Differences in the Infrared Stretching Frequency of Carbon Monoxide Bound to Abnormal Hemoglobins*

Winslow S. Caughey,† James O. Alben,‡ Sue McCoy,† Samuel H. Boyer, Samuel Charache, and Peter Hathaway§

ABSTRACT: The infrared stretching frequencies for carbon monoxide (ν_{co}) bound to various hemoglobins and myoglobins have been determined. The identity of ν_{co} was confirmed by spectra of $^{12}C^{18}O$ and $^{13}C^{16}O$ derivatives. For human hemoglobins A, F, H, and Chesapeake and sheep hemoglobins A, B, C, and lamb, one narrow CO absorption was found near 1951 cm^{-1}. However, two absorptions of similar area were found for hemoglobin M$_{\text{Emory}}$ ($\alpha_2{}^{A}\beta_2{}^{63\ \text{Tyr}}$), at 1970 and 1950 cm^{-1}, and for hemoglobin Zurich ($\alpha_2{}^{A}\beta_2{}^{63\ \text{Arg}}$), at 1958 and 1951 cm^{-1}. Thus substitution for the amino acid at position 63 of the β chain (the position of the distal histidine in β^{A}) markedly affected the ν_{co} for the CO bound to that chain but no such effect was evident when the amino acid substitution was at a location more remote from the ligand. These data suggest significant participation of the β^{63} residue in CO binding. Myoglobins from horse heart and sperm whale exhibited maximum CO absorptions at about 1944 cm^{-1}, consistent with stronger CO binding than is the case for hemoglobin A. However, these results demonstrate that the ν_{co}, a parameter related to the strength of the iron to CO bond, does not necessarily parallel over-all O_2 or CO affinities.

In infrared spectra of human carbonyl hemoglobin A, both within or isolated from the red blood cell, a single narrow absorption band at 1951 cm^{-1} was found (Alben and Caughey, 1966, 1968). The assignment of this absorption to the stretching frequency (ν_{co}) of carbon monoxide was confirmed by shifts in frequency found with isotopically labeled carbon monoxide. Interpretations of these data included consideration of (1) the frequency, which was consistent with relatively strong bonding between CO and iron, (2) the presence of a single absorption band, which reflected an essentially equal strength of CO binding to each heme iron, and (3) the band width of only 8 cm^{-1} at half-peak height, which was indicative of a rather nonpolar environment. The present report of infrared spectra for Hb A and nine other hemoglobins and for two myoglobins considers effects of known changes in protein structure upon CO and O_2 binding, as measured by ν_{co}. Some of this work has appeared in preliminary reports (Alben and Caughey, 1966, 1968; Caughey *et al.*, 1968).

Experimental Section

Blood was obtained from carriers of Hb Chesapeake[1] (Charache *et al.*, 1966), Hb Zurich (Rieder *et al.*, 1965), and Hb H (Rigas *et al.*, 1956). In each case the cells were washed with isotonic saline solution and hemolyzed with carbon tetrachloride. The resulting Hb solutions, centrifuged free of cell debris, were applied directly to starch blocks for electrophoretic separation of the abnormal Hbs from the Hb A component. Electrophoresis was carried out at 4° and 8 V/cm. For separation of Hb H phosphate buffer, $\Gamma/2 = 0.10$ (or 0.05 M) (pH 6.9), was used. Under these conditions Hb H migrated toward the anode; Hb A moved toward the cathode. The other two abnormal Hbs were separated in 0.05 M barbital buffer, pH 8.6, where Hb Chesapeake moved faster and Hb Zurich slower than Hb A. In each case the bands were eluted from the starch block with the buffer used and the protein contained in the band was concentrated to about 10^{-3} M by ultrafiltration in dialysis tubing under reduced pressure.

To obtain Hb M$_{\text{Emory}}$ blood cells from a carrier[2] (Gerald and Efron, 1961) were washed with isotonic saline solution and hemolyzed with CCl_4. The resulting Hb solution was centrifuged free of cell debris, equilibrated with carbon monoxide, and added to a Bio-Rex 70 column in the phosphate buffer system described by Konigsberg and Lehmann (1965). The dark band containing Hb M$_{\text{Emory}}$ separated from the red band of CO Hb A. The proteins were eluted from the column and concentrated as described above.

Packed red cells or solutions of hemoglobin from

---

* From the Departments of Physiological Chemistry and Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received July 5, 1968.* This investigation was supported by U. S. Public Health Service Grants HE-06079, HE-02799-12, HD-02508, and GM-11067.

† Present address: Department of Chemistry, University of South Florida, Tampa, Fla. 33620.

‡ Present address: Department of Physiological Chemistry, The Ohio State University College of Medicine, Columbus, Ohio 43210.

§ Present address: Division of Human Genetics, The Mount Sinai Hospital, New York, N. Y. 10029.

[1] Abbreviations used are Hb, hemoglobin; Mb, myoglobin.

[2] We gratefully acknowledge the assistance of Dr. Benjamin Gendel of The Emory University School of Medicine, who located the patient of S. H. Jones, A. G. Churchwell, and A. H. Gaston (1961), quoted in Gerald and Efron (1961), and sent a sample of her blood to us.

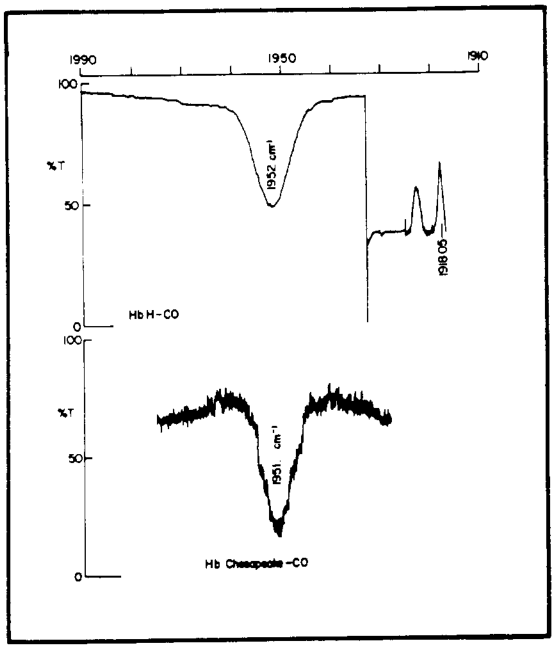

FIGURE 1: Infrared difference spectra of aqueous solutions of carbonylhemoglobin minus human oxyhemoglobin A and water vapor calibration peaks. Upper: Hb H. Lower: Hb Chesapeake.

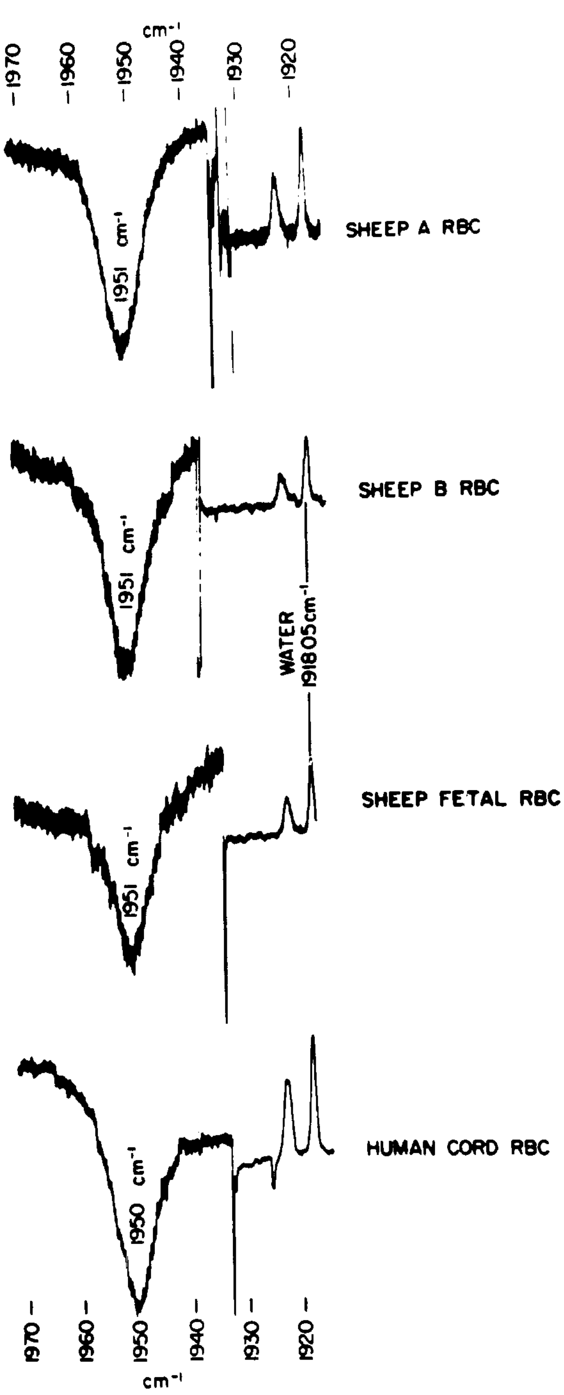

FIGURE 2: Infrared difference spectra of carbonylhemoglobin minus oxyhemoglobin A in packed red blood cells, with water vapor calibration peaks. Top to bottom: sheep Hb A, sheep Hb B, lamb Hb, and human cord Hb. The spectra of solutions of sheep Hbs A, B and C are not shown but were identical with these spectra.

homozygotes were used to obtain the spectra of the sheep and lamb hemoglobins (Boyer *et al.*, 1966).

Sperm whale and horse heart metmyoglobins were obtained from Mann Research Laboratories and were not further purified.

In each case the carbon monoxide complex was obtained by reducing the protein with sodium dithionite in a carbon monoxide atmosphere.

The infrared spectra were obtained as previously described for Hb A and normal human red cells (Alben and Caughey, 1968). As before, the CO Hb (or CO Mb) was placed in the sample cell and oxy Hb (or met Mb) was placed in the reference cell to give a CO Hb (or CO Mb) minus oxy Hb (or Met Mb) infrared difference spectrum. In many cases visible spectra for both carbonyl and oxy or Fe^{3+} protein derivatives were determined on a Cary 11 spectrophotometer both before and after the infrared spectrum was recorded. The use of CaF_2 windows allowed both the visible and the infrared spectra to be obtained on the same loaded cell. The visible spectra suggested that under these conditions even rather extended exposure to infrared radiation did not adversely affect the proteins.

Results

Several hemoglobins were examined which had no substitutions at the distal histidine in each chain. Data for normal human Hb A were reported earlier (Alben and Caughey, 1966, 1968). Spectra for carbonyl derivatives of Hb H ($\beta_4{}^A$) (Rigas *et al.*, 1956) and Hb Chesapeake ($\alpha_2{}^{92\ \mathrm{Leu}}\beta_2{}^A$) (Clegg *et al.*, 1966) are shown in Figure 1. The latter protein has an amino acid substitution (compared with Hb A) which is relatively remote from the heme. In Figure 2 similar spectra are recorded for human F (Schroeder *et al.*, 1963), sheep A, sheep B (Boyer *et al.*, 1967), and lamb hemoglobins (Wilson *et al.*, 1966), all of which have extensive amino acid replacements relative to the normal α and β chains of human Hb A but all of which contain both a proximal

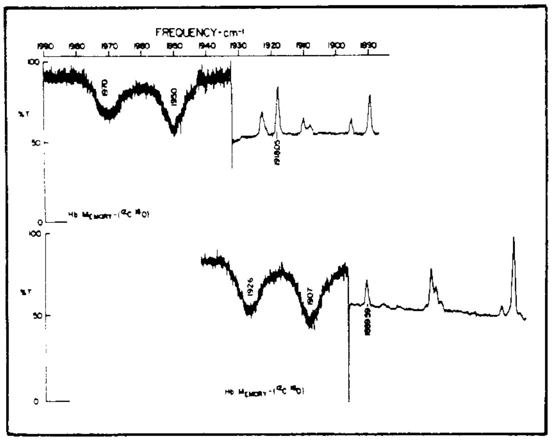

FIGURE 3: Infrared difference spectra of aqueous solutions of carbonylhemoglobin M_{Emory} *vs.* oxyhemoglobin A with water vapor calibration peaks. Upper: $^{12}C^{16}O$. Lower: $^{12}C^{18}O$.

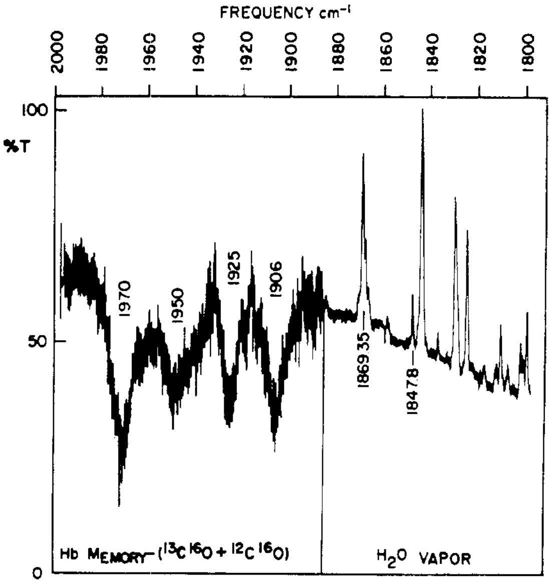

FIGURE 4: Infrared difference spectrum of aqueous solutions of $^{12}C^{16}O$ and $^{13}C^{16}O$ carbonylhemoglobin M_{Emory} *vs.* oxyhemoglobin A with water vapor calibration peaks.

and a distal histidine. Thus all of the hemoglobins studied which are known to have a distal histidine (as well as a proximal histidine) in both α and β chains exhibited ν_{co} values for bound $^{12}C^{16}O$ within 1 cm^{-1} of 1951 cm^{-1}.

Two of the hemoglobins studied here have an amino acid replacement in the β chains at position 63, occupied by the distal histidine in β^{A} chains. Carbonyl Hb M_{Emory} ($\alpha_2{}^{\mathrm{A}}\beta_2{}^{63\,\mathrm{Tyr}}$) (Gerald and Efron, 1961) spectra are shown in Figure 3. With $^{12}C^{16}O$ an absorption band appeared at 1950 cm^{-1} (attributable to the normal α-chain sites), together with a second absorption band of similar area at 1970 cm^{-1}. The 1970-cm^{-1} band is assigned to the CO bound to the abnormal β chains. Isotopic shifts in ν_{co}, which occur when $^{12}C^{18}O$ or nearly equimolar $^{12}C^{16}O$ and $^{13}C^{16}O$ are used (Figures 3 and 4), confirm the assignment of both peaks as ν_{co} bands. Spectra for Hb Zurich ($\alpha_2{}^{\mathrm{A}}\beta_2{}^{63\,\mathrm{Arg}}$) (Muller and Kingma, 1961) complexed with $^{12}C^{16}O$ and isotopically labeled CO appear in Figure 5. Here the 1951-cm^{-1} band was assigned to the normal α-chain sites and the 1958-cm^{-1} band to the abnormal β-chain sites.

The carbonyl derivative of myoglobin from sperm whale or horse heart both showed a major band at about 1944 cm^{-1}. These spectra are complicated by a second, smaller band (shoulder) at about 1935 cm^{-1}. The origin of this second band is unclear; experiments with electrophoretically homogeneous proteins are planned.

Discussion

The findings reported here are in accord with a direct interaction of the amino acid residue at β^{63} upon the carbonyl ligand bound to iron. With histidine at β^{63}, it can be inferred from X-ray studies (Kendrew, 1962) that hydrogen bonding or other interactions between this residue and bound CO are stereochemically reasonable. Such evidence is not presently available for the stereochemical relationships of the CO and the β^{63} residue with tyrosine or arginine at β^{63}. However, it is certainly reasonable to expect differences in interactions between the CO ligand and histidine, tyrosine, or arginine residues. The magnitude of the effects of β^{63} residues upon the ν_{co} values are sufficiently large to suggest an important contribution of the distal histidine to the binding

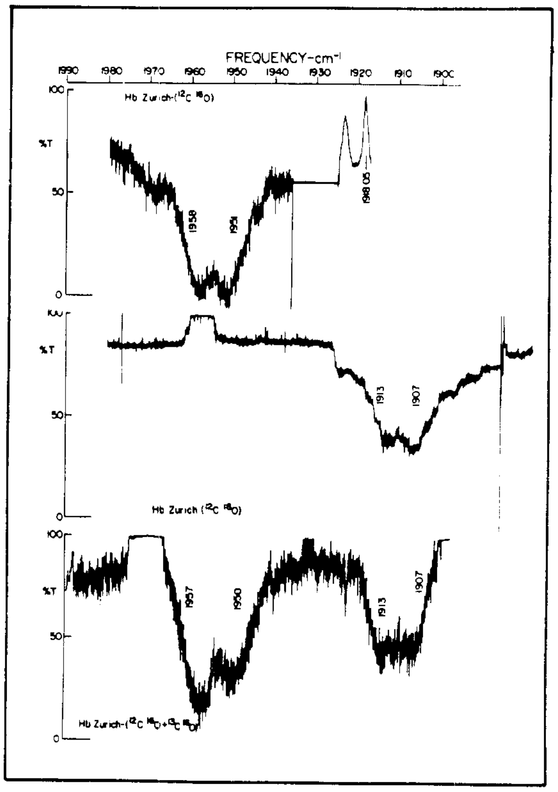

FIGURE 5: Infrared difference spectra of aqueous solutions of carbonylhemoglobin Zurich minus oxyhemoglobin A with water vapor calibration peaks. Upper: $^{12}C^{16}O$. Center: $^{12}C^{18}O$. Lower: $^{12}C^{16}O$ and $^{13}C^{16}O$.

of CO and, by reasonable analogy, the binding of O_2 and other ligands to hemoglobin and myoglobin (as has been suggested (Kendrew, 1962; Pauling, 1964; Caughey *et al.*, 1965)). However, it does not appear possible with present data to evaluate quantitatively the relative contributions of changes in strength of binding between ligand and iron and of changes in interaction between bound ligand and "solvent environment" to the observed ν_{co} differences.

Amino acid substitution at loci other than that of the distal histidine can also influence ν_{co}: sperm whale and horse heart myoglobins exhibited major ν_{co} values near 1944 cm^{-1}. Although similar in many respects to the α and β chains of Hb A, these myoglobins do contain extensive amino acid differences from Hb A.

No direct correlation between ν_{co} and the affinities for CO or O_2 was found. Many of the Hbs studied here differ markedly in O_2 affinity from Hb A (Charache *et al.*, 1966; van Vliet and Huisman, 1964; Benesch *et al.*, 1961) and yet have essentially the same ν_{co} value. The carbonyl stretching frequency is more closely related to the strength of the CO-to-iron bond *per se* than is the over-all affinity constant. While the affinity of a heme protein for CO is determined by enthalpic and entropic factors of all reactants and products involved in the equilibrium, the ν_{co} values reported here reflect only the condition of the ligand bound within the heme protein. Comparison of effects of amino acid substitutions upon ν_{co} values thus provides a means for evaluating effects of amino acid substitutions upon the *bonding* of CO at the heme site in contrast to effects of such substitutions upon the over-all affinities for CO. The lack of correlation between affinity and ν_{co} adds further support to the importance of such factors as changes in conformation (Muirhead and Perutz, 1963), α–β chain interactions (*e.g.*, Antonini *et al.*, 1965; Guidotti, 1967), and effects of 2,3-diphosphoglyceric acid (Benesch and Benesch, 1968), in addition to the strengths of ligand–heme binding, in the determination of over-all equilibrium constants for ligand binding.

References

Alben, J. O., and Caughey, W. S. (1966), *in* Hemes and Hemoproteins, Chance, B., Estabrook, R. W., and Yonetani, T., Ed., New York, N. Y., Academic, p 139.

Alben, J. O., and Caughey, W. S. (1968), *Biochemistry 7*, 175.

Antonini, E., Bucci, E., Fronticelli, C., Wyman, J., and Rossi-Fanelli, A. (1965), *J. Mol. Biol. 12*, 375.

Benesch, R., and Benesch, R. E. (1968), *Science 160*, 83.

Benesch, R. E., Raney, H. M., Benesch, R., and Smith, G. M. (1961), *J. Biol. Chem. 236*, 2926.

Boyer, S. H., Hathaway, P., Pascasio, F., Bordley, J., Orton, C., and Naughton, M. A. (1967), *J. Biol. Chem. 242*, 2211.

Boyer, S. H., Hathaway, P., Pascasio, F., Orton, C., and Bordley, J. (1966), *Science 153*, 1539.

Caughey, W. S., Alben, J. O., and Beaudreau, C. A. (1965), *in* Oxidases and Related Redox Systems, King, T. E., Mason, H. S., and Morrison, M., Ed., New York, N. Y., Wiley, p 97.

Caughey, W. S., Eberspaecher, H., Fuchsman, W. H., McCoy, S., and Alben, J. O. (1968), *Ann. N. Y. Acad. Sci.* (in press).

Charache, S., Weatherall, D. J., and Clegg, J. B. (1966), *J. Clin. Invest. 45*, 815.

Clegg, J. B., Naughton, M. A., and Weatherall, D. J. (1966), *J. Mol. Biol. 19*, 91.

Gerald, P. S., and Efron, M. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1758.

Guidotti, G. (1967), *J. Biol. Chem. 242*, 3673.

Kendrew, J. C. (1962), *Brookhaven Symp. Biol. 15*, 216.

Konigsberg, W., and Lehmann, H. (1965), *Biochim. Biophys. Acta 107*, 266.

Muirhead, H., and Perutz, M. F. (1963), *Nature 199*, 633.

Muller, C. J., and Kingma, S. (1961), *Biochim. Biophys. Acta 50*, 595.

Pauling, L. (1964), *Nature 203*, 182.

Rieder, R. F., Zinkham, W. H., and Holtzman, N. A. (1965), *Am. J. Med. 39*, 4.

Rigas, D. A., Koler, R. D., and Osgood, E. E. (1956), *J. Lab. Clin. Med. 47*, 51.

Schroeder, W. A., Shelton, J. R., Shelton, J. B., Cormick, J., and Jones, R. T. (1963), *Biochemistry 2*, 992.

van Vliet, G., and Huisman, T. H. J. (1964), *Biochem. J. 93*, 401.

Wilson, J. B., Edwards, W. C., McDaniel, M., Dobbs, M. M., and Huisman, T. H. J. (1966), *Arch. Biochem. Biophys. 115*, 385.