of the total number of water molecules, the overall polarity of the local environment is most likely unaffected by ligand binding. The spectra of Figure 1B show that the peak frequency and integrated intensity of the Cys92/S−H groups display only slight changes in the mutant with respect to the native protein and that these are in the opposite direction, i.e., there is a slight increase in intensity and a decrease in frequency in the ligand-bound protein with respect to the deoxygenated protein. The small effects of heme ligation on the SH infrared spectrum of the Phe97→Leu mutant are consistent with the preservation of the interface polarity upon ligand binding but cannot readily be ascribed to specific structural changes. Whatever the observed effects, it allows the direct probing of the subunit interface independently of the heme environment. It is of interest to discuss the present data in the framework of the reported destabilization of the T state in the Phe97→Leu mutant, based on kinetic and thermodynamic measurements (*6*). The infrared absorption band of deoxy Phe97→Leu HbI is very similar to that of native (T-state) HbI, except for a 10−15% increase in the integrated absorption intensity of the SH group. In contrast, large differences are seen when comparing the infrared spectra of the ligand-bound species of the mutant and of the native protein. The present findings, taken together with spectroscopic data of Pardanani et al. (*6*), indicate that the substitution of the Phe97 with Leu has different effects in two different districts of the protein, namely, at the heme pocket and at the subunit interface. In the deoxy derivative, slight changes in the overall heme symmetry occur, which point to a reduced proximal strain in the mutant with respect to the native protein, as indicated by the CD spectrum (*6*). In turn, in the CO and O_2 derivatives, the heme pockets are not affected by the Phe97→Leu mutation as indicated by the visible absorption and CD spectrum and by FTIR measurements on the CO derivatives (Figure 2). The Phe97→Leu substitution, therefore, brings about changes in the heme pocket by reducing the proximal iron−histidine strain. Such changes are not transmitted to the interface, thus weakening the overall cooperative effect.

A final comment concerns the S−H absorption bands in the HbII tetramer. The absorption profiles relative to Cys92

in HbII, as expected, are superimposable to those observed in the HbI dimer. The observed ligand-linked changes in the Cys9 band deserve further analysis since the HbII−CO crystal structure shows that the Cys9 residue is exposed to solvent and, therefore, should be insensitive to ligand-linked quaternary changes. However, the A helix region, where Cys9 is located, has been proposed to participate in the formation of intermolecular salt bridges during the ligand-linked polymerization of the deoxy A_2B_2 tetramer into $(A_2B_2)_4$ and $(A_2B_2)_8$ species (*1*, *19*). The decrease in the stretching frequency and the increase in intensity of the Cys9/SH group upon deoxygenation could well be accounted for by the formation of a hydrogen bond bridge with a hydrogen bond acceptor in the contralateral molecule. The confirmation of this hypothesis, however, must await the resolution of the deoxy derivative crystallographic structure.

In conclusion, the sensitivity of the sulfhydryl infrared spectra in detecting small changes in the polarity of the medium has provided the first heme independent probe for the ligand-linked structural transitions occurring at the interface of HbI. In more general terms, the present findings are relevant for the understanding the behavior of protein SH groups in different local environments.

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