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THE OHIO STATE UNIVERSITY, PH.D., 1979

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A FOURIER TRANSFORM INFRARED STUDY OF
HEMOGLOBIN STRUCTURE AND FUNCTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Patrick Pel-Yung Moh, B.S.

* * * * *

The Ohio State University
1979

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ACKNOWLEDGEMENTS

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To my Parents and sisters, I owe the early knowledge of life and compromise.
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Scientific Sessions.

FIELDS OF STUDY

Major Field: Biochemistry

Application of Fourier Transform Infrared Spectroscopy; Physical
and Biochemical Methods in Biological Material
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GENERAL INTRODUCTION AND QUESTIONS ASKED

The three dimensional structures of some of the liganded and unliganded forms of hemoglobin and of numerous variants have been resolved (17,30,35,43,50,56,66,67). The binding of ligands to hemoglobin as an allosteric protein have recently been defined in crystallographic terms. The allosteric theory of Monod, Wyman and Changeux (48) explains the cooperative (heme-heme interaction) and the Bohr effect of hemoglobin in terms of a reversible transition between a low (T or deoxy) and a high (R or oxy) oxygen affinity structures, i.e., two extreme quarternary forms of a conformational equilibrium. This theory implies that within any of the possible quarternary structures, the ligand affinities of any of the subunits should be independent of the state of ligation of the other subunits. In terms of Perutz's model of deoxy (T) (whose 6 salt-bridges together with other bonding forces, especially at the α1β2 interface, hold the tetramer in a "high energy state") (56,68) and oxy (R) (a "lower energy state" with all 6 salt-bridges broken) conformational state equilibrium, there will be no cooperative effect if there is no transition in quarternary structure. The difference in the total bond energy and distribution of the 2 quarternary states would then be described by the concentration ratio of the T and R structures in the absence of oxygen (without heme ligand), and the ratio of the dissociation constants of oxygen from the subunits in the T and R states (68).
Structural results obtained from comparative measurements of atomic coordinates of human deoxy, horse met and human carbonmonoxy hemoglobin, show that upon ligand binding to deoxy Hb, the structural movements involve the tertiary structure changes within the \( \alpha_1\beta_1 \) dimer, and quarternary structure alterations in the packing of \( \alpha_1\beta_1 \) and \( \alpha_2\beta_2 \) dimers. Movements, thus significant in structural terms, occur in the outer parts of the dimer, where the hemes, F helices and FG corners of both subunits move towards the center of the molecule. The changes that occur in both subunits result in a F-helix translation across the face of heme by \( \sim 1 \) Å. This moves the heme-linked histidine F8 from a position that is asymmetric with respect to the porphyrin nitrogens in deoxy to a more symmetric position in liganded hemoglobin (9). Gelin and Karplus (39) and Warshel (87) have also suggested from the energy calculation of the \( \alpha \) hemes of deoxy Hb that the strain caused by the steric effect of \( N_e \) of proximal histidine against the porphyrin and ligand favors the five-coordinated high-spin ferrous iron atom to be in its optimal out-of-porphrin plane position. Warshel (87) shows that for a 6-coordinated low-spin complex, as in HbCO, the most favorable geometry of a heme-ligand complex is for the iron to be either in or very close to the porphrin plane. The preferred geometry is influenced by the size of the iron atom and the axial ligands to which the iron is bonded. The steric repulsion between the porphyrin nitrogens and the axial ligand prevents either ligands from approaching the plane too closely. The exact position of the iron atom is that
which minimizes the total steric replusion. If the iron atom is prevented from taking up this most favorable position, the major contribution to the strain energy comes from the steric repulsion between the porphyrin nitrogen and the closer ligand, either the proximal $N_e$ or the axial ligand e.g. CO. The overall F-helix conformation in both high and low affinity form of Hb must also contribute to the steric strain balance at the heme thru its different conformational arrangements and the transmission of steric effect to the F-8 proximal histidine. The overall movement of the $\beta$ hemes towards the molecular center upon ligand binding removes the ligand-binding site from the vicinity of Val$\beta$E11 which hinders ligand binding in deoxy Hb. The possible steric involvement of distal histidine on O$_2$ or CO binding and releasing affinities and kinetics was supported by the finding that the distal histidine is situated at close range with the bound CO in myoglobin CO (58) and Horse HbCO (43).

Interaction between the distal histidine and bound CO had been shown by IR measurements of $\nu_{CO}$ values of abnormal high affinity Zurich (His E7 (63) $\beta$+Arg) ($\beta\nu_{CO}$ stretch shifts to 1958 cm$^{-1}$) (82). The involvement between the ValE11 (67) in CO binding were also shown by IR measurement of $\nu_{CO}$ values of abnormal HbSidney (Val E11 (67) $\beta$+A1a) ($\beta\nu_{CO}$ stretch shift to 1955 cm$^{-1}$) (81). The $\nu_{CO}$ OF legHb-CO was found to be at 1947.5 cm$^{-1}$ at neutral pH. The Fe-CO geometry was suggested to resemble that of protein free heme Fe-CO geometry upon protonation of the distal histidine and that the heme pockets of legHb appears to be more flexible than the heme pockets of myoglobin and Hb (37).
The sudden transition point from one quaternary structure to another, the event which was first reported by Christian Bohr (16) as the well-known sigmoid oxyhemoglobin dissociation curve, is still open to question. Thermodynamic evidences, however, have supported the fact that the quaternary structure of the tetramers with either 3 or 4 liganded hemes are similar.

EPR studies of NO-ligated Hb Kansas showed the α and β subunits to behave in opposite directions (83). The IHP effect on the visible and ultraviolet spectra of liganded valency hybrids (23, 59, 60) and EPR spectrum of NO derivatives (71, 78), also indicate a nonequivalence between the hemoglobin subunits. Nonequivalence therefore exists between α and β subunits.

Mechanism of oxygen binding in hemoglobin: Hoard (45, 46) observed that the low-spin heme compounds have their iron roughly coplanar with the heme porphyrin, and high-spin complexes have their iron out of the heme plane towards the distal side of the heme. Perutz examined the Fe atom position in deoxyhemoglobin at 2.8 Å resolution, and found it to be 0.75 Å out of the mean porphyrin nitrogen plane. In methemoglobin, it was 0.3 Å out of the plane. The α and β hemes of deoxyhemoglobin was found to be 0.60 and 0.63 Å out of plane (34) respectively, and 0.07 and 0.218 in methemoglobin respectively (50). In low-spin derivatives, the iron atom radius is smaller than high-spin derivatives, as a result the iron could go inner towards the plane. From these crystallographic measurements along with other visible, UV and valency hybrid spectral data, a mechanism which related
the spin state to quaternary structure equilibrium was proposed. The forced iron movement in and out of the proprhin ring was suggested to be the major pathway by which the strain of oxygenation was transmitted to the rest of the molecule. Briefly, therefore, the mechanism stated that the T state enhances high-spin and diminish oxygen affinity. A transition to R state releases tension on the heme Fe, lower the spin state and contribute to the high oxygen affinity of the R state. The mechanism suggests that the position of the iron being slightly out of the heme plane in methemoglobin would facilitate its transition to the T state upon IHP addition. Further visible and UV perturbation study by IHP addition to metHb led to the controversial interpretation that IHP binding to metHb causes an increase in the spin state of the ferric d electrons and facilitates the unliganded, low affinity T state conformation (64). This is an important proposal because it suggests a functional role of metals in biochemical systems, and the detailed mechanisms of action of hemoglobin as an allosteric protein.

The recent observation of a similar (±0.02 Å) Fe-porphyrin-nitrogen distances for the low affinity HbA and high affinity HbKempsey by Extended-X-ray absorption fine structure spectra by Eisenberger et al. (32) does not support the concept that change in spin state is the main direct driving force for conformational change.

The recently supported hemoglobin heme-heme interaction mechanism however, suggests that the steric strain is the primary force for decreasing the deoxy quaternary structure relative to liganded
quaternary structure, thus increases the liganded form of hemoglobin \cite{9,38,39,87}. Upon ligand binding, such a steric strain originates from the particular deoxy conformational position of the F-helix and of HisF8 relative to the heme, and the subsequent quaternary structural change to the high affinity form induces tertiary structural changes that reposition the F-helix and HisF8 relative to the heme in an energetically stable conformation, and thus dissipates the strain on the ligand binding \cite{9}.

The $\alpha_1\beta_2$ interface undergoes drastic changes between the liganded and deoxy forms. The $\beta 93$ cysteine is an invariant residue located at the $\alpha_1\beta_2$ interface on the F helix of each $\beta$ subunit of the tetramer and involved in structural changes upon ligand binding. The sulfhydryl group of this residue exchanges position sometimes with the HC2(141)$\beta$ Tyrosine, whose equilibrium position in deoxyHb is different from that of liganded hemoglobins \cite{43,61}.

Major questions asked in this thesis:—

1. (a) Can the $\beta 93$ cysteine sulfhydryl (-SH) be observed in the IR, and that could the band be resolved and baseline flattened to be used as a conformational probe. If so, (b) Does ligation (with or without ligand binding) at the heme link to the conformation of the $\beta 93$ -SH? Further, what is the relative equilibrium conformation of $\beta 93$ -SH in different liganded species (low spin e.g. CO, O2 and CN, low and high spin mixture e.g. met+, and high spin e.g. deoxyhemoglobin)? (c) If the -SH conformational equilibrium is ligand dependent, is it tertiary structure related, i.e., is it not related to the direct change of the quaternary structure (cooperative effect) $\beta_4$ tetramer does not
undergo allosteric changes upon ligand binding. A study of its β93 -SH could be an indicative answer to this question.

2. IHP (Inositol hexophosphate, a polyanionic phosphate) is known to bind and stabilize the T structure, e.g. deoxyHb, as 2,3-DPG does, but stronger. HDO resonance NMR data on IHP derivatives of human fluoro- and aquomethemoglobin and the crystallization of aquomethemoglobin + IHP supports the evidence that IHP induces change from the quarternary oxy (R) to the deoxy (T) structure in methemoglobin (35,68).

The EPR and crystallographic data had shown that IHP binding would change HbNO (R state Hb) to a T state structure (65). The proximal HisNϵ-Fe bond was shown to be broken under conformational stress from R to T transition (24,65). These works show that IHP shifts R state hemoglobin to T state to the limiting point as far as the heme-ligand structure would yield. No IHP effect on HbCO was observed under the same IHP binding condition (24). IR study on IHP perturbation on hemoglobin may help answer some questions: (a) Does IHP binding to HbCO and HbO₂ change the β93 -SH equilibrium conformation, thus change the related structural orientation such as the Tyrβ145(HC2), the F helix and the proximal histidine. If not then it would mean that the IHP complex does not have a different conformation at this site, that it may not change the critical conformation of β-chain F-helix of the HbCO and/or HbO₂, R state tertiary structure, or further that it does not change or shift the high affinity quarternary structural equilibrium of HbO₂ and HbCO. If the answer is yes, then what's the direction of the shift, more toward a high or a low affinity 3° structure.
How much "relatively" is the change of conformation at this site? (b) It's well documented that IHP binding shifts R and T equilibrium of methemoglobin towards the T structure (44, 64). The reactivity of the β93 -SH increases upon addition of IHP (63). The finding on whether IHP changes the conformation of the β93 -SH group in methemoglobin towards the T state tertiary structure, would help in understanding the concept of the R and T equilibrium. (c) It would be of future importance to see if there is any other new conformational related bands observable.

3. Mercurial derivatives bind at the β93 -SH site, have a greater reactivity towards liganded hemoglobin, increase the hemoglobin affinity for ligands, and cause dissociation into dimers in dilute solution (5, 31, 73, 74). On the contrary, IHP favors unliganded, low affinity T state hemoglobin. The heme liganded $^{12}$C$^{16}$O stretch band has long been well defined by Alben and Caughey (2, 3). Changes in Fe ligand field or radius would directly affect the Fe-C bond, and thus the polarization and bond structure of the CO ligand. By carefully studying the change of the $^{12}$C$^{16}$O and $^{13}$C$^{16}$O stretch bands of HbCO derivatives and the CO marker (1% CO) in HbO$_2$, caused by mercury binding at the β93 site, and of IHP at the β1β2 phosphate binding site (6, 7), one could get some insight for understanding (1) the perturbation on the F helix and the consequent effect at the heme, (2) the results of the quarternary structural change by IHP on the tertiary heme ligand binding. The binding site of Zinc was found to be related to β93
sulfhydryl groups, and Zn binding was found to increase oxygen affinity (70). Trace CuII (amount as in the blood stream) had been shown to enhance hemoglobin oxidation and metHb disulfide formation. This oxidation enhancement had been shown to be dependent both on the presence of oxygen and the binding of β93 -SH (65,88). The study of perturbation of the CO ligand at the heme site by Hg binding to β93 -SH groups, could be correlated to the Zn and CuII binding effects on hemoglobin.

In short, the mercurial binding effect of the β93 -SH on the CO heme, could provide some information about the effect of metal binding on the F9 cysteine of the peptidyl F helix on the heme ligand thru probably the perturbation of the Fe atom which is covalently bonded to the proximal histidine Nε. The binding of IHP would provide information about the organic phosphate binding between the β chains and its resulting effect on the heme ligands of both α and β chains. The direct binding effect could be caused by perturbation at various parts of the molecule. On the other hand, the IR study of the β93 absorption band of hemoglobin with different types of ligands could provide information about tertiary structural relationship between the binding and the type of ligands at the heme, and their relative effects on the conformation of the F9β93 -SH, thru tertiary structure change or the rotation of the F helix. Studies on the β93 -SH of β4 tetramer and Hb+IHP derivatives could provide information on the relationship of the quarternary structure on the β93 -SH conformation which could reflect not only the structure of the F helix, but also the local
environment in the vicinity of the ψ93 cysteine. The carbon monoxide in the hemoglobin derivatives with ψ1% subunits CO liganded, would be randomly distributed among the tetramers, and could provide information on the effect (if any) of the neighboring subunit on the CO liganded heme.

The β4 tetramer (in vivo could be found in α-thalassemic patients called hemoglobin H) is noncooperative in oxygen binding. The structural and functional evidences of the tetramer are at best conflicting (11,12,57), in that the tetramer has both R and T type properties. A study of ligation effect on the ψ93 -SH group, and of Hg and IHP perturbation on this isolated chain tetramer with comparison to that of α2β2 tetramer, warrants some new information about its structure.

IR works related to biological materials had also been done in recent years, but mainly with dispersive instruments (37,77). A preliminary IR study on Hb derivatives in D2O was done. The amide I mode absorption band frequencies of the derivatives were compared and related to their α-helical tertiary structures.

The CO stretch band in purified heart cytochrome c oxidase was reported to be observed (89). A comparison of the CO absorption of isolated and intact heart cytochrome oxidase were made.
METHODS AND MATERIALS

I. Preparation of α₂β₂ hemoglobin tetramer derivatives

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 to four times with 1% saline and hemolyzed with 1 volume of water and 0.5 volume of toluene. After centrifugation at 12,000 rpm, hemolysates (30-35 ml) separated from the stroma were passed through a 2.5 X 100 cm Sephadex G25 (Pharmacia) column equilibrated with 0.1 M chloride. Samples of the hemoglobin eluates were concentrated by pressure ultrafiltration (Amicon) to a desired heme concentration (17-24 mM). Deoxyhemoglobin was obtained by repeated evacuation and equilibration with nitrogen or argon (99.998% pure). HbCO and HbO₂ derivatives were prepared in the same way as deoxyHb except that pure carbon monoxide (Matheson) and oxygen gases were used respectively instead of nitrogen or argon. Samples were exposed to the corresponding gases for 12 hours before use. Methemoglobin was prepared by oxidation on Hb with a slight excess of K₃Fe(CN)₆ (J. T. Baker). Cyanomethemoglobin was prepared from methemoglobin by addition of a slight excess of KCN (J. T. Baker). Excess K₃Fe(CN)₆ and KCN were removed by passing through a Sephadex column, equilibrated with the pH 7.1 bis-tris chloride buffer. IHP (1.2 per tetramer) and mercuric chloride (1.1 and 1.5 per β-chain) (Sigma) were added to the hemoglobin samples before the ultrafiltration step. Samples of different pHs were
prepared by either equilibrium dialysis or by equilibration through a sephadex column. All reactions were done between 4-10 degree C.

II. Preparation of \( \beta_4 \) tetramer derivatives

Stripped HbA(CO) solutions were reacted with p-chloromercurobenzoate (PMB) (Sigma) solutions (dissolved in NaOH and titrated with acetic acid) as first described by Rosemeyer and Huehns (75), except that 35% by volume of saturated sucrose was added prior to the final mixing of the well chilled reaction mixture. The reaction must be carried out under ice-cold and constant-stirring conditions. The reaction solution was dialyzed before column chromatography. \( \beta^{\text{PMB}} \) chains were separated from alpha\(^{\text{PMB}} \) chains or other undissociated dimers essentially using the method of Geraci \textit{et al.} (40). After the removal of PMB with 15 mM ethanethiol on a short column (for 400 mg of \( \beta^{\text{PMB}} \) chains, a (2 x 15 cm) DEAE 52 Whatman column is sufficient), one column volume of 0.1 M tris (pH 8.5) buffer was quickly eluted before the \( \beta \) chains were eluated from the column with 0.1 M Bis-tris buffer (pH 7.1).

The \( \beta_4 \text{CO} \) tetramer was concentrated using pressure ultrafiltration. The \( \beta_4 \text{O}_2 \) derivative was prepared by flushing a \( \beta_4 \text{CO} \) aliquot with pure oxygen in ice under light for five or six consecutive times right after each evacuation step. The deoxy \( \beta_4 \) sample was prepared by repeated evacuation and equilibration with nitrogen of \( \beta_4 \text{O}_2 \) aliquot, followed by an addition of 1:1 heme equivalent of sodium dithionite (Baker). A 2.2 tetramer equivalent of IHP was added to \( \beta_4 \) tetramer to obtain the polyanionic IHP derivative. The \( \beta_4 \text{S}CO \) sample was prepared in the same way as \( \beta_4 \text{CO} \).
Determination of reactive SH groups of hemoglobin tetramer and subunit preparations was done using the method of Boyer (18) in 0.1 phosphate buffer (pH 6.0), and the reaction solution measured at 255 μm.

III. Electrophoresis

Hemoglobin samples were characterized with 1% agar gel electrophoresis at pH 6.0 (Citrate buffer) (Bare, Alben et al.) and 7% polyacrylamide gel disc electrophoresis at pH 8.3 (Tris-glycine buffer). The similar polyacrylamide gel disc electrophoresis technique performed was first described by Davis, B. J. and L. Ornstein. Twelve milliamperes/tube and 3 mamp/tube were used for the agar (10 cm gel) and polyacrylamide (6 cm gel) gel electrophoresis.

The polyacrylamide gels were scanned in a Gilford gel-scanner attached to a Beckman DU monochromator, and recorded on a Sargent recorder at 540 μm (CN derivatives) and 556 μm for unstained ones, and at 280, 420 and 558 μm for stained gels. The relative mobilities of the protein samples were measured by reading from ruler of variable scales and by measuring from the scanned plots with references to either an internal or external standard. Purified adult human HbA and sickle cell hemoglobin were used as standards. Either 40% sucrose or Nujol were used for maintaining a flat meniscus before the gel started to polymerize.

(A) HbS and HbF Preparation for Electrophoresis

HbS (Sickle) and HbF (Fetal) were prepared from ion-exchange column chromatography (DEAE and CMC self-packed columns), and dialyzed to pH 8.0 before application for acrylamide gel electrophoresis.
Riboflavin, NNN'N'-Tetramethyl ethylenediamine (Temed), Tris and Bis-tris were purchased from Sigma. Acrylamide from Eastman and Ammonium sulfate from Baker.

IV. Preparation of D$_2$O Hemoglobin Derivatives

For D$_2$O samples, hemolysates were prepared as H$_2$O samples described above, except that the H$_2$O hemolysate was first concentrated to less than 1/2 its volume by pressure ultrafiltration and then diluted to its original volume with D$_2$O. This concentration and dilution step was repeated several times until an estimated greater than 90% D$_2$O replacement was achieved. No observable H$_2$O band was observed at 1.43 μm in the near infrared with a pathlength of 15-25 μ.

V. Heme Concentration Determination

Hemoglobin concentrations were measured as HbCN assuming $\varepsilon_{540} = 11 \times 10^3$ (M heme)$^{-1}$ cm$^{-1}$.

VI. Measurement of Visible, Near-Infrared and Infrared Spectra

Both visible and near-infrared spectra for water content (1.43 μm) and hemoglobin purity and concentration determination were done with a Perkin-Elmer Model 4000A split double beam scanning spectrophotometer. Visible spectra were also done with a Cary 15 spectrophotometer.

VII. Miscellaneous Sample Preparation

Reduced CO purified cytochrome oxidase (0.1 M, pH 7.4 potassium phosphate buffer, containing less than 1% Tween-80) was precipitated on a cell-window (.4 mm spacer), wetted and packed. A reduced sample was used for background subtraction. Cytochrome oxidase samples were obtained from Dr. J. Rieske and Dr. D. Wharton.
Beef heart tissue was sliced, soaked in CO saturated 1% saline and packed in a demountable infrared cell by Dr. J. O. Alben. A water pathlength of .2 mm was measured from the near infrared spectrum.

Isolated rat heart was perfused in normal isotonic solution saturated with carbon monoxide gas by Dr. R. Altschuld. The heart tissue was sliced and packed with a .2 mm pathlength guide.

VIII. Spectroscopy

Infrared spectra at 1 cm\(^{-1}\) resolution (1 cm\(^{-1}\) to 2 cm\(^{-1}\) optical resolution) were taken of samples in demountable infrared cells with CaF\(_2\) windows for H\(_2\)O hemoglobin samples (0.03 cm and 0.01 cm pathlengths were used for the sulfhydryl and carbonyl regions respectively). A Digilab Model FTS-14D infrared interferometer equipped with either liquid N\(_2\) cooled InSb detector or a (Hg-Cd)Te detector was used with sample temperatures of 10-12 degree C.

Besides the spectra of HbCO in D\(_2\)O, for which a 14 bit A/D converter was used, all other interferograms were collected at 1 cm\(^{-1}\) resolution and INT=1, with a 15 bit A/D converter plus a gain ranger for the centerburst. A HeNe Laser frequency of 15801.5660 cm\(^{-1}\) in dry air was used as a "frequency" reference for the interferometer during data collection. All the spectra were plotted out using a HeNe Laser frequency of 15798.0112 cm\(^{-1}\) (in vacuum) and thus convert all the frequencies in dry air to that of vacuum. All data points were signal-averaged into a double precision word (16 bit X 2 = 32 bits). The fast Fourier transform and the subsequent computations on the spectra were done in double precision. Data analysis was done on a Data General Nova 2/10
computer with 16 K words of memory, interfaced with an Alpha Data
128 K word Fixed Head Disk memory. Instrumental artifacts (mainly
optical) were eliminated by removing the secondary centerbursts, and
by using difference spectroscopy (Appendix). Isolated band areas
were determined both by planimetry and computer integration. Center
frequencies were measured by direct reading from the absorption peak
maximum of the baseline-corrected spectra, and by taking the measure­
ment after interpolating linearly the corrected spectra with an eleventh­
degree polynomial. The spectra were plotted out by a Houston Instruments
DP-1 digital plotter. Band shapes were defined by the bandwidth at
half of the maximum band height.

The advantages of Fourier transform infrared interferometric
spectroscopy could briefly be described to be due to its
capability in measuring weak radiation intensity of a set
of spectral frequencies simultaneously (Fellgett's
advantage) with much greater signal to noise ratio than
a monochromator could achieve. The greater signal to noise
ratio is achieved mainly because of (1) the increased
signal at the detector (the throughput or Jacquinot's
advantage) and (2) easy signal averaging process made
possible by interferometer linked computer systems.
There is no slit dependent stray light.
The optical refractive artifacts in FTIR had been known to cause artifacts in spectra, which was found to appear as secondary center-burst in the interferogram. These artifacts if present would make detailed quantitation difficult. The optical artifacts were removed from the interferogram before mathematical transformation. The operation itself was not found to have any observable effect on the transformed spectra at the resolution (1 cm\(^{-1}\)) and signal to noise ratio used in this dissertation.

The integrated area absorptivity obtained from the absorption envelope for HbCO and HbO\(_2\) were curve-fitted with Lorentzian function \(A=a/((v-v_o)^2 + b^2)\); \(A=\) Absorbance at any chosen frequency; \(a\) and \(b\) are related to experimentally observable quantities by: \(A_{\text{max}}\) (Maximum absorbance) = \(a/b^2\), \(\Delta v_\frac{1}{2}\) (half-bandwidth) = 2\(b\) and the center frequency is \(v_o\). The integrated intensity enclosed by this function is \(\int A \, dv = (\pi/2) \left( A_{\text{max}} \times \Delta v_\frac{1}{2} \right)\), was chosen to be always greater than that integrated from the manually resolved 893 band with a confidence level >99%. The difference in the averaged integrated area absorptivity lies between the boundary of 0.02 and 0.14.
SECTION I

POLYACRYLAMIDE GEL ELECTROPHORESIS

(A) Results

Hemoglobin samples used for spectroscopic studies were characterized for purity by polyacrylamide gel disc electrophoresis. Relative mobilities ($R_f$) were measured using either purified HbA or HbS as both internal and external standards. Distance migrated was measured from the gel meniscus to the mid cross-section of each band. The $R_f$ values were listed in Table 1. Special attention was given to the polyacrylamide gel disc electrophoresis, because it gives better resolution especially for $\beta$-chain (tetramer) separation than electrophoresis on 1% agar gel. The $R_f$ values for cyanide derivatives are listed in Table II. The cyanamet, carboxy and oxy hemoglobins, all fully-liganded R state tetramers, were found to have similar electrophoretic migration pattern.
TABLE 1

Relative Mobilities of Oxy and Carboxy* Hemoglobin Derivatives (Distance Migrated by the Sample/Distance Migrated by the Reference [either HbA or HbS]) in 7% Polyacrylamide Gel Disc Electrophoresis at pH 8.3 in Tris-Glycine Buffer.

<table>
<thead>
<tr>
<th>**Oxy Hb Sample</th>
<th>( R_f_{\text{sample}} ) A</th>
<th>( R_f_{\text{sample}} ) SCN</th>
<th>( \sigma )</th>
<th>N</th>
<th># of Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )</td>
<td>.54</td>
<td>.64</td>
<td>±.02</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>( \beta )</td>
<td>1.34</td>
<td>1.58</td>
<td>±.02</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>( \text{HbS} )</td>
<td>.84</td>
<td>±.02</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>( \text{HbA} )</td>
<td>1.00</td>
<td>1.19</td>
<td>±.03</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>( \text{Kansas} )</td>
<td>.95</td>
<td>1.13</td>
<td>±.02</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>( \text{HbF} )</td>
<td>.90</td>
<td>1.07</td>
<td>±.02</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>( A_2 )</td>
<td>0.50</td>
<td>0.60</td>
<td>±.02</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

**\( R_f \) values measured using internal standards.

Oxy derivatives otherwise stated.

\( R_f A \) (External standard used: HbAoxy)

| \( \alpha \) chain | .54 | ±.07 | 8 | 4 |
| \( \beta \text{CO chain} \) | 1.36 | ±.01 | 3 | 1 |
| \( \beta \) chain | 1.30 | ±.03 | 8 | 4 |
| \( S \)          | .84 | ±.01 | 18| 9 |
| \( A\text{CO} \) | 1.0 | 2   | 1 |
| \( \text{Kansas} \) | .96 | 2   | 1 |
TABLE 2

Relative Mobilities of Cyanide metHb Derivatives (Distance Migrated by the Sample/Distance Migrated by the Reference (either HbACN or HbSCN))

Rf values measured using internal standards:

<table>
<thead>
<tr>
<th>CN Hb Sample</th>
<th>Rf(sample)_{ACN}</th>
<th>Rf(sample)_{SCN}</th>
<th>σ</th>
<th>N</th>
<th># of Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.00</td>
<td>1.17 ±0.03</td>
<td>19</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>0.84</td>
<td>1.00 ±0.03</td>
<td>15</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>0.51</td>
<td>0.64 ±0.04</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>β</td>
<td>1.9</td>
<td>2.4 ±0.1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Kansas</td>
<td>1.12</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1.10</td>
<td>±0.03</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
Plate 1: Electrophoresis pattern of unstained human B chain in gel 1 and B chain with internal standards of A & A₂ in gel 2. The samples were run on 7% polyacrylamide gel at pH 8.3 in Tris-glycine buffer.
SECTION II
FTIR STUDY OF β93 SULFHYDRYL GROUPS OF BOVINE Hb, AND
ITS CONFORMATIONAL RELATION TO HEME LIGATION

(A) Introduction

The rate of reaction of the sulfhydryl group of Cys F9(93)β with
the reagent N-ethylmaleide (NEM) was first shown to differ in liganded
and deoxyhemoglobin by Riggs (74). Early attempts to investigate this
group using spectroscopic techniques had been done by electron para­
magnetic resonance (EPR) studies of spin-labelled reagents attached to
β93 (51,54), and NMR studies with a reagent labelled with 19F attached
to the sulfhydryl group (47,48). The conformation of the β93 -SH of
HbCO, HbCN, Fluoromet and aquomet Hb had been shown to be related to
the conformation of the β-terminal Tyr 145 and to heme ligation (29,30,43).
When CN− was bound to the oxidized β-chains of α2β2 hybrids, the
deoxygenation of the α-chains was found not to affect the reaction of
the F9(93)β sulfhydryl with p-mercuribenzoate (PMB) (20 ). The
conformation of the β93 sulfhydryl group may therefore be related to
tertiary structure if not directly to quarternary structure. The
fourier difference crystallographic data of heme liganded and unliganded
hemoglobins show that ligand binding involves a tertiary structure
change, that is related to the overall quarternary structural changes
(61 ). This tertiary structure change involves an exchange of the
equilibrium positions between the β93 cysteine -SH and the Tyr 145.
In HbCO, the $\beta_{93}$ -SH is essentially in a non-polar pocket between F and H helices where tyr $\beta$-145 would be positioned in case of deoxy Hb (43). The interchange of $\beta_{93}$ cysteine -SH and tyr $\beta$-145 accompanies a rotation and a translation towards the heme by the F-helix. This involves the F8 proximal histidine which is covalently bound to the heme iron (61). Perutz further suggests that while in the non-polar pocket between F and H helices, the F$\beta_{93}$ cyteine may be H-bonded to the F5 ser. In order to study the relationship of $\beta_{93}$ cysteine structural alterations with heme ligation (of HbO$_2$ especially) in concentrated aqueous solution without an artificial label attached to the site of interest, the FTIR technique was chosen. Bovine hemoglobin contains cysteine only at the $\beta_{93}$ position and therefore was selected for this study.

The $\alpha_{104}$ and $\beta_{112}$ cysteine sulfhydryl group absorption bands had been previously identified by comparison with spectra of animal hemoglobins and isotopic substitution in D$_2$O (10).

(B) Results and Discussion

The sulfhydryl groups of cysteine residues of hemoglobin especially $\beta_{93}$ cysteine are observed in good detail by subtraction of a similar reference spectrum of hemoglobin that is lacking these sulfhydryl groups. Such a reference absorption spectrum can be obtained by use of hemoglobin in the presence of silver or mercury salts, which form argentide or mercaptide bonds with cysteine sulfhydryl groups. An absorption spectrum of human hemoglobin using
silver as -SH reacting group for the reference is shown in Figure 1, where absorption bands due to cysteine sulfhydryls at α-104, β-112, and β93 were clearly observed. The spectra of the β93 cysteine -SH in Carboxy (HbCO), Oxy (HbO2), Cyanamet (HbCN) and aquomet (metHb+) hemoglobin are in Figure 2, and their infrared measurements were listed on Table 3. Only one -SH absorption band was observed corresponding to the fact that only one type of cysteine, the F9β93 cysteine, is present in bovine hemoglobin. The β93 -SH on the two β chains are structurally similar. No frequency shift was observed among these absorption bands. The integrated intensity of the -SH absorption band at 2592 cm⁻¹ had the order, HbCO was greater than that of HbO2 and HbCN, which was much greater than that of aquomet and deoxy hemoglobin, which were not observed under these instrumental conditions for the bovine derivative. The integrated absorption intensity with oxy and cyanamet hemoglobin was about 3/4 that observed with HbCO. The center frequency of the β93 band (2592 cm⁻¹) was similar to that of 2-methylbutane-2-thiol (2591 cm⁻¹) (36). Table 4 shows the relation between the absorption intensity of the cysteine -SH groups and nearest neighbor molecular interactions. The absorption frequency of Bovine β93 cysteine is much higher than that of the α-104, β-112 -SH and any of the -SH frequencies of the ethanethiol in H-bond acceptor solvents. This rules out any hydrogen bonding of the β93 SH. The half-bandwidth of the β93 SH absorption is similar to that of the mercaptol (EtSH) in CCl4, but much narrower than that of EtSH in hydrogen bond acceptor
solvents. It is broader than the half-bandwidth of the α-104 or β-112 cysteine SH absorptions, reflecting the less restricted motion at the surface of the protein. A low intensity SH absorption of non-hydrogen bonded SH group would normally be expected from comparison with the small molecule studies. Nevertheless a fairly large integrated intensity with bovine carboxy Hb was observed, where the SH cannot be H-bonded since the absorption center frequency of 2592 cm⁻¹ is too high for such a bonding. This large intensity is explained in terms of steric distortion of the C-S-H bonds in a rather restricted non-polar space.
Figure 1: Infrared absorbance spectrum of SH stretching vibrations of aqueous human carboxy-hemoglobin A (23 mM heme) after absorbance subtraction of the protein contribution (carboxyhemoglobin A solution containing 5 moles Ag^+ per mole heme).
Figure 2: Infrared absorbance spectra of Bovine 893 sulfhydryl groups normalized to 19.6 mM heme concentration and 0.3 mm optical pathlength. All references and sample spectra were collected in double beam mode with respect to (reference spectrum) with 1024 scans at resolution=1 cm$^{-1}$. 
### TABLE 3

IR Data of Bovine β93 Sulfhydryl Groups of HbCO, HbO₂ and HbCN

<table>
<thead>
<tr>
<th>Sample</th>
<th>$v_{max}$ cm$^{-1}$</th>
<th>$\Delta v$ cm$^{-1}$</th>
<th>$a_{mM}$ mM$^{-1}$ cm$^{-1}$</th>
<th>$A_{(area)}$ mM$^{-1}$ cm$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbCO-HbCOHg</td>
<td>2592</td>
<td>19.9</td>
<td>0.041</td>
<td>0.84</td>
</tr>
<tr>
<td>HbO₂-HbO₂Hg</td>
<td>2592</td>
<td>19.9</td>
<td>0.029</td>
<td>0.52</td>
</tr>
<tr>
<td>HbCN-HbCNHg</td>
<td>2592</td>
<td>20.0</td>
<td>0.029</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>( \nu_{\text{SH}} \text{ (cm}^{-1} \text{)} ) (center)</td>
<td>( \Delta \nu ) (cm(^{-1}))</td>
<td>( a_{\text{mM}} \text{ (mM}^{-1}\text{cm}^{-1})</td>
<td>( A\text{(area)} \text{ (mM}^{-1}\text{cm}^{-2})</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------------------------------------------------</td>
<td>-------------------------------</td>
<td>--------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td><em>Human Carboxyhemoglobin</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*In H(_2)O: ( \alpha )-104</td>
<td>2552.6</td>
<td>13.0</td>
<td>0.17</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td>( \beta )-112</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow Carboxyhemoglobin ( \beta )-93</td>
<td>2592.0</td>
<td>19.0</td>
<td>0.041</td>
<td>0.80</td>
</tr>
<tr>
<td><em>Ethanethiol (0.1 M)</em></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>In Bromoform (0.12 M EtSH)</td>
<td>2572.8</td>
<td>17.0</td>
<td>0.0023</td>
<td>0.050</td>
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<tr>
<td>In Chloroform</td>
<td>2582.3</td>
<td>17.5</td>
<td>0.0027</td>
<td>0.061</td>
</tr>
<tr>
<td>In Carbon Tetrachloride</td>
<td>2579.8</td>
<td>23.0</td>
<td>0.0021</td>
<td>0.065</td>
</tr>
<tr>
<td>*In Water</td>
<td>2573.6</td>
<td>39.5</td>
<td>0.0073</td>
<td>0.210</td>
</tr>
<tr>
<td>*In Acetone</td>
<td>2564.8</td>
<td>30.2</td>
<td>0.0132</td>
<td>0.429</td>
</tr>
<tr>
<td>*In Dimethylacetamide</td>
<td>2534</td>
<td>58</td>
<td>0.021</td>
<td>1.35</td>
</tr>
</tbody>
</table>

\(^{a}\)Absorbance, \( A = \log \left( \frac{I_0}{I} \right) \), was used to calculate millimolar absorptivity, \( a_{\text{mM}} = \frac{A}{c_1} \), and the integrated absorption coefficient, \( A\text{(area)} = \int [1/c] A \text{ d}v \).  

*Data partially obtained from Ref. 10.
SECTION III

FTIR STUDY OF HORSE \( \beta 93 \) CYSTEINE SULFHYDRYL GROUPS

(A) Introduction

In the X-ray crystallographic study of horse HbCO-metHb* Fourier difference map, Heidner et al. (43) had observed that the \( \beta 93 \) cysteine sulfhydryl groups of the high-spin horse aquo-metHb spent some part of its equilibrium time in the pocket between the F and H helices, where the penultimate tyrosine \( \text{HC2(145)}\beta \) would have resided in the case of deoxyhemoglobin. In the IR spectra observed for Bovine metHb, no absorption band was observed (Figure 2). However, amino-acid sequence data had shown an important amino-acid residue difference between bovine hemoglobin and horse and human hemoglobins (28). The NH2-terminal valine, a possible DPG binding residue, present in both \( \beta \)-chains of adult human and horse hemoglobins were found to be absent in bovine \( \beta \)-chains. Instead, a methionine was present. Cow, sheep, goat, and cat had found to have low oxygen affinity hemoglobins, which interact weakly with 2,3-DPG, and exhibit low concentrations of red cell 2,3-DPG (21). The \( \beta \)-chain NH2-terminus of the hemoglobins of these animals are structurally different from that of high oxygen affinity hemoglobins of mammals like man and horse. In order to pursue this question, a \( \beta 93 \) sulfhydryl group study was extended to horse hemoglobin (horse Hb has an \( \alpha 104 \) sulfhydryl group on each \( \alpha \)-chain besides the two \( \beta 93 \) cysteine sulfhydryls).
It had been established that the iodoacetamide reaction involves only the β93 residue (42). It was also generally believed that of six sulfhydryl groups of human α2β2 hemoglobin, four, namely those at α-104 and β-112 are unreactive (or "masked") in relation to a variety of specific reagents, such as p-mercuribenzoate (PMB), and two namely those at β93, are reactive (or "free") (26).

(B) Results and Discussion

Figure 3 shows the spectra of the β93 cysteine -SH in Carboxy, Oxy, Cyanamet and Aquomet horse hemoglobins using horse HbCO+Hg (β93 -SH: HgCl2=1:1.2) as the reference. Table 5 includes the IR measurements of the absorption bands of horse sulfhydryl groups obtained using either HbCO+Hg or HbO2+Hg as reference spectra. The residual α-104 and (2554.6 cm⁻¹) was calculated to be mainly due to a reduction of free α-104 -SH in the reference, caused by the 2 -SH equivalent excess of mercuric chloride. The difference spectra of horse HbO2, HbCN and metHb⁺ v.s. HbCO+Hg derivative in Figure 3 also shows a band at 2610 cm⁻¹. By taking a difference spectrum between the horse HbO2 and HbCO (HbO2-HbCO), a 2610 cm⁻¹ band (Apparent absorptivity (aM)= 1 X 10⁻² mM⁻¹cm⁻¹ and half-band widthΔν₁=20 cm⁻¹), was better resolved (Figure 4a). The same band was not observed in the HbO2 v.s. HbO₂+Hg (Figure 5b) and HbCO v.s. (HbCO+Hg) (Figure 5a) spectra. The same phenomenon was observed with the HbCN derivative. The 2610 cm⁻¹ band is also present in the difference spectra between (HbO₂+Hg) and (HbCO+Hg) (Figure 4b). Thus the band is not due to a
sulfhydryl group absorption and appears to be absent from the CO derivatives. The negative band at 2554.6 cm\(^{-1}\) in Figure 4b is due to a 0.4 \(-\text{SH}\) equivalent excess of HgCl\(_2\) added to the HbO\(_2\)+Hg sample, resulting in a smaller \(\alpha-104\) total absorption in the (HbO\(_2\)+Hg) derivative. A similar 2610 cm\(^{-1}\) band was also observed in the human (\(\beta_\text{O}_2\))\(_4\) -deoxy \(\beta_4\) difference spectrum (Figure 11) described in a later section. As a result, the 2610 cm\(^{-1}\) band may well be an absorption band related to differences in the hemoglobin tertiary conformation.

The absorption band of HbCO (Figure 3 and 5) at 2592 cm\(^{-1}\) (Apparent absorptivity \((a_{\text{mM}})=0.04\) mM\(^{-1}\)cm\(^{-1}\); and Integrated area intensity \((A_{\text{area}})=0.80\) mM\(^{-1}\)cm\(^{-2}\)) corresponded exactly with the \(\beta_93\) -SH absorption band of Bovine hemoglobin, which had only one sulfhydryl group namely the \(\beta_93\) cysteine -SH on each \(\beta\)-chain. There was no observable frequency shift among the horse \(\beta_93\) absorption bands shown in Figures 3 and 5. A similar pattern of the \(\beta_93\) -SH absorptivity variations observed among bovine Hb derivatives was observed in horse Hb derivatives. The absorptivity of horse HbCO \(\beta_93\) -SH \(>\) HbO\(_2\) \(\sim\) HbCN \(>\) MetHb (Table 5). However, unlike that of cow aquomethemoglobin, the estimated integrated absorption intensity of horse aquometHb was slightly greater than 1/2 of that obtained for HbCO. This horse metHb datum correlates well with the horse crystallographic study of Heidner \textit{et al.} (43). Differences therefore exist between the bovine and horse aquometHb at the region near the \(\alpha_1\beta_2\) contact, namely the penultimate tyrosine HC2(145)\(\beta\) pocket.
In horse metHb, the F9β93 -SH was at an equilibrium between that of situating in a non-polar tyrosine β145 pocket (thus expel the tyrosine partially to the external), and that of free positioning in an external aqueous environment.

Figure 5 shows the difference spectra of horse HbCO v.s. (HbCO+Hg) in the top trace (a), and horse HbO₂, HbCN and metHb v.s. (HbO₂+Hg) in (b), (c) and (d) respectively. The 2610 cm⁻¹ band was not observed between (HbO₂ v.s. HbO₂+Hg) and (HbCO v.s. HbCO+Hg) difference spectra, showing that this difference band appears to be present between hemoglobin of different ligands with slightly different tertiary structures.
Figure 3: FTIR absorbance difference spectra of (from the top) horse carboxy, oxy, cyanomet and met hemoglobin v.s. mercuric carboxyhemoglobin derivative with water correction. The hemoglobin concentration were normalized to 19.02 mM (heme) and optical pathlength to 0.028 cm. The spectra were collected at Resolution = 1 cm⁻¹ and signal-averaged 1024 times.
Figure 4: The conformational dependent band at 2610 cm$^{-1}$ obtained by taking a difference spectra of horse HbO$_2$-HbCO (a) and (HbO$_2$+Hg)-(HbCO+Hg) (b). The heme concentration was normalized to 19.2 mM. The pathlength = 0.28 mm.
**Figure 5:** The absorption spectra of horse HbCO in the top trace (a) and horse HbO₂, HbCN and metHb in (b), (c) and (d), normalized to 19.2 mM heme concentration and 0.28 mm pathlength.
TABLE 5

IR Spectral Data of β93 -SH Band of Horse Hb Derivatives

<table>
<thead>
<tr>
<th>Sample -Reference</th>
<th>ν_{center} ± .1</th>
<th>Δν_{2} cm^{-1} ± .2</th>
<th>( a_m ) mmM^{-1} cm^{-1} ± .005</th>
<th>A(area) mmM^{-1} cm^{-2} ± .020</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbCO -(HbCO+Hg)</td>
<td>2591.5</td>
<td>20</td>
<td>0.040</td>
<td>0.80</td>
</tr>
<tr>
<td>HbCO -(HbO_2+Hg)</td>
<td>2591.0</td>
<td>20</td>
<td>0.040</td>
<td>0.84</td>
</tr>
<tr>
<td>HbO_2 -(HbO_2+Hg)</td>
<td>2591.0</td>
<td>20</td>
<td>0.038</td>
<td>0.80</td>
</tr>
<tr>
<td>HbCN -(HbO_2+Hg)</td>
<td>2591.0</td>
<td>20</td>
<td>0.035</td>
<td>0.75</td>
</tr>
<tr>
<td>MetHb -(HbO_2+Hg)</td>
<td>2591.0</td>
<td>20</td>
<td>0.021</td>
<td>0.46</td>
</tr>
</tbody>
</table>
SECTION IV

AN IR STUDY OF HUMAN SULFHYDRYL GROUPS: IHP EFFECT ON $\beta$93 -SH OF HbCO, HbO$_2$ AND MetHb ; RELATIVE MERCURIAL BINDING OF THE -SH GROUPS AT THE $\alpha_1\beta_1$ INTERFACE

(A) Introduction

IHP, an inositol polyanionic ester, has approximately 8 negative charges at pH 7.3, twice that of DPG (a heterotrophic regulator of hemoglobin in human red blood cells). It binds 10 times stronger to human deoxyhemoglobin than DPG. Complex formation of IHP and fully liganded oxyhemoglobin, was detected by Gibson and Gray (41), and verified by oxygen equilibrium data by Tyuma et al. (81). The dissociation constant for the (HbO$_2$+IHP) complex was found to be equal to $0.9 \times 10^{-4}$ at pH 7.3 in 0.1 M NaCl with a hemoglobin concentration at 0.4 mM (14). Kinetic data on the reaction of various human hemoglobins ($5 \times 10^{-5}$ M, 20°C) with p-Hgbenzoate ($2 \times 10^{-5}$ M) at a pH below 7.0 had shown that IHP reduces the apparent first rate constants of aquo- and fluoromethemoglobin by large factors, and those of the cyanomet, azidomet and carbonmonoxyhemoglobin by small factors (63). The human high-spin fluoromethemoglobin IHP crystal was isomorphous with deoxyhemoglobin, and has been shown to have a deoxy quarternary structure by the x-ray difference fourier synthesis study against deoxyhemoglobin A ($3.5 \, A^{-1}$) (35). The IHP was found to bind at
the same site between the β-chains as the deoxyhemoglobin. However, the structures of the IHP liganded aquomethemoglobin, HbCO and HbO\textsubscript{2} were still open to discussion.

(B) Results and Discussion

The β93 -SH absorption bands of HbCO±IHP and HbO\textsubscript{2}±IHP were all very similar to each other (Figure 6 and 7). The intensity of HbCO \((a_{\text{mM}}=0.040 \text{ mM}^{-1}\text{cm}^{-1} \text{ and } A_{\text{area}}=0.84 \text{ mM}^{-1}\text{cm}^{-2})\) > HbCO+IHP \((a_{\text{mM}}=0.035 \text{ mM}^{-1}\text{cm}^{-1} \text{ and } A_{\text{area}}=0.77 \text{ mM}^{-1}\text{cm}^{-2})\), and that of HbO\textsubscript{2} \((a_{\text{mM}}=0.037 \text{ mM}^{-1}\text{cm}^{-1} \text{ and } A_{\text{area}}=0.80 \text{ mM}^{-1}\text{cm}^{-2})\) > HbO\textsubscript{2}+IHP \((a_{\text{mM}}=0.032 \text{ mM}^{-1}\text{cm}^{-1} \text{ and } A_{\text{area}}=0.71 \text{ mM}^{-1}\text{cm}^{-2})\). The β93 cysteine -SH band was observed for human metHb+IHP. However, the baseline of this spectrum did not allow reliable area quantitation to be made.

Of all the samples listed in Table 6 and 7, the total amount of β112 -SH plus α104 -SH does not exceed the excessive amount of HgCl\textsubscript{2} (0.1 -SH; 1 mM, or 0.5 -SH; 5 mM equivalent of Hg) added. Therefore, it shows that all the β93 -SH in the mercurial reference samples used were fully reacted with Hg. The α-104 and β-112 sulfhydryl groups of human hemoglobin have known to be much less reactive than β93 -SH, thus the name 'masked' sulfhydryl groups had been given to these two types of α1β1 interface cysteine groups. With addition \((1.5 \text{ Hg/β93 -SH})\) of excess mercuric chloride to the reference sample, the residual β112 band at 2567 cm\textsuperscript{-1} of the (sample - Hg reference) difference spectra, always appeared to be larger than that with less mercury added \((1.1 \text{ Hg/β93 -SH})\) (Figure 9, Table 6 and 7). The excess mercuric chloride
added, reacted 3 times more with β112 -SH than α104 -SH as indicated by the residual β112 and α104 -SH bands (Table 6 and 7), showing that the β112 -SH was approximately 3 times more reactive than α104 -SH in the α2β2 tetramer.

The similar β93 sulfhydryl absorption band seen in the horse and bovine hemoglobin was observed in human HbCO, HbCO+IHP, HbO₂, HbO₂+IHP, MetHb and MetHb+IHP derivatives (Figure 6, 7, 8 and 9).

The β93 SH absorptivities of human liganded hemoglobins were in the same order as horse β93 cysteine -SH group, i.e., HbCO (aₘM⁻¹cm⁻¹; A(area)=0.04 mM⁻¹cm⁻²) > HbO₂ (aₘM⁻¹cm⁻¹; A(area)=0.037 mM⁻¹cm⁻²), > MetHb+ (aₘM⁻¹cm⁻¹; A(area)=0.024 mM⁻¹cm⁻²). The β93 SH absorption intensity of the methemoglobin is about 1/2 that of HbO₂. This correlates with the β93 -SH data of horse metHb, and the structural interpretation that in aquometHb, the F9893 cysteine sulfhydryls are in equilibrium between an external polar position and a non-polar environment between the F and H helices.
Figure 6: IR spectra of human carboxyhemoglobin with and without IHP v.s. mercuric chlordie derivatives as reference. (a) HbCO+IHP-(HbCOIHP+Hg); (b) HbCOIHP-(HbCO+Hg); (c) HbCO-(HbCO+Hg). The spectra were collected at 1 cm\(^{-1}\) resolution and normalized to 16.45 mM heme concentration and 0.0289 cm pathlength.
Figure 7: IR difference spectra of human oxy hemoglobin ± IHP v.s. mercuric chloride derivatives as reference. (a) HbO₂-(HbO₂IHP+Hg); (b) HbO₂+IHP-(HbO₂IHP+Hg). The spectra were collected at 1 cm⁻¹ resolution and normalized to 24 mM heme concentration and 0.0289 cm pathlength.
Figure 8: Difference spectra of human oxy hemoglobin v.s. human oxy hemoglobin with mercury, collected at 1 cm$^{-1}$ and normalized to 24 mM heme and 0.0289 cm pathlength. (a) HbO$_2$-(HbO$_2$ + Hg) (1.5 HgCl/β95 -SH); (b) HbO$_2$-(HbO$_2$ + Hg) (1.1 HgCl/β93 -SH).
Figure 9: Difference spectra of human oxy hemoglobin ± IHP and methemoglobin v.s. mercuric derivative of oxy hemoglobin normalized to 24 mM heme concentration and 0.0289 cm pathlength. (a) (HbO₂+IHP)-(HbO₂+Hg); (b) (HbO₂)-(HbO₂+Hg) (c) Hb⁺-(HbO₂+Hg); (d) Hb⁺-(HbO₂+Hg). The Hg:β93 -SH ratio in the reference samples were 1.5:1 in (a), (b) and (c), and 1.1:1 in (d).
TABLE 6

IR Spectral Data for Human Hemoglobin Derivatives in 0.05 Bis-tris, 0.1 Chloride Buffer at pH 7.05.

$\varepsilon_{\text{app}}$=apparent peak absorptivity in mM$^{-1}$cm$^{-1}$; A(area)-integrated area absorptivity in mM$^{-1}$cm$^{-2}$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>-Reference</th>
<th>Band</th>
<th>$\nu_{\text{max}}$ cm$^{-1}$</th>
<th>$\Delta\nu_{1/2}$ cm$^{-1}$</th>
<th>$\varepsilon_{\text{app}}$/mM$^{-1}$cm$^{-1}$</th>
<th>A(area)/mM$^{-1}$cm$^{-2}$</th>
<th>-SH Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbCO</td>
<td>(HbCO+Hg)</td>
<td>893</td>
<td>2591.5</td>
<td>21.0</td>
<td>0.040</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 Hg/893SH</td>
<td>Δ8112</td>
<td>2567.7</td>
<td>13.0</td>
<td>0.028*</td>
<td>0.36**/(0.45 mM, 8112)</td>
<td>0.054</td>
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<tr>
<td></td>
<td></td>
<td>Δα104</td>
<td>2550.3</td>
<td>13.0</td>
<td>0.037*</td>
<td>0.46**/(0.18 mM, α104)</td>
<td>0.022</td>
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<tr>
<td>(HbCO+IHP)-(HbCO+IHP+Hg)</td>
<td>(HbCO+IHP+Hg)</td>
<td>893</td>
<td>2591.7</td>
<td>21.0</td>
<td>0.035</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 Hg/893-SH</td>
<td>Δ8112</td>
<td>2567.6</td>
<td>12.5</td>
<td>0.025*</td>
<td>0.28**/(0.35 mM)</td>
<td>0.044</td>
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<tr>
<td></td>
<td></td>
<td>Δα104</td>
<td>2550.3</td>
<td>11.5</td>
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<td>0.019</td>
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<td>0.07**</td>
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<tr>
<td>HbO$_2$</td>
<td>(HbO$_2$+Hg)</td>
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<td>2591.5</td>
<td>21.0</td>
<td>0.037</td>
<td>0.80</td>
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</tr>
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<td>2567.1</td>
<td>13.0</td>
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<td>Δα104</td>
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<td>0.44**/(0.18 mM)</td>
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<tr>
<td>HbO$_2$</td>
<td>(HbO$_2$+Hg)</td>
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<td>2591.5</td>
<td>21.0</td>
<td>0.036</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 Hg/893SH</td>
<td>Δ8112</td>
<td>2567.0</td>
<td>13.0</td>
<td>0.041*</td>
<td>0.52**/(0.54 mM)</td>
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<td></td>
<td>Δα104</td>
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</tr>
<tr>
<td></td>
<td>1.5 Hg/893SH</td>
<td>Δ8112</td>
<td>2566.7</td>
<td>13.0</td>
<td>0.038*</td>
<td>0.51**/(0.64 mM)</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Δα104</td>
<td>2552.4</td>
<td>11.5</td>
<td>0.038*</td>
<td>0.45**/(0.19 mM)</td>
<td>0.016</td>
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</table>

$\Delta$=residual band

*Residual mMolar absorptivity

**Residual integrated area absorptivity; calc. using total heme concentration.
TABLE 7

IR Spectral Data for Human Hemoglobin Derivatives in 0.05 Bis-tris, 0.1 Chloride Buffer at pH 7.05.

$\alpha_{\text{mM}}$=apparent peak absorptivity in mM$^{-1}$cm$^{-1}$; $A(\text{area})$=integrated area absorptivity in mM$^{-1}$cm$^{-2}$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>-Reference</th>
<th>Band</th>
<th>$\nu_{\text{max}}$ cm$^{-1}$</th>
<th>$\Delta \nu_{\text{cm}^{-1}}$</th>
<th>$\alpha_{\text{mM}}$/mM$^{-1}$cm$^{-1}$</th>
<th>$A(\text{area})$/mM$^{-1}$cm$^{-2}$</th>
<th>-SH Equivalent</th>
</tr>
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<tbody>
<tr>
<td>Hb$^+$</td>
<td>-(HbO$_2$+Hg)</td>
<td>893</td>
<td>2591.2</td>
<td>21.0</td>
<td>0.024</td>
<td>0.024</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>1.5 Hg/893SH</td>
<td>Δ8112</td>
<td>2567.0</td>
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<td>0.047(0.85 mM)</td>
<td>0.074</td>
<td>0.024</td>
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<tr>
<td></td>
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<td>Δx104</td>
<td>2552.0</td>
<td>11.5</td>
<td>0.048(0.28 mM)</td>
<td>0.024</td>
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<tr>
<td>Hb$^+$</td>
<td>-(HbO$_2$+Hg)</td>
<td>893</td>
<td>2591.2</td>
<td>21.0</td>
<td>0.024</td>
<td>0.024</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>1.1 Hg/893SH</td>
<td>Δ8112</td>
<td>2567.0</td>
<td>13.0</td>
<td>0.031(0.58 mM)</td>
<td>0.051</td>
<td>0.019</td>
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<td>Δx104</td>
<td>2552.0</td>
<td>11.5</td>
<td>0.038(0.22 mM)</td>
<td>0.019</td>
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<tr>
<td>HbO$_2$</td>
<td>-Hb$^+$</td>
<td>893</td>
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<td>21.0</td>
<td>0.011</td>
<td>0.011</td>
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<tr>
<td>HbO$_2$</td>
<td>-(HbO$_2$IHP+Hg)</td>
<td>893</td>
<td>2592.0</td>
<td>21.0</td>
<td>0.035</td>
<td>0.035</td>
<td>0.75</td>
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<tr>
<td>HbO$_2$IHP</td>
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<td>893</td>
<td>2592.0</td>
<td>21.0</td>
<td>0.032</td>
<td>0.032</td>
<td>0.71</td>
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</table>
SECTION V

CONFORMATIONAL STUDY OF $\beta_4$ -SH groups, and IHP BINDING EFFECT ON THE
$\beta_{93}$ -SH OF THE NON-COOPERATIVE TETRAMER

(A) Introduction

The functional properties of the intact tetrameric hemoglobin differs from the isolated $\beta$ and $\alpha$ chains mainly due to the presence of interactions between the $\alpha$ and $\beta$ globin subunits. The tetrameric configuration of $\alpha_2\beta_2$ hemoglobin lowers the overall heme-ligand ($O_2$ and CO etc.) binding affinity of hemoglobin relative to free chains, and renders its efficient physiological role of oxygen loading and unloading possible. In contrast to $\alpha$-chains, which is mainly in monomer or dimer forms (70), isolated $\beta$ chains aggregate to form the $\beta_4$ tetramer (76). The $\beta_4$ tetramer forms \textit{in vivo} in thalassemia patients (hemoglobin H).

Isolated $\beta$ and $\alpha$ chains and myoglobin have long been known to have similar oxygen binding properties (13, 20, 81). They all have high oxygen affinity without heme-heme interaction or Bohr effect. Polyanions, such as 2,3-diphosphoglycerate (DPG), do not have the heterotropistic effect on their oxygenation as the anions do on hemoglobin tetramer. In case of $\alpha$-chains, 2,3-DPG simply does not bind (70). However, DPG had been shown to bind equally to both oxy and deoxy $\beta$ chain, supporting the fact that the interaction of DPG with $\beta_4$ is not oxygen-linked, and that the binding of organic phosphates needs $B_1B_2$ contact (6, 7). The reactivity of the sulfhydryl group at $\beta_{93}$ cysteine was found to be approximately
the same in oxy hemoglobin and oxy- or deoxy-B₄ (15,57). The β chains aggregate in tetramer form, yet the latter binds oxygen non-cooperatively with an affinity characteristic of oxy hemoglobin (12). Without α-chain, the B₄ tetramer obviously cannot have the αβ subunit structural interaction such as the interchain salt-bridges, which are essential for the cooperativity in α₂β₂ tetramer (67). Nevertheless, there are biochemical and crystallographic evidence that some properties of the B₄ tetramer resemble those of oxy hemoglobin, but others resemble those of deoxy hemoglobin (11). An infrared study of the B₄ tetramer would therefore serve two purposes. (1) A study of the β₉₃ -SH may provide information for answering the question whether the change of β₉₃ -SH absorption band properties, thus the alteration of the reactive β₉₃ cysteine conformation upon ligand binding, is directly related to tertiary structure change. (2) It may also answer some questions about the non-cooperative tetramer arrangement of the B₄ tetramer.

(B) Results and Discussion

The IR spectrum of B₄ β₉₃ cysteine sulfhydryl was obtained by the first time from the difference spectra of B₄CO - Horse HbCOHg (Figure 10). The band with an apparent center peak frequency of 2591.0 cm⁻¹ had the similar center frequency and apparent half-bandwidth and absorptivity as the β₉₃ -SH of hemoglobin α₂β₂ tetramer. The negative band at 2556 cm⁻¹ was due to the α-104 cysteine -SH of horse hemoglobin (10). Difference spectra of a B₄CO+PMB versus the horse HbCOHg showed no β₉₃ absorption
band in the sulfhydryl region, indicating a complete 'loss' of the $\beta_93$ -SH absorption in the mercurial derivative due to the reaction of the sulfhydryl group with the mercurial chloride. The $2591 \text{ cm}^{-1}$ band in the $(\beta\text{CO})_4$ was therefore assigned as the $\beta_93$ -SH band. Table 8 showed the IR data of $(\beta\text{CO})_4$, $(\beta\text{O}_2)_4,(\beta\text{CO})_4 + \text{IHP}$ and $(\beta\text{S}\text{CO})_4$. The $(\beta\text{CO})_4,(\beta\text{O}_2)_4,(\beta\text{CO})_4 + \text{IHP}$ and $(\beta\text{S}\text{CO})$ showed essentially the same apparent absorptivity $a_{\text{M}}$ (mM$^{-1}$cm$^{-1}$), and integrated area absorptivity $A_{\text{area}}$ (mM$^{-1}$cm$^{-2}$). These absorptivities were also very similar to that of bovine and human HbCO $\beta_93$ -SH absorption band. The total absorption intensity of the tetramer doubles that of $\alpha_2\beta_2$ tetramer with the same heme concentration. The $\beta_93$ -SH of the CO and CO+IHP derivatives had similar absorption intensity. Figure 11 shows the difference spectrum of $(\beta\text{O}_2)_4$ - deoxy $\beta_4$. A difference $\beta_93$ band was observed at $2592 \text{ cm}^{-1}$ along with a smaller and conformational related band at approximately $2610 \text{ cm}^{-1}$. A similar $2610 \text{ cm}^{-1}$ band was shown in Figure 4 and described earlier. The $2567 \text{ cm}^{-1}$ $\beta_{112}$ -SH band observed in human tetramer was not observed in any of these $\beta_4$ spectra indicating that either the $4 \beta_{112}$ -SH are in a much polar or random environment than in the $\alpha_2\beta_2$ tetramer, or they are simply not free sulfhydryl groups, which is very unlikely. The slightly lower frequency of $\beta_4$ -SH band listed in Table 8 as compared to that of human, horse and bovine hemoglobin, could be explained by the presence in the difference spectrum of a negative $\alpha$-104 -SH absorption band. The $\beta_93$ absorption intensities of $(\beta\text{CO})_4$ and $(\beta\text{O}_2)_4$ are essentially the same. The $\beta_93$ SH absorptivity and frequency in the $\beta_4$ and $\alpha_2\beta_2$ tetramers are similar. Since similar absorptivity/-SH is observed in both $\beta_4$ and
α₂β₂, the absorptivity of β93 in α₂β₂ is only dependent on the state of ligation of the β chain, thus tertiary-structure related.
Figure 10: The absorption difference spectrum of human β₄CO- (horse HbCO+Hg) in 0.1 M bis-tris chloride buffer at pH 7.1. The spectrum was normalized to a heme concentration of 20 mM and a pathlength of 0.029 cm.
<table>
<thead>
<tr>
<th>Sample</th>
<th>(v_{\text{max}})</th>
<th>(\Delta v_{\pm} , \text{cm}^{-1})</th>
<th>(a_{\text{M}} , \text{mM}^{-1}\text{cm}^{-1})</th>
<th>(A_{\text{area}} , \text{mM}^{-1}\text{cm}^{-2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>((\beta\text{CO})_4^-\text{[HbCOHg (horse)]})</td>
<td>2591.0</td>
<td>± .4</td>
<td>± .003</td>
<td>± .05</td>
</tr>
<tr>
<td>((\beta\text{O}_2)_4^-\text{[HbCOHg (horse)]})</td>
<td>2591.0</td>
<td>19.5</td>
<td>0.042</td>
<td>0.82</td>
</tr>
<tr>
<td>((\beta\text{O}_2)_4^-\text{[HbO}_2\text{Hg (horse)]})</td>
<td>2591.0</td>
<td>19.5</td>
<td>0.043</td>
<td>0.82</td>
</tr>
<tr>
<td>((\beta\text{CO})_4^-\text{+IHP-[HbCOHg (horse)]})</td>
<td>2591.0</td>
<td>19.5</td>
<td>0.039</td>
<td>0.80</td>
</tr>
<tr>
<td>((\beta^5\text{CO})_4^-\text{[HbCOHg (horse)]})</td>
<td>2591.5</td>
<td>19.7</td>
<td>0.037</td>
<td>0.83</td>
</tr>
</tbody>
</table>
Figure 11: The difference absorption spectrum of Oxyβ₄-deoxy β₄. The spectrum was normalized to a heme concentration of 11 mM and a pathlength of 0.0289 cm.
SECTION VI
MERCURY, SILVER AND IHP BINDING EFFECT OF THE CO LIGAND OF HUMAN HEMOGLOBIN
(A) Introduction

Mercurial and mersalyl binding data on human and horse hemoglobins showed an increase in the oxygen affinity of the hemoglobins (74). It is also known that mercurial binding to β93 sulfhydryl groups favors αβ dimer formation (31,75), which has higher oxygen affinity. However, the high hemoglobin concentration and buffer condition used in the IR experiments favor the tetrameric form.

Zinc binding has also been shown to induce an increase in the oxygen affinity of human hemoglobin (72). The reaction of CuII with hemoglobin is of considerable significance in normal erythrocyte metabolism. When present at a very low concentration, copper catalyses the autoxidation of oxyHb by a mechanism requiring oxygen, but otherwise similar to that of direct oxidation (88). One CuII ion/1000 heme groups significantly increases the rate of autoxidation, and the erythrocyte is thought to contain sufficient copper for this to be the main pathway of methemoglobin formation (88). The binding sites of zinc and copper appears to involve the β93 cysteine (73,88). A study of the influence on CO stretch band by mercurial binding of the peptide at the F9893 position of the F helix may elucidate the reciprocal relationships between the 93-SH conformational equilibrium and the Fe-ligand bonding properties.
The β93-SH absorption data described before correlate well with the X-ray crystallographic data (43,61). In Hbmet, β93-SH is partially out of the non-polar pocket between the F and H helices, and thus becomes more reactive to -SH reagents. The β93-SH of HbCO, HbO₂ and HbCN is held more firmly in the non-polar pocket. In deoxy Hb, the β94 Asp and 146His form a salt bridge which limits the accessibility of solutes to the -SH group. The structural differences among HbCO, Hbmet+, and deoxyHb at the heme have been described by Perutz and Ten Eyck, and Baldwin and Chothia (9,69). According to these authors, ligation causes a rotation of the F helices, anticlockwise as seen from the FG corner. Both the hemes and F helices shift towards the distal side with a probable shrinking in the distance between the β heme and F helix. Movements on transition from a high spin ferric (met) to a low-spin ferrous ligand (CO) also include a shortening of Fe-N bonds, and the β93-SH groups go into the non-polar pocket between the F and H helices (61).

(B) Methods

The shift of center frequencies were measured and compared by two methods. After correction for water and protein background absorptions, the plots of the interpolated 1 cm⁻¹ resolution spectra provide a center frequency measurement of the absorbance bands with an uncertainty around ± 0.05 cm⁻¹ for the ¹²C¹⁶O bands with a 600:1 signal to noise ratio and ± 0.1 cm⁻¹ for the ¹³C¹⁶O bands with a 65:1 signal to noise ratio. An alternative measurement of the frequency shifts was done by finding the maximum to minimum height of the difference spectrum of a pair of
absorbance spectra e.g., HbCO and HbCO+Hg, with normalized peak maximum (ΔA/A), followed by comparison with computed Lorentzian bands of similar (ideally same) band shape, which yields approximate frequency shifts between the two spectra (CO and CO+Hg). The ΔA/A values listed in Tables 9, 10, and 11, would therefore qualitatively provide a comparative measurement for the degree of frequency shift (greater the ΔA/A, greater the shift) (1).

(C) Results and Discussion

The center frequency of the 12C16O stretch band in D2O HbCO sample was measured at 1950.65 cm⁻¹, essentially the same as that in H2O sample, indicating that within the vicinity of the CO binding site there is unlikely any deuterium exchangeable proton.

All the 13C16O band of hemoglobin derivatives are due to the natural abundance of 13C16O (1.11% of total atmospheric CO thus equivalent to ≈1% of the total CO ligand) and trace of 12C18O (0.204% of total atmospheric CO, thus 15% of the 13C16O band). With 1.3% abundance of total CO ligands, therefore, the 13C16O and 12C18O are assumed to be randomly bound among all the subunits with neighboring subunits bound with 12C16O.

Figure 12-13, Table 9 (a) shows respectively the IR spectra and data of the 13C16O bands of bovine hemoglobin derivatives at pH 7.1. The center frequency of Hb13C16O band was measured to be 1906.3 cm⁻¹. With HgCl binding to the β93-SH group at the α1β2 interface of the bovine HbCO hemoglobin, the center frequency of the overall population of the heme iron-linked CO group shifts (Δνobs = 0.34 cm⁻¹) to a lower frequency.
At pH 6.1, $v_{CO}$ shift ($\Delta v_{obs} = 0.3 \pm 0.1 \text{ cm}^{-1}$) towards lower frequency caused by Hg binding was also observed for the $^{13}$C$^{16}$O band in human hemoglobin (Table 9b). The amount of shift is comparable to that of the $^{13}$C$^{16}$O band in bovine hemoglobin at pH 7.1. IHP binding causes a smaller CO frequency shift ($0.18 \pm 0.1 \text{ cm}^{-1}$) (Table 9b).

The $^{13}$C$^{16}$O in human $\beta_4$ absorbs at 1907.3 cm$^{-1}$, (Figure 15 and 16, Table 9c) approximately 1 cm$^{-1}$ higher than that in the $\alpha_2\beta_2$ tetramer. IHP binding shifts the CO band to a slightly lower frequency (Figure 17, 18 and 19, Table 9c).

The various $^{12}$C$^{16}$O absorption spectra of fully CO liganded human hemoglobin derivatives with or without Hg and IHP binding perturbations are shown in Figure 20 and Figure 22. The IR data are listed in Table 10 (a and b).

At pH 6.1, the $^{12}$C$^{16}$O band is also shifted ($\Delta v_{obs} = 0.39 \text{ cm}^{-1}$) to a lower $v$ by the HgCl binding of the 93-SH. A smaller but definite downward shift ($\Delta v_{obs}=0.18 \text{ cm}^{-1}$) in $v_{CO}$ is caused by IHP (Figure 20 and 21, Table 10a).

At pH 8.1, a significant shift ($\Delta v_{obs} = 0.28 \text{ cm}^{-1}$) due to Hg also exists (Figure 23 lower trace and Table 10b). Ag$^+$ binding also appears to give a shift $0.08 \pm 0.05 \text{ cm}^{-1}$ in the same direction but to a much lesser degree (Table 10b). Addition of IHP at this pH did not cause more than $0.02 \text{ cm}^{-1}$ shift in CO consistent with the fact that IHP binds much weaker at higher pH. However, a small but definite narrowing in bandwidth appears in the difference spectrum (Figure 23 upper trace; Figure 22 and Table 10b).
At pH 7.1, the difference spectrum of Hb$^{12}$C$^{16}$O-(HbCO+IHP) in Figure 14 also shows a narrowing of the overall bandshape by IHP addition in HbA.

At pH 7.1, the $^{12}$C$^{16}$O absorption band of partially (1%) CO liganded human HbO$_2$ of hemoglobin with neighboring subunits bound with O$_2$ ligands, also shows a Hg binding effect on the CO stretch (0.46 ± 0.05 cm$^{-1}$ downward shift) (Figures 24, 25 and Table 10c). IHP may also lower the $^{12}$C$^{16}$O absorption frequency by (0.09 to 0.05 cm$^{-1}$) (Table 10c). The relatively broader half-bandwidth (7.52 cm$^{-1}$) of the $^{12}$C$^{16}$O absorption (Table 10c) of the partial CO hemes of HbO$_2$ (human) indicates a random binding of CO to the $\alpha$ and $\beta$ chains with the presence of a competitive ligand like oxygen. Mercurial binding at the B93 site shifts the CO bands of the R state hemoglobin towards a lower frequency, i.e. towards a higher affinity form of ligand carbonyl. This correlates with the phenomenon of the rotation of the $\beta$ subunit F-helices, as described in the case of the conformational change from metHb to HbCO by Perutz et al. (61). The $\nu_{CO}$ of CO hemes having different substituents in positions 2 and 4 of the porphyrin ring, and the CO stretching frequency values of carbonmonoxy hemoglobins and myoglobins have been correlated with the strength of the CO bonding to the porphyrin-iron and to the intramolecular interactions occurring in the porphyrin by Alben and Caughey (2). In the case of 2,4-substituted deuterio hemoglobins of ethyl, hydrogen, vinyl and acetyl groups, the decrease in the absolute $\nu_{CO}$ of the substituted HbCO derivatives in the order ethyl > hydrogen > vinyl > acetyl has been interpreted as due to a decrease in $\Pi$-donor capacity of the porphyrin-iron
atom caused by a decrease in basicity of the tetrapyrrole nitrogens, due to the increased electron withdrawing power of the 2,4-substituents (ethyl < hydrogen < vinyl < acetyl). In a very simplified valence bond terms then, the stronger the Fe-(CO) bond, the greater will be the bond order of the Fe-(CO) bond (i.e. greater the iron ligand field) the smaller will be the CO bond order, and the weaker will be the CO bond, i.e. the $\nu_{\text{CO}}$ frequency can be expected to decrease as the bond order of the CO bond decrease (2). The greater the $\Pi$-bond character of Fe-(CO), the more polarized the CO oxygen atom would be. The CO bond is strengthened thus a higher $\nu$, if the ability of the iron atom to serve as a $\Pi$ donor decreases.

Other possible factors that might affect the $\nu_{\text{CO}}$ may include the solvation shell effect results from heme pocket size variation and nearest neighbor steric effects which could affect the ($\text{Fe}=\text{C}=\text{O}$) bond angle and the polarization of the ligand. Upon HgCl binding and thus may be Zn and Cu(II) binding too at the F9893 -SH, the HgCl may cause stress on the F helix to favor a position which conformationally would appear to rotate slightly anticlockwise as seen from the FG corner. Such a position would favor the proximal histidine $N_\varepsilon$ linked heme Fe of the $\beta$ chain to be pushed further into the porphyrin, and results in its increase in ligand field or $\Pi$ bond donating ability. This would strengthen the ligand bonding affinity, thus a more oxy type tertiary structure, resulting in the enhancement of ligand affinity of hemoglobin by mercurial binding of the F9893 residue. The weaker CO bond is
manifested as a lower $\nu_{CO}$ frequency. By the same token, the enhancement of oxygen affinity by Zinc binding of hemoglobin may also be explained.

A relatively great downward frequency shift caused by Hg binding, suggests a weaker CO bond, stronger Fe ligand field and more polar oxygen atom. This if apply to oxygen liganded hemoglobin means a greater tendency for the heme $O_2$ to form superoxide in the presence of CuII. If so, then the oxygen and $\beta93$ -SH dependent enhancement of hemoglobin oxidation by CuII could be understood in terms of the steric properties of the F-helix. The much greater effect of HgCl$_2$ than Ag$^+$ may be due to the fact the S-Hg-C1 rotates into the non-polar pocket and results in greater strain of the F helix than that caused by S-Ag.

IHP has been shown to shift Hbmet$^+$ equilibrium from R to T quarternary states (64), and also constrained HbNO to a T state globin conformation (65). The Raman spectroscopy shows no observable IHP effect on R state hemoglobin (8). The $\beta93$ -SH absorption intensities of HbCO with and without IHP are similar, i.e. either no or less than 10% decrease in $\beta93$ absorption intensity, and thus IHP either has no effect on $\beta93$ -SH conformation or reduces its occurrences in the non-polar pocket between F and H helices by less than 10%. The binding of IHP to liganded hemoglobin shows some conformational alteration at the heme as indicated by a shift of $^{12}C^{16}O$ and $^{13}C^{16}O$ bands towards a lower frequency (by 0.1 to 0.2 cm$^{-1}$) at low pH. At higher pH when IHP is bound less "tightly", a narrowing of the CO band shape is still observable. At pH 7.1, the IHP
shifts the CO in $\beta_4$CO towards a lower frequency ($\Delta \nu = 0.12 \pm 0.1 \text{ cm}^{-1}$) (Figures 17-19, Table 9c). While at the same pH the $^{12}$C$^{16}$O of the partial CO heme in the $\beta_4$ (deoxy+CO) hybrid, has a higher frequency ($\Delta \nu = 0.60 \pm 0.05 \text{ cm}^{-1}$) than that in the $\beta_4$ (O$\text{}_2$+CO) hybrid (closer to high affinity tertiary structure) mixture. This indicates that the IHP has shifted $\beta_4$ CO in the direction opposite to that of $\beta_4$ (deoxy+CO) hybrid.

The addition of IHP therefore may have shifted the heme ligand carbonyl $\beta$ chains in the tetramer towards a lower frequency or weaker CO bond and presumably the $\alpha$ chain CO towards a higher frequency in a less accentuated fashion as seen by a narrowing of the half-bandwidth in Figure 14 and Figure 22 upper trace. This piece of data implies that in HbCO and maybe HbO$_2$ low spin samples, IHP's effect seems to involve a change of $\beta$ chain ligand towards a tertiary oxy or higher affinity state like CO bond, and that its effect on the $\alpha$ chain would be just the opposite and with less pronounced alterations of the CO bond (as shown by a narrowing of the CO band).

$\beta_4$CO has a CO center frequency always greater than 1951 cm$^{-1}$, while that of the $\alpha$CO slightly lower than 1951 cm$^{-1}$ (Alben and Bare, unpublished results). The tetramer of both chains however, has a center frequency in between them. This implies that the CO band of $\alpha_2\beta_2$ tetramer is a composite band of $\alpha$CO and $\beta$CO absorptions, yet the composite band is not a normal distribution of energy absorptions, but one from 2 non-equivalent $\alpha$ and $\beta$ bands. The true implication still needs to be tested.
The half-bandwidth of the CO absorption band due to the 1% CO heme in HbO\textsubscript{2} (7.52 cm\textsuperscript{-1}) (Figure 24, Table 10c) is broader than that of isolated α and β chains, thus suggests that CO might be randomly bound to the α and β chains. Otherwise somewhat narrower bandwidth would be anticipated if an overwhelming preference of one type of chain is preferred over the other.

The metHb reduction to HbO\textsubscript{2} by ascorbic acid was found to be accelerated as much as 10 times by IHP presence (79). However, the IHP stimulatory effect was shown to be restricted to the β chains of the protein (80). This again supports the CO data that IHP has greater effect on the β chains of the tetramer. Its effect on the heme ligand site was manifested by the shift in the ν\textsubscript{CO}. These small shift of ν\textsubscript{CO} to a lower frequency in HbCO, β4CO and HbO\textsubscript{2}CO hybrid caused by the addition of IHP may be interpreted to indicate a stronger Fe-(CO) bond, or a bond that is bent further from a plane that is perpendicular to the heme plane. Nevertheless, no significant IHP effect of the F9 cysteine residue was observed in R state hemoglobin. Its effect on the heme may not be transmitted thru the conformational change of the F-helices in the β chains but rather thru other regions of the molecule, or due to an overall slight conformational change such as a narrowing of the β heme pocket. In short, the possible cause of the effect of mercurial and IHP binding to the protein on the heme Fe liganded CO includes a possible stress exerted on the steric orientation of the F-helix. Such a stress could be initiated by the change in the interplay of the conformational equilibrium positions of β93 -S-Hg-Cl or β93 -SH and HCβ145Tyr in the
vicinity of the F and H helices, as demonstrated by the correlation of the crystallographic and FTIR data on the β93 -SH groups' conformation in various Hb derivatives. Upon ligand (HgCl and IHP) binding on the protein, especially IHP binding, the possible steric factors that may be involved includes direct steric contact interaction with the ligand solvation shell due to groups like the imidazole group of E7 histidine and the γ-methyl group of E11 valine, which are situated at close range to the heme bound ligand. Absence of the E11 valine steric effect had been shown to shift νCO to higher frequency (82). The effect of the HgCl2 on heme-liganded CO, however, may be due to the conformational stress exerted on the F helix, and thus possibly affects the proximal histidine by the HgCl binding to the F9393 cysteine sulfhydryl group. In order to release such a stress, the tertiary structure of the HgCl bound β chain has a tendency to go to a high affinity conformation.

Figure 26 and Table 11 show a higher νCO (1951.10 cm⁻¹) for 13% CO in oxy β4 and 1951.70 cm⁻¹ for 13% CO in deoxy β4, in the β chain tetramer than that of α2β2 tetramer (1950.77 cm⁻¹, Figure 24c, Table 10c). The CO of the CO hemes (13%) mixed in deoxy β4 sample, has a higher ν than that of oxy β4 sample (Table 10), thus show a frequency shift in the difference spectra between deoxy β4 v.s. oxy β4 (Figure 26). There are conflicting data on the functional and structural properties of β4 tetramer in the literature. The tetramer exhibited no significant cooperative effect for oxygen binding and was thus thought to be without quarternary structural change. However, the difference in frequency of
CO bound to 87% deoxy v.s. oxy-β₄ tetramer proves the existence of some quaternary structure dependent control of the heme-carbonyl complex. The $\nu_{CO}$ shift caused by IHP in $\beta_4CO$ may be due to a very small quaternary structural change. The $\nu_{CO}$ shift does not relate to the interchain salt bridges in $\alpha_2\beta_2$ tetramer, since the salt bridges are absent in $\beta_4$ tetramer. Further it suggests that different tertiary structures may exist in the $\beta_4$ non-cooperative tetramer.
Figure 12: Absorption spectra of bovine carboxyhemoglobin and HbCO+Hg at pH 7.1. The Hb$^{13}$C$^{16}$O band (1) at 1906.2 cm$^{-1}$ is at a slightly higher frequency than Hb$^{13}$C$^{16}$O+Hg. The small band (2) at 1967 cm$^{-1}$, which is less than 1% of the major $^{12}$C$^{16}$O stretch band at 1951.0 cm$^{-1}$, may be due to a minor population of Hb$^{12}$C$^{16}$O with a different heme environment. The spectra were collected at 1 cm$^{-1}$ resolution, with a heme concentration of 19.3 mM and a pathlength of 0.3 mm.
Figure 13: Upper trace: The difference absorption spectra between bovine Hb\textsuperscript{13}C\textsubscript{16}O and Hb\textsuperscript{13}C\textsubscript{16}O+Hg (1), and between bovine Hb\textsuperscript{12}C\textsubscript{16}O and Hb\textsuperscript{12}C\textsubscript{16}O+Hg derivatives. Lower trace: The same difference spectra with less vertical expansion but a wider frequency range. The hump at 2592 cm\textsuperscript{-1} is the \( \beta93 \)-SH absorption band. The spectra were collected at 1 cm\textsuperscript{-1} resolution, with a pathlength of 0.03 cm, and a heme concentration of 19.3 mM (pH 7.1).
Figure 14: The difference spectra of human Hb\textsuperscript{12C\textsuperscript{16O}}-HbCOIHP at pH 7.1. The spectrum shows a slight or no center frequency shift between the two samples. However, the IHP derivative shows a narrower bandwidth. Heme concentration = 16.45 mM; pathlength = 0.0289 cm.
Figure 15: The absorption spectrum of human $(\beta CO)_4$ (20 mM heme, 0.29 mm pathlength) with baseline correction using water, and $(\beta O_2)_4$ as the protein reference. The heme liganded $^{13}C^{16}O$ and $^{12}C^{18}O$ band at 1907.53 cm$^{-1}$ was shown in the dotted circle. The $^{13}C^{16}O$ (1.11% of total CO) and $^{12}C^{18}O$ (0.204% of total CO) reacted with $\beta_4$ were of natural abundance. The overshoot band at approximately $1951.5 \pm 0.2$ cm$^{-1}$ is the major heme liganded $^{12}C^{16}O$ band.
Figure 16: The absorption of the mainly $^{13}\text{C}^{16}\text{O}$ band of human (BCO)$_4$. An expanded version of the band in the dotted circle of Figure 15.
Figure 17: The difference absorption spectra of human hemoglobin \((\beta CO)_4\) v.s. \((\beta CO)_4 + IHP\). The spectra were normalized to 20 mM heme concentration and 0.3 mm pathlength. Difference spectra of the \(^{12}\text{C}^{16}\text{O}\) and the \(1.11\% \ ^{13}\text{C}^{16}\text{O}\) bands were observed.
Figure 18: The difference spectrum of human $(\beta CO)_4-((\beta CO)_4+IHP)$ with a normalized heme concentration of 20 mM and a pathlength of 0.3 mm.
Figure 19: An expanded difference spectrum of human $\beta_4^{13}\text{C}^{16}\text{O} - (\beta_4^{13}\text{C}^{16}\text{O} + \text{IHP})$, showing a shift of the $^{13}\text{C}^{16}\text{O}$ band of the IHP derivative towards a lower frequency. The data were collected at 1 cm$^{-1}$ resolution with a normalized heme concentration of 20 mM and a pathlength of 0.03 cm.
Figure 20: The absorbance spectra of human Hb$^{12}$C$^{16}$O+IHP, Hb$^{12}$C$^{16}$O+Hg and Hb$^{12}$C$^{16}$O (from top to lower trace) at pH 6.1, from which the difference spectra shown in Fig. 21 were derived. The individual $^{13}$C$^{16}$O band could also be observed at approx. 1906 cm$^{-1}$. 
Figure 21: The difference absorbance spectra at pH 6.1 of human 
(Hb^{12}C^{16}O+Hg)-Hb^{12}C^{16}O in the upper trace, and of (Hb^{12}C^{16}O+IHP)-Hb^{12}C^{16}O 
in the lower trace. The spectra were normalized to a heme concentration of 
14.23 mM. The spectra show clearly that both the mercuric and IHP complex 
of human HbCO have a lower $^{12}C^{16}O$ stretching frequency than that of HbCO. 
However, the mercuric complex shifts to a much lower frequency (greater 
peak to peak height) than the IHP complex.
Figure 22: The absorbance spectra of human (Hb$^{12}$C$^{16}$O+Hg), (Hb$^{12}$C$^{16}$O) and (Hb$^{12}$C$^{16}$O+IHP) at pH 8.1 (top to bottom trace) from which the difference spectra shown in Figure 23 were derived. The individual 13C16O band could also be observed at approx., 1906.1 cm$^{-1}$. 
Figure 23: The difference absorbance spectra of human (Hb\textsuperscript{12}C\textsuperscript{16}O+IHP)-HbCO in the upper and (HbCO+Hg) - HbCO in the lower trace. The sample were at pH 8.1., and with a normalized heme concentration of 13.3 mM.
Figure 24: The absorbance spectra of the 1% CO HbO₂ samples, from which the difference absorbance spectra in Figure 25 were derived (Table 10c).
Figure 25: The $^{12}$C$^{16}$O difference absorbance spectra of human (HbO$_2$+Hg) (with 1% CO heme) versus HbO$_2$ (with 1% CO heme), and (HbO$_2$+IHP) (with 1% CO heme) versus HbO$_2$ (with 1% CO Heme). The spectra were normalized to 24.48 mM total heme and a pathlength of 0.29 mm. The spectra show that the stretching frequencies of the 1% CO in both the mercuric and IHP derivatives at pH 7.1 are lower than that of HbCO. The (HbCO+Hg) has the lowest frequency among the derivatives.
Figure 26: The spectra of 13% CO in Oxy ε4 and deoxy ε4 are shown in a and b. The CO in the Oxy sample had a lower center frequency. The absorbance difference spectrum is shown in the lower trace.
TABLE 9
IR Data of the Various $^{13}\text{C}^{16}\text{O} (\& ^{12}\text{C}^{18}\text{O})$ Bands

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>$v^\ast$ center cm$^{-1}$</th>
<th>$\Delta v$ obs. center shift cm$^{-1}$</th>
<th>$\Delta v_\parallel$ cm$^{-1}$</th>
<th>$\frac{\Delta A}{A_{\text{max}}}$</th>
<th>$a_{\text{mM}}$ (mM$^{-1}$ cm$^{-1}$)</th>
<th>A(area) (mM$^{-1}$ cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. BOVINE HbCO</td>
<td>7.1</td>
<td>1906.34</td>
<td>0.34</td>
<td>7.3</td>
<td>6.0 $\times$ 10$^{-2}$</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1906.00</td>
<td></td>
<td>0.34 (0.43)***</td>
<td>0.17</td>
<td>6.3 $\times$ 10$^{-2}$</td>
<td>0.44</td>
</tr>
<tr>
<td>b. HUMAN HbCO+Hg</td>
<td>6.1</td>
<td>1905.97</td>
<td>0.30</td>
<td>7.2</td>
<td>4.6 $\times$ 10$^{-2}$</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>1906.27</td>
<td>0.18</td>
<td>7.2</td>
<td>5.2 $\times$ 10$^{-2}$</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>1906.09</td>
<td></td>
<td>7.2</td>
<td>6.6 $\times$ 10$^{-2}$</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>c. HUMAN $\beta_4$CO</td>
<td>7.1</td>
<td>1907.53</td>
<td>0.12</td>
<td>7.2</td>
<td>4.1 $\times$ 10$^{-2}$</td>
<td>0.30</td>
<td></td>
</tr>
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<tr>
<td></td>
<td></td>
<td>1907.41</td>
<td></td>
<td>7.0</td>
<td>4.6 $\times$ 10$^{-2}$</td>
<td>0.32</td>
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</table>

*Lines parallel to the baseline were drawn intersecting the absorption band. The extrapolation to the absorption peak of the averaged mid pts. of at least six of these parallel lines gives the measurement of $v^\ast_{\text{center}}$ of the band.

**Extinction coefficients obtained using total CO heme concentrations of $^{12}\text{C}^{16}\text{O}$, $^{13}\text{C}^{16}\text{O}$ and $^{12}\text{C}^{18}\text{O}$; $a_{\text{mM}}$ stands for the apparent peak absorptivity; A(area) stands for integrated area absorptivity.

***From comparison with difference of computed Lorentzian functions.
### TABLE 10

**IR Data of the Various Human $^{12}$C$^{16}$O Bands**

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>$v^*_{center}$ cm$^{-1}$ ± 0.03</th>
<th>$\Delta v_{obs.}$ center_shift cm$^{-1}$ ± 0.05</th>
<th>$\frac{\Delta A}{A_{max}}$ cm$^{-1}$ ± 0.05</th>
<th>$\Delta v_\parallel$ cm$^{-1}$ (mM$^{-1}$cm$^{-1}$) ± 0.05</th>
<th>$A$ (area) (mM$^{-1}$cm$^{-2}$) ± 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a. HbCO+Hg</strong></td>
<td>6.1</td>
<td>1950.40</td>
<td>0.39 (0.38)*</td>
<td>0.101</td>
<td>7.28</td>
<td>4.63</td>
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<tr>
<td>HbCO</td>
<td></td>
<td>1950.79</td>
<td>0.18 (0.15)*</td>
<td>0.061</td>
<td>7.32</td>
<td>4.68</td>
</tr>
<tr>
<td>HbCO+IHP</td>
<td></td>
<td>1950.61</td>
<td></td>
<td></td>
<td>7.28</td>
<td>3.86</td>
</tr>
<tr>
<td><strong>b. HbCO+HgCl$_2$</strong></td>
<td>8.1</td>
<td>1950.50</td>
<td>0.28 (0.27)*</td>
<td>0.090</td>
<td>7.04</td>
<td>4.89</td>
</tr>
<tr>
<td>HbCO</td>
<td></td>
<td>1950.78</td>
<td>0.0 (0.004)*</td>
<td>(≤ .02)</td>
<td>7.10</td>
<td>4.88</td>
</tr>
<tr>
<td>HbCO+IHP</td>
<td></td>
<td>1950.78</td>
<td>0.1 (0.005)*</td>
<td>(≤ .02)</td>
<td>7.09</td>
<td>5.02</td>
</tr>
<tr>
<td>HbCO+Ag$^+$</td>
<td></td>
<td>1950.70</td>
<td>0.08 (0.10)*</td>
<td>0.039</td>
<td>7.07</td>
<td>5.00</td>
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<tr>
<td><strong>c. 1% HbCO in HbO$_2$</strong></td>
<td></td>
<td>7.1</td>
<td>1950.31</td>
<td>0.46 (0.50)*</td>
<td>0.170</td>
<td>7.35</td>
</tr>
<tr>
<td>HbCO</td>
<td></td>
<td>1950.77</td>
<td>0.09 (0.12)*</td>
<td>0.044</td>
<td>7.52</td>
<td></td>
</tr>
<tr>
<td>HbCO+IHP</td>
<td></td>
<td>1950.68</td>
<td></td>
<td></td>
<td>7.49</td>
<td></td>
</tr>
</tbody>
</table>

*From comparison with difference of computed Lorentzian functions.*

a and b fully saturated with CO.
c with 1% CO in O$_2$ liganded Hb.
TABLE 11

IR Data of $^{12}$C$^{16}$O in Human Oxy and Deoxy $\beta_4$ (Data from Figure 26)

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>$v_{\text{center}}$ cm$^{-1}$ ± 0.03</th>
<th>$\Delta v$ obs. center shift cm$^{-1}$ ± 0.05</th>
<th>$\Delta A/\Delta A_{\text{max}}$ ± 0.05</th>
<th>$\Delta v_2$ cm$^{-1}$ ± 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. 13% CO in Oxy $\beta_4$</td>
<td>7.1</td>
<td>1951.10</td>
<td></td>
<td>0.60 (0.72)*</td>
<td>0.337</td>
</tr>
<tr>
<td>b. 13% CO in deoxy $\beta_4$</td>
<td>7.1</td>
<td>1951.70</td>
<td></td>
<td></td>
<td>7.08</td>
</tr>
</tbody>
</table>

*From comparison with difference of computed Lorentzian functions.
SECTION VII

FURTHER DISCUSSION ON β93 -SH STUDIES

The β93 sulfhydryl group absorption band was observed for the
liganded human, horse and bovine hemoglobins, by using proper mercurial
references. The relative intensity pattern of the β93 absorption
HbCO > HbO₂ ∼ HbCN > methHb >> Hbdeoxy was also observed for α-104
-SH (1). The intensity results agree with and extend the crystallo-
graphic data (43,61). The crystallographic data of horse HbCO and
methHb done by Heidner et al. suggest that the sulfhydryl reactivity
of the β93 cysteine could be interpreted by the change in tertiary
structure in the immediate vicinity of the heme. The conformational
change from HbCO to methHb includes a slight rotation of the globin
helix F and an interplay of the side-chain positioning between the
β145 tyrosine and β93 cysteine -SH (61). HbO₂ crystal structure has
not been available because of the instability of HbO₂ to the X-ray
beam. In short, in deoxy hemoglobin, the tyrosine residue is positioned
in a pocket between the F and H helix, and all the β93 -SH groups are
external. In HbCO, the reverse situation is true. The β93 -SH is held
firmly in the non-polar pocket between the F and H helices and the
tyrosine is expelled to the external surface. In methemoglobin, there
is an equilibrium between the two alternate conformations. In HbCN
and HbO₂, the equilibrium is somewhere in between that of HbCO and
methemoglobin. The β93 -SH conformational change can cause indirect
steric effect at the heme and distal histidine, presumably through the
anti-clockwise rotation of the F helix (upon ligand binding) as seen from the FG corner described by Perutz (61). The high absorption frequency (2592 cm\(^{-1}\)) of the \(\beta_93\) -SH of the hemoglobin in solution, rules out the possibility that -SH might form a hydrogen bond to the carbonyl group of F5 ser while in the non-polar tyrosine pocket.

The similarity of the \(\beta_93\) -SH absorption in \(\beta_4\) tetramer derivatives suggests two points: (1) Ligand related \(\beta_93\) -SH conformational change depends primarily upon tertiary structure associated with ligation at the heme. (2) The tertiary structure of the \(\beta_4\) tetramer in the vicinity of the \(\beta_93\) cysteine is similar to that of \(\alpha_2\beta_2\) tetramer.

The \(\beta-112\) cysteine sulfhydryl group at the \(\alpha_1\beta_1\) interface was found to be 3 times more reactive than the \(\alpha-104\) -SH.

The bovine methemoglobin tertiary conformation was found to be different from horse and human methemoglobin. This correlates with the fact that bovine hemoglobin differs from horse and human hemoglobin in DPG binding. It does not have the \(\text{NH}_2\) -terminal valine which is presumably essential for DPG binding. \textit{In vivo}, bovine hemoglobin does not bind DPG and has a much lower oxygen affinity than the other two hemoglobins.
SECTION VIII

(1) A BRIEF COMPARATIVE STUDY OF PURIFIED AND INTACT TISSUE CYTOCHROME OXIDASE CO BANDS*

(A) Introduction

Cytochrome oxidase, a mitochondrial enzyme which oxidizes ferro-cytochrome c and in turn itself oxidized by molecular oxygen, is a lipoprotein containing prosthetic groups of heme a and copper in an equimolar ratio. This copper heme protein of the inner mitochondrial membrane is the site of major oxygen utilization and an associated energy coupling for oxidative phosphorylation (25). Carbon monoxide has been an important inhibitory probe for the cytochrome c oxidase as well as other heme proteins (49, 85). As in most heme proteins, it is reasonable to assume that CO competes with oxygen for a common binding site. A better understanding may provide insight for oxygen binding.

Because of its membranous lipoprotein character, cytochrome oxidase is not readily purified in a water soluble form. Most purified preparations have been obtained extracting the membranes of heart mitochondria with bile salts or synthetic detergents. To maintain the solubility of the oxidase in aqueous solutions, detergents must always be present. An absorption band frequency of 1963.5 cm\(^{-1}\) (\(\Delta \nu_1=6\) cm\(^{-1}\)) had been reported for purified heart cytochrome c oxidase.

*Refer to the Method and Material Section for other workers involved.
(89). The CO stretching vibrational absorptions of cytochrome c oxidase in both purified wet pellet and intact heart tissues of bovine and rat were studied and compared.

(B) Results and Discussion

A single oxidase pellet (pH 7.4, 0.1 M potassium phosphate buffer) (Figure 27). The center frequency at 1963.3 ± 0.2 cm⁻¹ is approximately 100 cm⁻¹ lower than the frequencies noted for Cu(I) carbonyls (e.g. the hemocyanin (6 cm⁻¹) carbonyl (4)), and is comparable to that obtained by Yoshikawa, Caughey and et al., yet the half-bandwidth (4.8 cm⁻¹) is narrower than that reported (89). The unperfused beef-heart tissue shows a cytochrome oxidase CO band (1964.0 ± 0.2 cm⁻¹; Δν₁ = 3.4 ± 0.2 cm⁻¹) and a composite band at 1951 cm⁻¹ (Figure 28). By subtracting a bovine hemoglobin band (pH 6.0) from this composite band, a band with a center frequency (1944 ± 0.5 cm⁻¹) and half-bandwidth (9.5 ± 0.5 cm⁻¹) comparable to that of purified myoglobin was isolated (Figure 29). The resulting spectra (Figure 29) resemble all the features of that of the perfused rat heart tissue sample which were well free of hemoglobin (Figure 30). The composite band is therefore a combination of myoglobin and hemoglobin heme liganded CO stretching absorption. With the perfused rat heart tissue sample, an oxidase CO band at 1964.4 ± 0.2 cm⁻¹ (Δν₁ = 3.5 ± 0.2 cm⁻¹) and a myoglobin band at 1944.0 ± 0.5 cm⁻¹ (Δν₁ = 9.9 ± 0.5 cm⁻¹) were observed (Figure 30). The cytochrome to myoglobin ratio of 0.44:0.02 and 0.37:0.02 were estimated for perfused rat heart tissue and unperfused beef heart tissue respectively.
The CO stretch bands for CO liganded to purified cytochrome oxidase, unperfused beef heart tissue in CO saturated saline and perfused rat heart tissue in CO saturated isotonic buffer were observed. The absorption frequencies are rather similar, and half-bandwidths comparably the same. The much narrower half-bandwidth of cytochrome c oxidase CO than that of hemoglobin (7-8 cm\(^{-1}\)), could be interpreted as that the heme (heme A) environment around the oxidase bound CO is more restricted or non-polar than the one around protoporphyrin IX of hemoglobin.
Figure 27: The CO stretch band of beef heart cytochrome oxidase (reduced and with CO addition) in wet pellet. The spectra were collected at 1 cm$^{-1}$ resolution and signal averaged 2000 scans. A reduced sample without CO addition was used for protein subtraction.
Figure 28: Infrared absorption bands of carbon monoxide bound to hemeproteins in hemoglobin-containing beef heart. The beef heart spectrum was obtained by the spectroscopic methods described in the Method and Material Section.
Figure 29: Infrared absorption bands of carbon monoxide bound to heme-protein in unperfused bovine heart tissue obtained by subtracting a bovine hemoglobin band (pH 6.0), of 0.017, 0.018 and 0.019 mM heme concentration respectively, from the spectrum in Figure 28. The sample and reference spectra were collected at 1 cm$^{-1}$ resolution, and signal averaged 2048 scans for the sample and 1024 scans for the bovine hemoglobin sample.
Figure 30: Infrared absorption bands of carbon monoxide bound to heme-proteins in rat heart tissue. Rat heart was perfused with normal saline saturated with carbon monoxide gas. Fragments of ventricular tissue were pressed between CaF₂ windows and mounted in the infrared cell.
(2) A COMPARATIVE STUDY OF THE AMIDE I BAND OF HEMOGLOBIN DERIVATIVES IN D₂O

(A) Introduction

In pioneering days of Infrared Biospectroscopy, the Amide I and Amide II (1535 cm⁻¹) were useful for preliminary protein structural assignments. The amide I mode consists of the carbonyl stretching vibration with small contributions from the C-N-H in plane bending and the C-N stretching vibrations (33,53). The frequency of the amide I mode for the alpha helical conformation ranges from 1650-1657 cm⁻¹. The early work on polypeptides by Miyazawa had found that the amide I band of helical polypeptides had a strong intensity maximum at 1650 cm⁻¹ and a medium intensity maximum at 1652 cm⁻¹ (52). Disordered conformations (e.g. random coil) and beta sheets appeared to have an amide I apparent band frequency (1656 cm⁻¹) higher than that of alpha helical structures (53).

IHP had recently been shown to bind and shift HbNO to a T state structure (24,65), with the proximal Histidine Nε-Fe bond broken under the conformational stress arised from the R to T transition. A brief study of the amide I band of HbNO+IHP and various hemoglobins of known quarternary and spin states was done in D₂O solution in order to see any possible tertiary structural differences and amide I absorption mode patterns that were quarternary or tertiary structure related.
(B) Results and Discussion

The absorption spectra of various hemoglobin samples (HbCO, deoxyHb, HbO₂, azidoHb, HbCN and fluorometHb) in D₂O solution were obtained by signal-averaging 1024 times using D₂O as reference (Figure 31). A heme concentration of 3 mM and a pathlength range of 15-25 μ were chosen. The apparent amide I band frequencies (frequencies at the maximum band height) were listed in Table 12. All the amide I band frequencies (1694.4-1651.3 cm⁻¹) resembled that of alpha helical polypeptides. The HbNO+IHP had a slightly higher frequency than the other samples without IHP bound, which included both the R and T state hemoglobins. The tertiary structure of HbNO+IHP appeared to differ slightly from that of deoxyHb. The similarity of all the amide I band frequencies, however suggests that the overall amide I mode related tertiary structures were quite similar in all the hemoglobin derivatives studied.
Figure 31: Absorbance spectra of hemoglobin derivatives in D$_2$O.
Hemoglobin concentration of 3 mM heme and pathlengths of 1.5-2.5 μ were used.
<table>
<thead>
<tr>
<th>Hb Sample</th>
<th>$v_{\text{max}}$ in cm$^{-1}$ ± 0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (deoxy)$^\uparrow$ (High spin)</td>
<td>1649.7</td>
</tr>
<tr>
<td>HbF</td>
<td>1649.4</td>
</tr>
<tr>
<td>HbCN</td>
<td>1650.0</td>
</tr>
<tr>
<td>Hb$O_2$</td>
<td>1650.4</td>
</tr>
<tr>
<td>HbCO $^\uparrow$ (Low spin)</td>
<td>1650.6</td>
</tr>
<tr>
<td>Hb$N_3$</td>
<td>1650.6</td>
</tr>
<tr>
<td>HbNO</td>
<td>1650.8</td>
</tr>
<tr>
<td>HbNO+IHP (IHP modified)</td>
<td>1651.3</td>
</tr>
</tbody>
</table>
APPENDIX

Ghosts appearing as sine waves in the spectra due to interference fringes from the low pass optical filter and the detector window were of concern for sometime. These instrumental artifacts were found to be represented as secondary centerbursts in the interferogram before the fast Fourier transform. A nulling of the secondary centerbursts in digital space had successfully removed this type of periodic errors.

These instrumental artifacts and the effectiveness of its removal are demonstrated in Figure 32 and 33. Figure 32 shows an interferogram of signal-averaged 1024 scans and the appearance of a secondary and tertiary images of the primary centerburst upon an 8-fold expansion of the amplitude. Figure 33 shows a comparison of corrected and uncorrected infrared difference spectra in the SH absorbing region (2520 - 2620 cm⁻¹) of cytochrome oxidase (reduced, CO treated minus reduced). The periodical interference was removed in Figure 33. Figure 34 shows an amplified version of the corrected spectrum. The peak to peak noise level is approximately 0.0002 absorbance units with signal averaging of 1024 scans.
Figure 32:
Figure 32: FTIR interferogram after signal-averaging 1024 scans at 1.0 cm$^{-1}$ resolution. The upper trace shows the primary centerburst without an expansion in amplitude. The secondary and tertiary images of the primary centerburst became observable after an 8-9 fold scale expansion in amplitude as shown in the lower trace. The abscissa, R, indicates twice the distance from the stationary position in microns of the moving mirror of the interferometer.
Figure 33: FTIR difference spectrum in the SH stretching region of cytochrome oxidase (cyt ox-CO complex minus cyt. ox reduced; 0.3-0.4 mm light path). The spectrum was obtained at 2 cm\(^{-1}\) resolution and signal averaging of 1000 scans. Bottom scan is the uncorrected spectrum. Top scan is the same as the bottom scan except with the secondary interferogram shown in Figure 32 removed.
Figure 34: Corrected spectrum of Figure 33. (Top scan, frequency range between arrows) with amplitude expanded to show noise level. Mean peak to peak noise level is indicated by the span of arrows.
BIBLIOGRAPHY


42. Guidotti, G. and Konigsberg, W., (1964), J. Biol. Chem. 239, 1474.


