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Conformational Sensitivity of β-93 Cysteine SH to Ligation of Hemoglobin Observed by FT-IR Spectroscopy†

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ABSTRACT: The SH vibrational absorption of cysteine F9(β-93) in concentrated aqueous solutions of native liganded hemoglobin (human HbA, horse, and bovine) has been observed by use of Fourier transform infrared spectroscopy. The pattern of β-93 SH absorption intensity is ligand dependent. In bovine hemoglobin derivatives the SH absorption intensity pattern is (carbonmonoxy)hemoglobin (HbCO) > oxyhemoglobin (HbO_2) = cyanomethemoglobin (HbCN) ≫ aquomethemoglobin (metHb) and deoxyhemoglobin (deoxyHb). In horse and human hemoglobin derivatives the pattern is HbCO ≥ HbO_2 > HbCN > metHb. The bovine metHb β-93 SH shows a much lower absorptivity than that of horse or human metHb, and thus it has a different local tertiary equilibrium conformation than does horse or human hemoglobin. X-ray diffraction studies have shown the β-93 SH in carbon monoxide or oxygen bound hemoglobin to be situated within a nonpolar pocket between the F, G, and H helices. The higher than usual SH absorption frequency (2592 cm^{-1}) that we observe implies there is no hydrogen bonding for this thiol group while situated within this nonpolar pocket. A similar β-93 SH absorption has been observed in the β-chain tetramer (thalassemic hemoglobin H in vivo). The β-112 SH stretching band, previously observed in the $\alpha_2\beta_2$ tetramer, was observed for the first time in the β-chain tetramer. A band at 2610 cm^{-1} that is not due to SH was resolved and found to be ligand dependent.

Mammalian hemoglobin is a tetrameric protein consisting of two α and two β subunits that cooperatively bind heme ligands. The oxygen affinity of the isolated subunits is unaltered when they assemble into $\alpha_1\beta_1$ dimers, and ligand binding is noncooperative for both monomers and dimers (Mills & Ackers, 1979). Comparisons of the X-ray crystallographic structures of tetrameric hemoglobin (Baldwin & Chothia, 1979; Fermi et al., 1984) show that ligand binding causes large intersubunit changes about the hemes that extend to the $\alpha^1\beta^2$ contact region with only minor differences at the $\alpha^1\beta^1$ interface. X-ray diffraction does not allow individual bond lengths to be determined with sufficient precision to identify very small changes in large molecules, such as those that might be present at the $\alpha^1\beta^1$ contact. Detection of bond vibrations may enable one to do this, but the problem has always been to isolate and identify the individual vibrating group. We have previously shown that cysteine sulfhydryl vibrations absorb in an isolated spectral region and that FT-IR[1] spectroscopy has the sensitivity to measure the absorption from these groups in concentrated protein solutions (Bare et al., 1975). Cysteine SH groups have proven to be very sensitive molecular probes of protein conformation. The frequency of the SH vibrational absorption is decreased by H-bonding to a nucleophilic group, and the extent of this decrease is determined by the base strength of the associated nucleophile. In similar fashion, the area of the absorption band is proportional to the integrated absorption coefficient (B), which increases with H-bonding due to the greater change in SH

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[1] Abbreviations: FT-IR, Fourier transform infrared; Hb, hemoglobin; HbCO, (carbonmonoxy)hemoglobin; HbO_2, oxyhemoglobin; HbF, fluoromethemoglobin; HbCN, cyanomethemoglobin; HbN_3, azidomethemoglobin; HbNO, nitrosylhemoglobin; metHb and $HbOH_2$, aquomethemoglobin; deoxyHb, deoxyhemoglobin; HbOOCH, formatomethemoglobin; $(\beta CO)_4$, tetramer of carbonmonoxy β-chains; $(\beta O_2)_4$, tetramer of oxy β-chains; deoxyβ_4, tetramer of deoxy β-chains; $\alpha_1\beta_1$, α–β dimer; $\alpha_2\beta_2$, α–β tetramer; PMB, *p*-(chloromercuri)benzoate. Superscripts denote interfacial contacts between α- and β-chains, as in $\alpha^1\beta^1$ or $\alpha^1\beta^2$.

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dipole moment (μ) with the vibrational coordinate (Q), according to

$$B = k(d\mu/dQ)^2 \quad (1)$$

where k is a function of the frequency and number of vibrating dipoles. The SH absorptions of the two cysteine residues (α-104 and β-112) that occur in the nonpolar $\alpha^1\beta^1$ interface of human hemoglobin were measured by FT-IR spectroscopy (Alben & Bare, 1978, 1980; Bare et al., 1975; Alben et al., 1974, 1978) and provided detailed information about the dependence of hemoglobin conformation on the state of ligation at the heme. The α-104 SH frequency increased in the sequence HbCO < HbN_3 < HbNO < HbCN < HbO_2 < $HbOH_2$ < HbF < HbOOCH ≪ deoxyHb (Alben & Bare, 1980).

The α-104 SH was suggested to be H-bonded to a peptide carbonyl group of α-100 leucine, one turn back in the G helix (Bare et al., 1975). DeoxyHb has an α-104 SH frequency 4.0 cm^{-1} higher than that of HbCO. The higher α-104 SH frequency of deoxyHb (T state) corresponds to a longer SH···O=C distance and thus to a less tightly wound helix. These data are in agreement with analyses of crystallographic data by Baldwin and Chothia (1979) that show a rotation of the G helix with the R ↔ T transition. The infrared data also correlate quantitatively with single-crystal polarization data of Makinen and Eaton (1974), in that the α-104 cysteine SH center frequency increases in direct proportion to the amount of tilt of the heme (Alben & Bare, 1980). Similar dependence of the α-104 cysteine SH frequency on the Fe–ligand complex at the heme was observed with hemoglobins from horse and pig which contain only one cysteine (α-104) in the $\alpha^1\beta^1$ interface (Bare et al., 1975). The β-112 cysteine SH in human hemoglobin is H-bonded differently, such that its SH frequency decreases with the R ↔ T transition. Cow hemoglobin, which contains cysteine only at the β-93 position, was used as the reference for these studies in order to subtract the absorption of the protein and permit observations of the remaining Cys SH groups at α-104 and β-112. The result was that no information about β-93 Cys SH was obtained from these studies.

Dr. M. F. Perutz suggested that we examine β-93 Cys SH by FT-IR spectroscopy to see whether it might also be H-bonded to a group in the protein. Crystallographic studies of horse hemoglobin by Heidner et al. (1976) indicated the β-93 cysteine sulfur was in a pocket between the F and H helices. In horse metHb, the β-93 cysteine appeared as a mixture of states, in and out of the F–H pocket. In human HbO_2 (Shaanan, 1983) the β-93 cysteine sulfur was observed mainly in the F–H pocket, but it was also in a second conformation. It appears that low-spin hemes favor an internal β-93, while high-spin hemes favor an external one, with aquometHb having the highest proportion of exposed β-93 (Shaanan, 1983). This is consistent with the results of Perutz et al. (1974) that reactivity of β-93 Cys with PMB increased in the order HbCO < HbO_2 < metHb. According to Shaanan (1983) the β-145 Tyr is in the F–H helix pocket in both the R and T states, but with a slightly different conformation. When a ligand binds at the heme, the Tyr phenyl ring appears to rotate by about 90°. The sulfur of the internal β-93 Cys then may come in contact with the face of the β-145 Tyr ring. When the β-93 Cys is rotated out of the nonpolar F–H helix pocket, it is exposed to the aqueous solvent. The random associations with the solvent water molecules would produce a very broad and weak absorption that is not observed under our measuring conditions. Internal SH groups have a more consistent environment (decreased random polar interactions) and narrower bandshapes. In this paper we report that the β-93 Cys SH absorbs at 2591.5 cm^{-1} and that it has no significant frequency change with heme ligation (to within 0.5 cm^{-1}). The higher than usual SH frequency suggests that this group cannot be hydrogen bonded to another protein residue. The β-93 SH band intensity varies with ligation as the fraction of internal β-93 Cys reported from X-ray crystallography and inversely with the reactivity of PMB with β-93 Cys: HbCO ≥ HbO_2 ≫ metHb. These observations are all consistent in that the lower infrared absorptivity and the greater PMB reactivity should both correspond to the protein conformation in which the β-93 SH has a higher probability of being in the external position. We also observe an absorption band at 2610 cm^{-1} that responds to heme ligation, but is not due to a cysteine SH vibration. Its cause has not yet been identified, but it may correspond to an overtone or combination mode due to the heme or adjacent protein group.

Experimental Procedures

Hemoglobin Preparations. Fresh human, horse, and cow (Holstein and Jersey) blood samples were collected in heparin or 0.013 M sodium citrate. Red cells were washed three or four times with 1% saline and hemolyzed with 1 volume of water and 0.5 volume of toluene. After centrifugation, hemolysates separated from the stroma were passed through a Sephadex G25 column equilibrated with 0.1 M chloride. The purified hemoglobin was concentrated by pressure ultrafiltration to a desired heme concentration (17–24 mM). DeoxyHb was obtained by repeated evacuation and equilibration with nitrogen or argon (99.998% pure). HbCO and HbO_2 derivatives were prepared in the same way except that pure carbon monoxide and oxygen gases were used, respectively. Samples were exposed to the corresponding gases for 12 h before use. MetHb was prepared by oxidation of Hb with a slight excess of $K_3Fe(CN)_6$. HbCN was prepared from metHb by addition of an excess of KCN. Excess $K_3Fe(CN)_6$ and KCN were removed by passage through a Sephadex column equilibrated with pH 7.1 [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris) chloride buffer. Mercuric chloride was added to the hemoglobin samples to be used as des-sulfhydryl references with the final pH checked to be 7.1. All reactions were done between 4 and 10 °C.

β_4 tetramers (hemoglobin H in vivo) were prepared from stripped HbA(CO) solutions reacted with PMB (dissolved in NaOH and titrated with acetic acid) as first described by Rosemeyer and Huehns (1967), except that 35% by volume of saturated sucrose was added prior to the final mixing. The reaction must be carried out under ice-cold and constant-stirring conditions. After dialysis the β^{PMB} chains separated from the α^{PMB} chains and other undissociated dimers by using the column chromatography method of Geraci et al. (1969). PMB was removed from the separated chains by washing the column with ethanethiol. The $(\beta O_2)_4$ derivative was prepared by repeatedly evacuating a $(\beta CO)_4$ aliquot (in ice) under a tungsten lamp and then flushing with pure oxygen. The deoxy-β_4 derivative was obtained by repeated evacuation and equilibration with nitrogen of the $(\beta O_2)_4$ aliquot followed by the addition of 1:1 heme equivalent of sodium dithionite.

The separated chains were characterized for purity with 7% polyacrylamide gel disk electrophoresis at pH 8.3 (Tris–glycine buffer) using 6-cm gels and 3 mA/tube (Davis & Ornstein, 1959). Relative mobilities (R_f) were measured with respect to purified HbA, with oxy α-chains at 0.54 and oxy β-chains at 1.34, relative to oxyHbA at 1.00.

Reactive SH groups of the protein samples were determined by the method of Boyer (1954) in 0.1 M phosphate buffer at

Table I: Infrared Absorption Bands of Sulfhydryl Groups in (Carbonmonoxy)hemoglobin from Humans and Ethanethiol in Several Solvents[a]

	ν_{SH} (cm^{-1})	$\Delta\nu$ (cm^{-1})	a_{mM} (mM^{-1} cm^{-1})	B (mM^{-1} cm^{-2})	ref
human (carbonmonoxy)hemoglobin in H_2O					
α-104[b]	2552.6	13.5	0.17	2.43	Bare et al. (1975)
β-112[b]	2566.3	12.5	0.055	0.80	Bare et al. (1975)
β-93[c]	2591.5	19.5	0.046	0.88	this work
ethanethiol (0.1 M)					
in bromoform (0.12 M EtSH)	2572.8	17.0	0.0023	0.050	this work
in chloroform	2582.3	17.5	0.0027	0.061	this work
in carbon tetrachloride	2579.8	23.0	0.0021	0.065	this work
in water	2573.6	39.5	0.0073	0.210	Bare et al. (1975)
in acetone	2564.8	30.2	0.0132	0.429	Bare et al. (1975)
in dimethylacetamide	2534	58	0.021	1.35	Bare et al. (1975)

[a] Absorbance, $A = \log (I_0/I)$, was used to calculate millimolar absorptivity, $a_{mM} = A/(cl)$, and the integrated absorption coefficient, B(area) = $(1/cl)\int A\, d\nu$. [b] Band parameters were determined by deconvolution through curve fitting with two Lorentzian bandshapes. [c] Band parameters were determined from the observed band envelope.

pH 6.0. The reaction solution was measured at 255 nm. This provided direct identification of the β-chains during chromatographic separation. Heme concentrations were measured as the cyanide derivative assuming $a_{540} = 11 \times 10^3$ (M heme)$^{-1}$ cm^{-1}.

Spectroscopy. Both visible and near-infrared spectra to determine water content (1430 nm) and hemoglobin purity and concentration were measured with a Perkin-Elmer Model 4000A split beam scanning spectrophotometer. Visible spectra were also measured with a Cary 15 spectrophotometer.

Infrared spectra were obtained at 1-cm^{-1} resolution with a Digilab Model FTS-14D infrared interferometer fitted with a germanium-coated KBr beamsplitter and a liquid N_2 cooled photovoltaic InSb detector. The optical bandpass was limited to 3000–1800 cm^{-1} by the detector, the absorption of solvent water, and a 3.5-μm low frequency pass optical filter. Interferograms were signal averaged and Fourier transformed to obtain single-beam spectra which were ratioed with a similarly obtained water spectrum to remove the instrumental contribution. A residual spectral component due to solvent water was removed by fractional absorbance substraction of a spectrum obtained from the ratio of two water samples of different path lengths. A reference protein absorbance spectrum was similarly corrected for solvent water. A fraction of the reference absorbance was then subtracted from the sample spectrum to leave the SH absorbance difference spectra. No other spectral smoothing or averaging procedures were used.

Results

Absorbance difference spectra of (carbonmonoxy)-hemoglobin preparations from human, horse, and cow were obtained with hemoglobin reference preparations that were similar except they were treated with a slight excess of $HgCl_2$, which readily reacts with the β-93 SH and more slowly with SH groups that are buried inside the protein (Figure 1). Strong, broad absorption bands due to β-93 cysteine SH are observed near 2592 cm^{-1}. Absorption bands at 2555 and 2565 cm^{-1} were previously assigned to cysteine SH groups at α-104 and β-112 in human hemoglobin and at α-104 (2555 cm^{-1}) in horse HbCO. In comparison with SH spectra of ethanethiol in several solvents (Table I) the high absorption frequency precludes the possibility that β-93 cysteine SH in HbCO might be H-bonded to protein or solvent. The spectra are consistent with crystallography of horse HbCO (Heidner et al., 1976) in which β-93 cysteine sulfur was observed in a pocket between the F and H helices. The broad bandwidth (19 cm^{-1}, compared with 14 cm^{-1} for α-104 SH) is consistent with the greater thermal motion of a surface group as compared with the motion of an internally H-bonded group at the nonpolar $\alpha^1\beta^1$ interface.

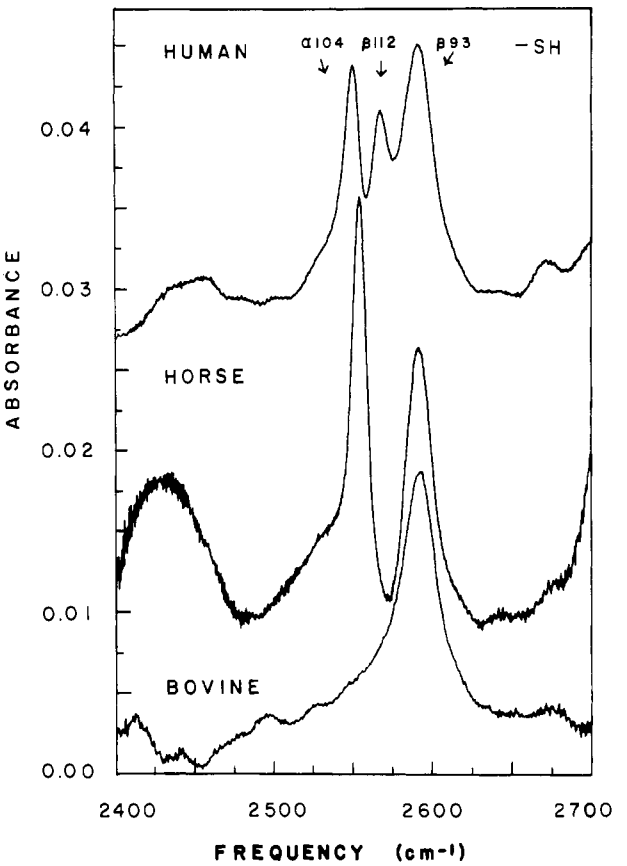

FIGURE 1: Cysteine SH vibrational absorption spectra from human, horse, and bovine (carbonmonoxy)hemoglobin obtained with 0.029-cm optical path and all normalized to 24 mM heme. Reference proteins were the corresponding (carbonmonoxy)hemoglobins treated with $HgCl_2$. Interferograms from human HbCO were averaged from 2048 scans, and those from horse and bovine HbCO were each averaged from 1024 scans. Spectra have been arbitrarily displaced on the absorbance scale to facilitate viewing.

With only one cysteine residue per symmetrical half-molecule, bovine hemoglobin presents the simplest SH spectrum. The effects of heme ligation on β-93 Cys SH are illustrated in Figure 2. No difference in SH frequency is observed, only a large difference in apparent absorptivity of the SH band, with HbCO > HbO_2 = HbCN. No absorption due to β-93 SH was observed with bovine metHb or deoxyHb. This was rather surprising in view of Heidner's observation that in horse metHb the β-93 Cys sulfur appeared to consist of a mixture of states, in and out of the F–H pocket. If we assume a model in which the β-93 SH infrared absorption band is observed only for that fraction that is rotated into the F–H pocket and define HbCO as 100%, then in bovine HbO_2 and HbCN β-93 SH is approximately 66% and 64%, respectively, in the F–H

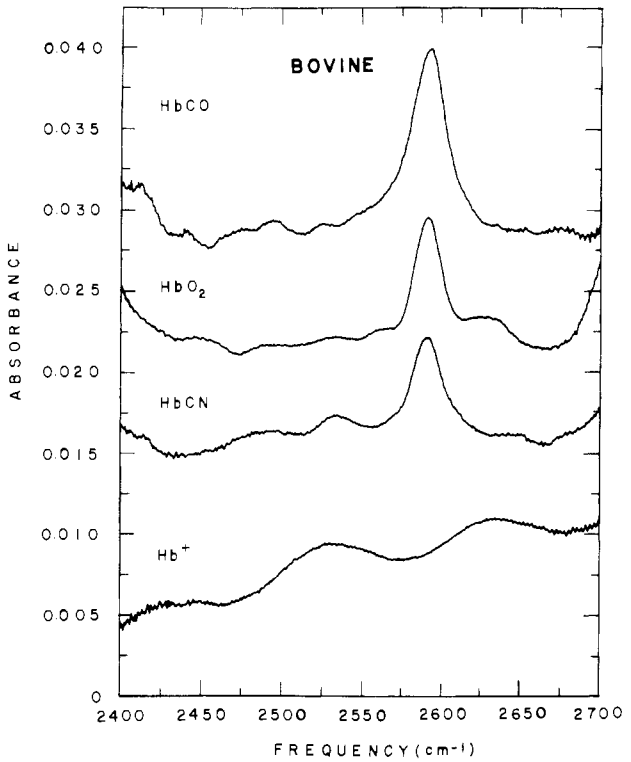

FIGURE 2: Infrared absorbance spectra of β-93 cysteine SH groups in bovine hemoglobin derivatives carbonmonoxy, oxy, cyanomet, and acid aquomet. Sampling conditions were normalized to 19.9 mM heme and 0.03-cm optical path length. Reference spectra were the Ag^+- or Hg^{2+}-treated hemoglobin derivatives. Interferograms were each averaged from 1024 scans.

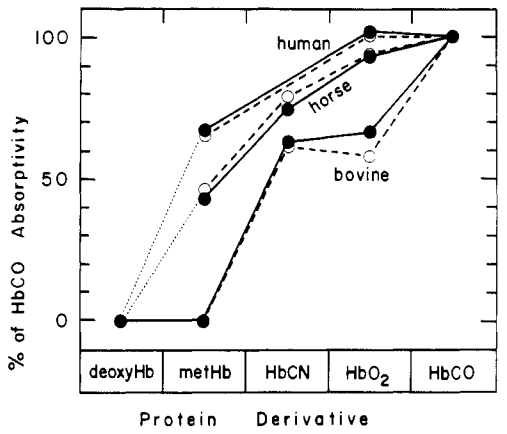

FIGURE 3: Percent of HbCO infrared absorptivity (-●-) and integrated absorptivity (--O--) for bovine, horse, and human (carbonmonoxy)-, oxy-, cyanomet-, aquomet-, and deoxyhemoglobin derivatives.

pocket and in metHb it is excluded from the F-H pocket. The absorptivities of the metHb, HbCN, HbO_2, and HbCO derivatives are listed in Table II and graphed as a percent of HbCO in Figure 3.

Preparations of horse hemoglobin were therefore examined in similar fashion (Figure 4). In this case, absorption bands due to α-104 cysteine SH indicate that a significant amount of this group had also reacted with $HgCl_2$ in the reference hemoglobin preparation. However, the relative intensities of the β-93 SH absorption bands may still be compared and yield 93%, 74%, and 43% of HbCO for HbO_2, HbCN, and metHb, respectively (see Figure 3 and Table II). These data appear to be in good agreement with the crystallographic observations of Heidner et al. (1976) for horse HbCO and metHb in which metHb, HbO_2 (Shaanan, 1983), and presumably HbCN appear to be mixtures of conformational states of the β-93 sulfhydryl.

Table II: Vibrational Absorption of β-93 Cysteine SH[a]

sample hemoglobin	ν_{SH} (cm^{-1})	a_{mM} (mM^{-1} cm^{-1})	B (mM^{-1} cm^{-2})
human			
HbCO	2591.5	0.046	0.88
HbO_2		0.047	0.89
metHb		0.031	0.58
β-chains			
$(\beta CO)_4$	2591 ± 1.5	0.047	0.88
$(\beta O_2)_4$		0.045	0.85
horse			
HbCO	2591.0	0.047	0.87
HbO_2		0.044	0.82
HbCN		0.035	0.69
metHb		0.020	0.40
bovine			
HbCO	2592.0	0.044	0.89
HbO_2		0.029	0.52
HbCN		0.028	0.54
metHb		0	0
deoxyHb		0	0

[a] Infrared spectral data for hemoglobin derivatives in 0.05 M Bis-Tris and 0.1 M chloride buffer at pH 7.05.

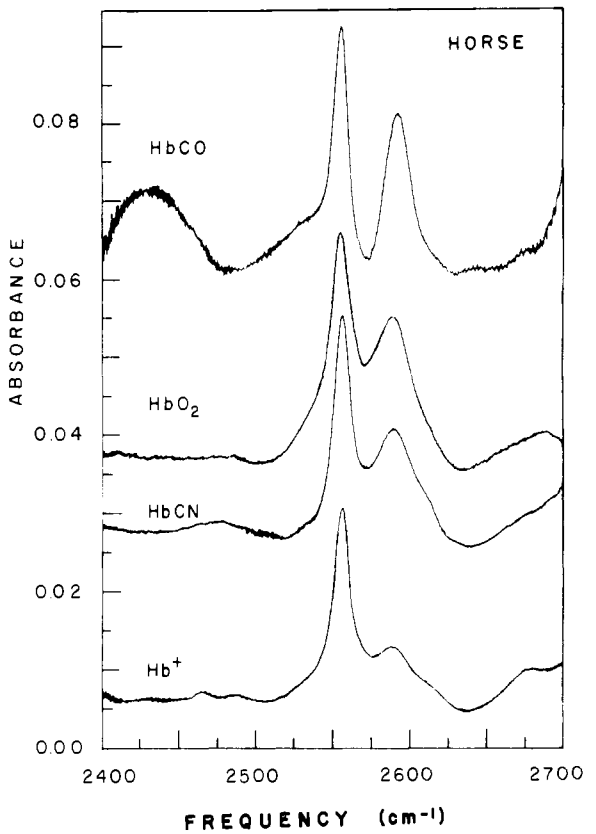

FIGURE 4: Vibrational spectra of cysteine SH groups in horse hemoglobin derivatives. (Carbonmonoxy)hemoglobin was compared with $HgCl_2$-treated HbCO, while other derivatives (oxy, cyanomet, and acid aquomet) were compared with $HgCl_2$-treated HbO_2 as reference. Spectra were normalized to 24 mM heme and 0.028-cm optical path length. Interferograms were each averaged from 1024 scans.

Human hemoglobin displays more complicated SH spectra (Figure 5) due to the presence of three partially overlapped absorption bands from cysteines at α-104, β-112, and β-93 in the symmetrical half-molecule ($\alpha_1\beta_1$). However, previously described data patterns are again observed here, with narrow absorption bands due to α-104 and β-112 SH and a broader absorption band due to β-93 SH. Peak frequencies of the α-104 and β-112 SH groups converge in the sequence

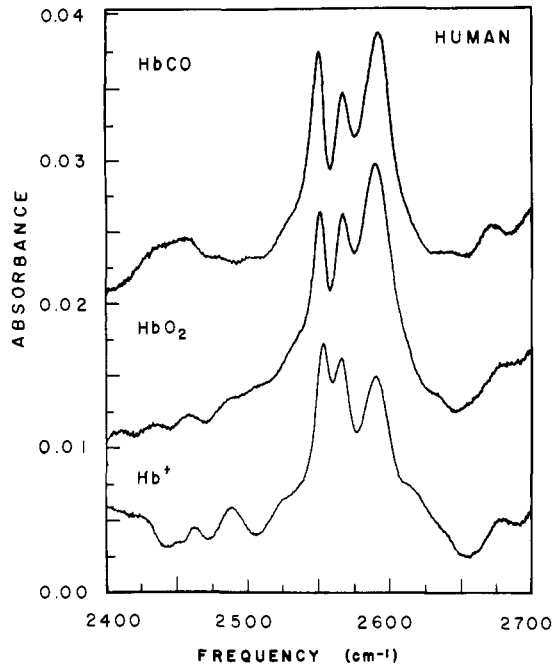

FIGURE 5: Infrared spectra of cysteine SH groups in derivatives of human hemoglobin A. Reference spectra were $HgCl_2$-treated CO and O_2 derivatives of HbA as in Figure 4.

HbCO:HbO_2:metHb, whereas that for the β-93 SH remains constant at 2592 cm^{-1}. The apparent intensities of the β-93 SH band relative to that of human HbCO are 102% and 67% for human HbO_2 and metHb, respectively (see Figure 3 and Table II).

Note that the $HgCl_2$-treated reference hemoglobins in Figures 2–5 were prepared from HbO_2 for all sample spectra except HbCO, for which HbCO($HgCl_2$) was used. The $HgCl_2$-treated deoxyHb and metHb were unstable and quickly denatured, while the HbO_2 and HbCO derivatives remained in solution and appeared quite stable. The Hg^{2+}-treated HbCO was not suitable as a reference for the other derivatives, however, since its use led to the appearance of another absorption band at 2610 cm^{-1} in the absorbance difference spectrum [Figure 6 (top and middle)]. This band is not due to an SH absorption since it disappears when the same hemoglobin derivative treated with Hg^{2+} is used as the protein reference. The absorption at 2610 cm^{-1} is clearly observed in the absorbance difference between the two reference proteins, Hg^{2+}·HbO_2 and Hg^{2+}·HbCO [Figure 6 (bottom)]. It must therefore be due to an overtone or combination mode that is more intense in Hg^{2+}·HbO_2 than in Hg^{2+}·HbCO.

Isolated β-Chains (β_4). The effects of ligation on β-93 Cys SH were examined with isolated β-chains that, at the high protein concentration required to observe these weak bands by IR spectroscopy, exist as the β_4 tetramer. Absorbance difference spectroscopy of four β_4 derivatives, $(\beta CO)_4$, $(\beta O_2)_4$, deoxyβ_4, and [Hg^{2+}·$(\beta CO)_4$], were determined (Figure 7 and 8). Only the $(\beta CO)_4$ was reacted with $HgCl_2$ because of the instability of other derivatives toward heme oxidation and precipitation. The absorbance difference spectra of $(\beta CO)_4$ or $(\beta O_2)_4$ minus [Hg^{2+}·$(\beta CO)_4$] (Figure 7) clearly reveal absorptions at 2565 and 2590 cm^{-1} due to Cys SH at respectively β-112 and β-93 in addition to a small shoulder at 2610 cm^{-1}. This confirms the assignment of the 2565-cm^{-1} band to β-112 Cys SH and suggests that it may be H-bonded within the same β-chain, in a manner similar to that suggested for α-104 cysteine (Bare et al., 1975). The $(\beta O_2)_4$ minus deoxyβ_4 difference spectrum (Figure 8) exhibits the β-93 absorption at 2592 cm^{-1} and the 2610-cm^{-1} band.

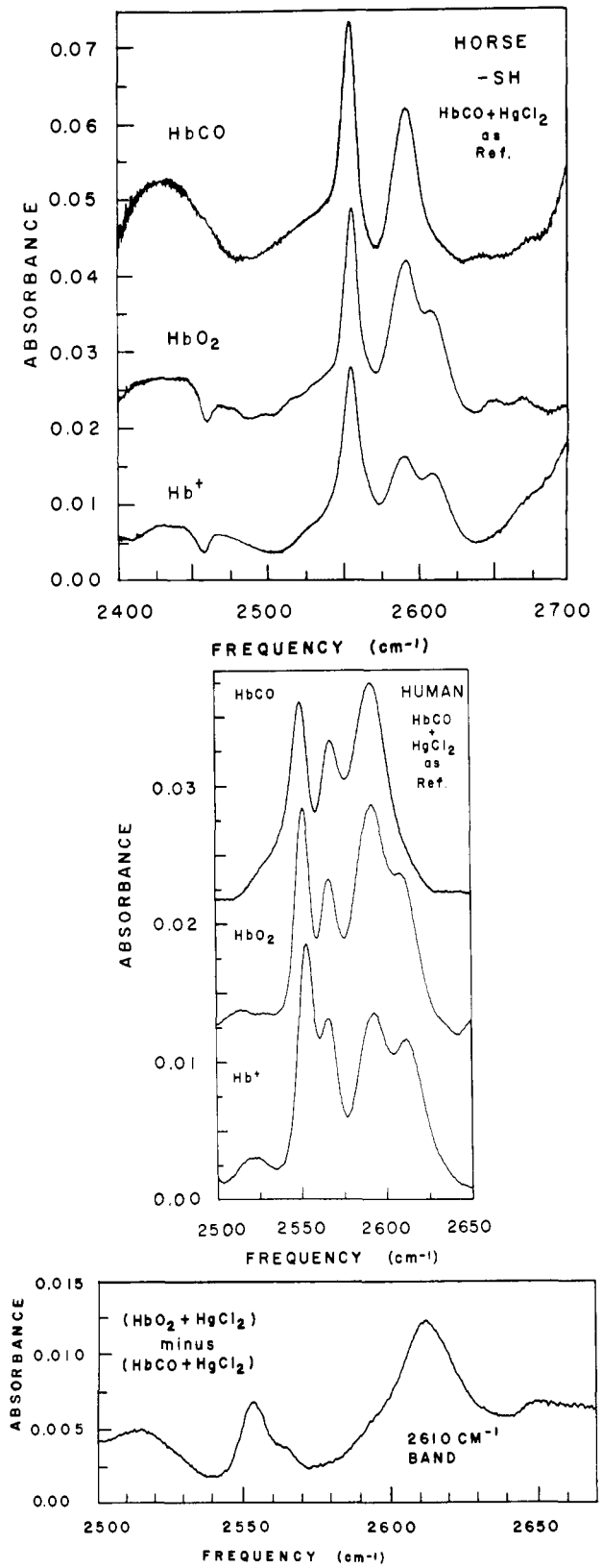

FIGURE 6: Infrared spectra from (top) horse and (middle) human hemoglobin derivatives compared with corresponding $HgCl_2$-treated HbCO derivatives as reference. The additional absorption at 2610 cm^{-1} is also present in the absorbance difference of the reference spectra (bottom) $HgCl_2$-treated O_2 minus CO derivatives of human hemoglobin A and is therefore not due to a sulfhydryl absorption.

The β-93 SH absorption in the β_4 tetramer exhibited a relatively wide variation in center frequency, 2589.5–2592.0 cm^{-1} for $(\beta CO)_4$ compared to that for $\alpha_2\beta_2$ (carbonmonoxy)hemoglobins at 2591.0–2592.0 cm^{-1}. Otherwise, the β-chain tetramer data are in good agreement with observations

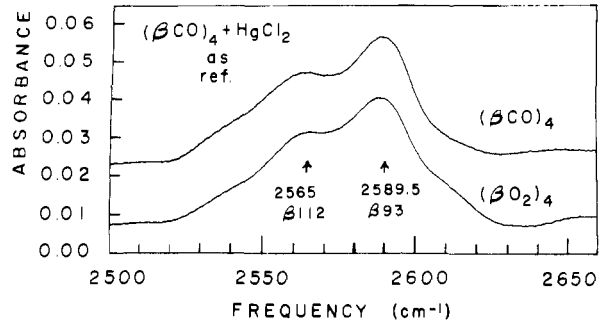

FIGURE 7: Infrared spectra of carbonmonoxy and oxy derivatives of tetramers of human β-chains referenced to $HgCl_2$-treated carbonmonoxy β-chain tetramers.

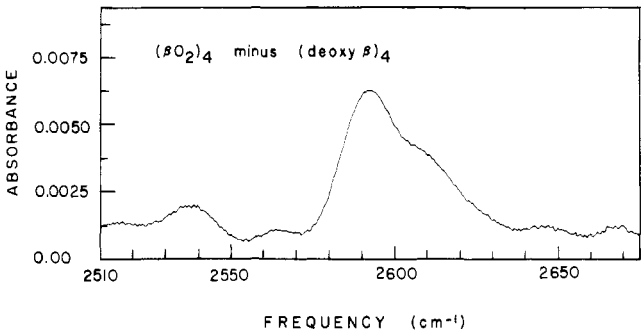

FIGURE 8: Infrared absorbance difference spectrum of oxy minus deoxy human β-chain tetramers. The spectra were normalized to a heme concentration of 11 mM and an optical path length of 0.0145 cm.

from the hemoglobin tetramer, and several conclusions may be drawn from them. (1) The absorption band at 2592 cm^{-1} due to β-93 cysteine depends only upon ligation of the β-chain heme and its effect upon β-chain tertiary structure, especially rotation of the F helix. (2) In human hemoglobins and β-chains, rotation of β-93 Cys SH into the F–H pocket appears to be complete for both the CO and O_2 complexes. (3) The β-93 SH center frequency is insensitive to hemoglobin quaternary structure. In contrast, while the frequency shift of the α-104 cysteine SH is greatly accentuated by the R ↔ T transition in the $\alpha_2\beta_2$ tetramer (Bare et al., 1975; Alben & Bare, 1980), no such enhancement is observed at the β-93 cysteine. (4) The absorption at 2610 cm^{-1} is not due to an SH group, but is sensitive to β-chain conformation and especially to local differences between CO and O_2 complexes. The near identity of the β-93 SH absorption for these complexes suggests that the β-chain tertiary structures are also very similar near this site, and major differences must be limited to the heme pocket. (5) Since the β-112 absorbance band does not appear in the difference spectrum $(\beta O_2)_4$ and $(\text{deoxy}\beta)_4$, frequency shifts at this location appear to be dependent on $\alpha_2\beta_2$ quaternary interactions, as previously found for α-104 cysteine SH (Alben & Bare, 1980).

Discussion

Infrared spectroscopy of hemoglobins from cow, horse, and human (HbA) consistently show an absorption near 2592 cm^{-1} that disappears upon reaction with Hg^{2+} or Ag^{+} salts. The band frequency is consistent with a non-hydrogen-bonded thiol surrounded by noninteracting groups (as in a nonpolar solvent), and the bandwidth is consistent with thermal motion of a group near the protein surface. However, both the absorptivity at the band maximum and the integrated band absorptivity are much greater than those observed for simple thiols in nonpolar solvents. Integrated absorptivity is a function of the change in dipole moment with the vibrational coordinate and may be increased with bond length as in H-bonding or by an increase in charge density. Frequency of a harmonic oscillator varies with force constant and inversely with mass. H-bonding would be expected to decrease the SH force constant, and thereby the frequency, as observed for the α-104 and β-112 Cys SH groups. The large absorptivity and high SH frequency of the β-93 are consistent with an increased charge density on the sulfur associated with a small increase in SH force constant. This may reasonably be associated with polarization induced by the phenyl ring of the β-145(HC2) Tyr. This tyrosine is in van der Waals contact with the β-93 Cys sulfur in the nonpolar pocket formed by the F and H helices in HbO_2, as observed by X-ray crystallography (Shaanan, 1983).

The apparent absorptivity of the β-93 SH may then serve as a measure of rotation of the F helix, caused by ligation at the β-heme. This is a direct effect of the state of the F8 His–Fe–porphyrin complex on the F9 Cys(β-93), F helix, and β-chain tertiary structure. The effect is fully as large in isolated β-chain complexes as in the $\alpha_2\beta_2$ tetramer and therefore affects only tertiary structure and is independent of quaternary interactions. This is in sharp contrast to SH groups located at the hydrophobic $\alpha^1\beta^1$ interface (α-104 and β-112), where sensitivity to heme ligation is greatly accentuated by $\alpha_2\beta_2$ quaternary structure.

Unlike the low oxygen affinity bovine hemoglobin, the horse and human aquomethemoglobins showed significant β-93 Cys SH absorptivity. This indicates a much less exposed equilibrium conformation of β-93 SH in and out of the nonpolar pocket between the F, G, and H helices. Bunn (1971) has reported that mammalian hemoglobins that exhibit relatively high oxygen affinity when stripped of organic phosphates, such as horse and adult human hemoglobins, react strongly with diphosphoglycerate (DPG), causing a considerable decrease in their oxygen affinity. Those with low oxygen affinity, such as cow, were reported to interact weakly with DPG, resulting in only a slight further reduction of their oxygen affinity. Perutz (1983) has reported that the main amino acid substitution that distinguishes the high and low oxygen affinity hemoglobins is at position NA2β. In high oxygen affinity hemoglobins this site is occupied by hydrophilic residues: His, Gln, or Asn. In low oxygen affinity hemoglobins this site contains a large hydrophobic residue: Leu, Met, or Phe. The NH_2-terminal residues Val(NA1β) and His(NA2β) of high oxygen affinity hemoglobins form salt bridges with DPG (Arnone, 1972; Perutz, 1983), which pull the two A helices toward the center of the molecule and lower the oxygen affinity by stabilizing the T structure. In many low oxygen affinity hemoglobins (such as cow) the NA1β residue is missing and methionine is at position NA2β. The hydrophobic side chain of methionine may play the same role in stabilizing the bovine hemoglobin T structure as DPG does in the human and horse hemoglobins (Perutz, 1983). This stabilized T structure might explain the differences we see between certain derivatives of bovine and human or horse hemoglobins. The high oxygen affinity hemoglobins show similar absorptivities for the low-spin heme R state derivatives (HbCO and HbO_2), with a $^1/_3$ to $^1/_2$ decrease in absorptivity for the metHb structure (Table II, Figure 3). The low oxygen affinity bovine hemoglobin shows a 40% decrease on going from HbCO to HbO_2 or HbCN and no observed sulfhydryl absorbance for metHb or deoxyHb.

Translation of infrared absorptivities into fractional occupancy of the β-93 SH in the nonpolar F–H pocket deserves some comment. The values obtained for absorptivities and integrated absorptivities, respectively, are the same within

experimental uncertainty for CO complexes for the three species tested (human, horse, and cow). These appear to be limiting values since they are the same as those obtained for CO and O_2 derivatives of human β-chain tetramers and human HbO_2 and are only slightly greater than those obtained for horse HbO_2. If these values are taken to represent maximal interaction (100% occupancy) between β-93 Cys and β-145 Tyr in HbCO, then the infrared data suggest a similar 100% occupancy in human HbO_2. This contrasts with Shaanan's (1983) observation of only 70% occupancy by X-ray crystallographic measurements. It appears unlikely that our quantitation could be in error by as much as 30%. Neither does it appear likely that the limiting maximal interaction of β-93 SH with β-145 Tyr could be associated with significantly less than 100% occupancy. We therefore suggest that the equilibrium orientation in the crystal may be somewhat different from that in aqueous solution as is reasonably expected from differences in anion composition of the solvent. Phosphate in the crystal binds more tightly to anion binding sites than does chloride used in this work (Nigen et al., 1980).

The effect of oxygenation of deoxyhemoglobin appears to be the following. Ligation changes the heme configuration from square pyramidal to square planar, forcing rotation of the F helix, which in turn triggers breakage of salt bridges. In the β-chains, this is a primary event observed at the β-93 SH that is maximal with CO as the coordinating ligand but is modulated by species-determined amino acid sequence and by fractional occupancy of anion binding sites. Movement of the β-chain F helix with ligation appears to be independent of quaternary structure but may trigger other movements at the $\alpha^1\beta^2$ interface that lead to cooperativity. A second motion directly associated with ligation is movement of the heme toward the G and H helices and concomitant rotation of these helices. This last effect is strongly sensed by the α-104 and β-112 SH groups. Since frequency shifts of the latter SH groups are greatly accentuated by the $\alpha_2\beta_2$ quaternary structure (Alben & Bare, 1980), this $\alpha^1\beta^1$ interface may play a significant role in information transfer and therefore in cooperativity. This conclusion differs from that of Pettigrew et al. (1982), who exhaustively studied the effects of interactions at the $\alpha^1\beta^2$ interface on the energetics of cooperativity. They have clearly demonstrated the importance of this region. However, they have not made a similar study of interactions at the $\alpha^1\beta^1$ interface, where quaternary structure has a major effect on H-bonding at the α-104 and β-112 cysteine residues.

Conclusions

The β-93 cysteine SH in hemoglobin absorbs at 2592 cm^{-1}. This is too high a frequency to be explained by hydrogen bonding of the SH with another protein residue and indeed is slightly higher than the observed frequency of EtSH in nonpolar solvents. This implies that the sulfur has an increased charge density, probably induced by the phenyl ring of the adjacent β-145 tyrosine. The SH absorptivity strongly depends upon heme ligation and provides a good measure of the probability of finding the β-93 cysteine within the nonpolar pocket formed by the F, G, and H helices, where it appears to interact strongly with the phenyl ring of the HC2 Tyr. For the HbCO (R state) derivative of all three mammalian hemoglobins tested, 100% of the β-93 SH is in the pocket, while for the T-state deoxyHbs there is a very low probability of finding it within the pocket. Low oxygen affinity bovine hemoglobin has a greater tendency toward T structure when in the aquomet state than do the high oxygen affinity human and horse aquomethemoglobins, as measured by β-93 SH absorptivity. The β-93 absorptivity changes with oxygen or carbon monoxide binding were just as large in the β_4 tetramer, which does not show cooperativity in ligand binding, as in $\alpha_2\beta_2$ hemoglobin. This indicates that the environment about the β-93 SH is affected by tertiary, not quaternary, protein structure in these complexes. Conversely, factors that affect β-chain tertiary structure such as ligation, anion binding, and species differences may also contribute to cooperative control of oxygen binding.

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Registry No. HbA, 9034-51-9; HbH, 9034-79-1; HbO_2A, 9062-91-3; HbCOA, 9072-24-6; metHbA, 12646-21-8; L-cysteine, 52-90-4.

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