Spectroscopic Studies of the Nature of Ligand Bonding in Carbonmonoxyhemoglobins: Evidence of a Specific Function for Histidine-E7 from Infrared and Nuclear Magnetic Resonance Intensities†

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ABSTRACT: Infrared spectra of carbon monoxide ligated hemoglobins from human, horse, and rabbit donors have been examined. A single vibrational frequency at 1951 cm^{-1} is observed for CO bound to the heme in horse and human hemoglobins. Studies of the isolated α-CO and β-CO subunits of human hemoglobin reveal that the observation of a single frequency in the intact tetramer is the result of a superposition of the α-CO and β-CO vibrational frequencies. The apparent integrated absorption intensities of these CO vibrations are shown both to have values of 1.0×10^5 M^{-1} cm^{-2} within experimental error. For rabbit CO-Hb two vibrational frequencies appear (Caughey, W. S., et al. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 552) and are assigned to CO bound to the β (1951 cm^{-1}) and α (1928 cm^{-1}) subunits within the intact tetramer. The β-CO subunit exhibits both frequency and intensity similarities with horse and human hemoglobins. The rabbit α-CO subunit, however, exhibits a markedly lower frequency and much smaller intensity compared with the other CO-hemoglobins. These data are interpreted in terms of a specific role for the distal histidine (E7) in rabbit α subunits, in which this histidine functions as a nucleophilic donor to coordinated CO.

Carbon monoxide has proved to be a useful spectroscopic probe of the environment experienced by a ligand bound to the heme in proteins such as myoglobin and various hemoglobins. Both ^{13}C magnetic resonance (Moon & Richards, 1972, 1974; Matwiyoff et al., 1973; Moon et al., 1974) and infrared techniques (Alben & Caughey, 1968; Caughey et al., 1969; Maxwell et al., 1974) have been applied. The work of this paper concentrates on infrared studies of these interactions in which both the frequencies and intensities of the various absorptions have been determined for human, horse, and rabbit hemoglobins. These results are then discussed in conjunction with ^{13}C magnetic resonance observations with particular reference to the interaction between the distal histidine (E7) and bound ligands. This work suggests that the distal histidine acts as an electron donor to the heme bound CO.

Carbon monoxide bound to various heme proteins (Alben & Caughey, 1968; Maxwell et al., 1974) absorbs in the infrared region between 2000 and 1900 cm^{-1}, with nearly all normal hemoglobins exhibiting a single absorption within one or two wave numbers of 1951 cm^{-1}. Unlike ^{13}C magnetic resonance spectra, the IR absorptions for CO bound either to the α or to the β subunits of most hemoglobins are superimposed. Mutant human hemoglobins (Caughey et al., 1969; Caughey, 1970) and rabbit hemoglobin (Caughey et al., 1973; Matwiyoff et al., 1973) provide the only exceptions. In the human hemoglobin variants, Hb Zurich (β 63 E7 His → Arg) exhibits stretching frequencies for the CO ligands at 1958 (β) and 1951 (α) cm^{-1}, while COHb M Emory (β 63 E7 His → Tyr) absorbs at 1970 (β) and 1951 (α) cm^{-1}. Rabbit carbonmonoxyhemoglobin has absorptions at 1951 and 1928 cm^{-1}.

The substitution of histidine-E7 by some other residue not only alters the IR frequency for CO bound to this subunit but also changes markedly the chemical properties of the subunit. Hemoglobins in which histidine-E7 is replaced by tyrosine (M. Emory, M. Saskatoon, and M. Hamburg) suffer especially facile oxidation of the altered subunits to the ferric (met) state with a net loss in the oxygen carrying capacity of the protein. Not surprisingly, therefore, histidine-E7 on the distal side of the heme, in close proximity to the ligand binding site, is highly conserved in all normal mammalian hemoglobins (Dayhoff, 1976). One notable exception is opossum hemoglobin with His-E7 replaced by glutamine in the α subunits.

In spite of the dependence of physical and chemical properties on the nature of the distal residue E7, the exact role of this histidine in the function of normal hemoglobins remains undefined. Collman et al. (1976) have suggested that the primary role of amino acids lining the heme pockets and situated within a van der Waals radius of the bound ligand is to provide a steric destabilization of the bound ligand. Since the integrated intensities of ligand CO have been shown to be sensitive indicators of the extent of (metal) $d\pi \rightarrow (CO)\pi^*$ bonding (Darensbourg, 1972a,b), we have considered the intensities of the infrared absorptions together with the ^{13}C chemical shift data for carbonmonoxyhemoglobins (rabbit, horse, and human) in an attempt to define the role of histidine-E7.

Experimental Section

Hemoglobin Preparation. Samples of hemoglobins from horse, human, and rabbit (New Zealand white) donors were prepared from freshly drawn, citrated whole blood. The red cells were separated from the plasma by centrifugation at 2000 rpm and washed a minimum of three times with 0.15 M sodium chloride. Lysis was carried out with distilled water for 15–20 min at 4 °C. After removal of cell debris by centrifugation at 15 000 rpm for 1 h, the hemoglobin was purified by chromatography on a Sephadex G-75 (Sigma) column, equilibrated with 0.01 M phosphate buffer, pH 8.0. Next, samples were

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dialyzed for a minimum of 12 h against three changes of 6 L each of either 0.15 M NaCl (pH 6.8–7.3) or 0.15 M NaH_2PO_4 (pH 7.0–7.3). Following this initial preparation the hemoglobin was either concentrated by ultrafiltration (Amicon UM-10 or PM-10) and used for spectroscopic samples, or it was used for further experimental manipulations (vide infra).

Chain Separation. The α and β subunits of normal human hemoglobin were prepared as previously described (Geraci et al., 1969; Moon & Richards, 1974). The isolated, PMB labeled subunits were employed in the infrared spectroscopy experiments described later in this section. All experiments involving the isolated chains were initiated within 6 h from the time that the chains were eluted from the cellulose column.

Exhaustive Deuteration. In order to exhaustively deuterate protons in the heme pocket the heme group was extracted from rabbit hemoglobin and the apoprotein maintained in twice distilled D_2O (99.8% Stohler) for up to 48 h prior to reconstitution with heme. The method employed for apohemoglobin formation was that of Rossi-Fanelli et al. (1958). Extreme care was taken to assure that all manipulations involving the protein were carried out at 0 °C. Reconstitution of the protein with hemin chloride (Sigma) was accomplished by the method of Winterhalter & Huehns (1964). The ferric protein was reduced with sodium dithionite (Baker) and ligated with carbon monoxide (Matheson, CP). The sample was maintained in D_2O/0.15 M NaCl throughout this process and prior to spectroscopic experiments it was pressure dialyzed with three times its volume of D_2O/0.15 M NaCl to remove extraneous ions generated in the reduction procedure.

Sample Preparation for Infrared Spectroscopy. Depending upon the conditions under which the IR spectra were to be obtained, the hemoglobin was prepared in different ways. Following the column purification and dialysis described above, the hemoglobin solution was concentrated by ultrafiltration (Amicon UM-10, PM-10). Initially the infrared spectra were collected from samples of hemoglobin-CO in H_2O/saline or H_2O-phosphate buffer at regulated pHs using the difference spectra technique of Caughey et al. (1974). However, we soon found it suitable to collect spectra in D_2O/saline or D_2O/deuterium phosphate buffer with only the requirement of a cell window/D_2O reference sample. Preparations of samples in D_2O/saline was accomplished by pressure dialysis of the hemoglobin solution in the ultrafiltration device with D_2O/saline. Deuterium phosphate buffered solutions were obtained in the same manner using NaD_2PO_4/D_2O buffer, NaD_2PO_4 was obtained by several cycles of dissolving NaH_2PO_4 (Mallinckrodt) in D_2O followed by lyophilization. The pH of these solutions was adjusted with NaOD obtained by dissolving sodium metal (Alpha) in D_2O.

Once the correct solvent conditions for the hemoglobin samples had been met, they were deoxygenated by successive flushing with prepurified nitrogen gas (Linde). Carbon monoxide ligated hemoglobin was obtained by exposing the unligated Hb to CO gas. In certain cases quantitative deoxygenation was determined by the criteria at Benesch et al. (1965). After the formation of carbonmonoxyhemoglobin, the concentration was redetermined from the extinction coefficients of the characteristic CO-Hb Soret band.

Infrared Spectra. Infrared spectra were obtained using a Beckman IR-4240 spectrometer interfaced with a Fabri-Tek 1062 Instrument computer. Spectra were collected from single scans for the purposes of integration and comparison with other samples, and from multiple, signal averaged scans when peak detection was critical. All spectra were obtained at a resolution of 1.5 cm^{-1} and the sample temperature in the beam path was regulated at 29 ± 1 °C by means of a cooled compressed air stream impinging upon the sample.

Because of the nature of this study, extreme caution was taken during preparation of the NMR and IR Hb-CO samples. The samples of Hb-^{12}CO or Hb-^{13}CO in gas tight syringes were taken into an inert atmosphere glove box. Infrared samples were prepared from disassembled cell holders (Beckman), polyethylene cell windows, and lead spacers whose thickness was predetermined by micrometer measurements. After construction the sample cells were sealed with Apiezon W grease to prevent gas exchange with the atmosphere. The samples were used immediately after preparation.

NMR samples were made in a similar manner. NMR tubes were flushed with prepurified nitrogen to remove all traces of oxygen, sealed, and were then taken into the glove box. The hemoglobin-^{13}CO solution was introduced into the tubes by syringe. The ^{13}C NMR spectra were obtained using the pulse Fourier transform mode on a Varian XL-100-15 spectrometer equipped with a Varian 520/i computer. Spectra were accumulated at 32 °F using a 3500-Hz bandwidth and calibrated with an external reference.

The effect of an inert atmosphere upon the tendency of CO to dissociate from hemoglobin was determined by varying the composition of the inert atmosphere within the glove box. It was found that using prepurified nitrogen, argon, or carbon monoxide as the internal atmosphere caused no alteration in the relative intensities of α-CO and β-CO vibrations in rabbit Hb-CO, *as long as oxygen was excluded.* Moreover, despite the demonstrated lability of the α-CO subunit of rabbit hemoglobin-CO (vide infra), prolonged exposure of rabbit Hb-^{13}CO samples to the nitrogen atmosphere of the glove box (up to 1 h) revealed no changes in the extent of ligation as determined by both NMR and IR. For these reasons nitrogen was generally used as the internal glove box gas.

Gaseous Exchange Experiments. Gas tight syringes loaded with 15 cm^3 of Hb-CO (rabbit) at known concentrations were exposed to 35 cm^3 of gases potentially capable of exchanging with coordinated CO. In separate experiments the gases oxygen (Matheson) and the atmosphere were added to samples of Hb-^{12}CO. In another experiment ^{12}CO was used to exchange with Hb-^{13}CO. The time course of the exchange reactions was monitored by IR spectroscopy.

Integration to Determine Apparent Intensities. The apparent integrated absorption intensities, B, were calculated from eq 1 and are reported in units of 10^4 M^{-1} cm^{-2} as suggested by Brown & Darensbourg (1967). Infrared peaks from single scans of the CO stretching region were used for integration to determine the apparent intensities. The spectra were obtained under the conditions previously described using an expanded horizontal scale and scan speeds of 2, 5, or 20 cm^{-1}/min. Spectra obtained at any of these speeds were superimposeable with all others. Peaks were repeatedly integrated either graphically or by weight and the averaged values employed in the calculations were determined to be reproducible within 5%. Moreover, values calculated from different samples of the hemoglobin from the same species were found to agree within 10%. The weights of the integrated peaks were converted to the required units by separate determinations of the weight per cm^2 of the paper.

Results

The integrated absorption intensity, which is proportional to the area under the absorption band, provides another useful parameter in understanding the nature of an infrared absorption in addition to the more common spectral parameters of frequency and line width. Table I collects these data for rabbit, horse, and human carbonmonoxyhemoglobins as well

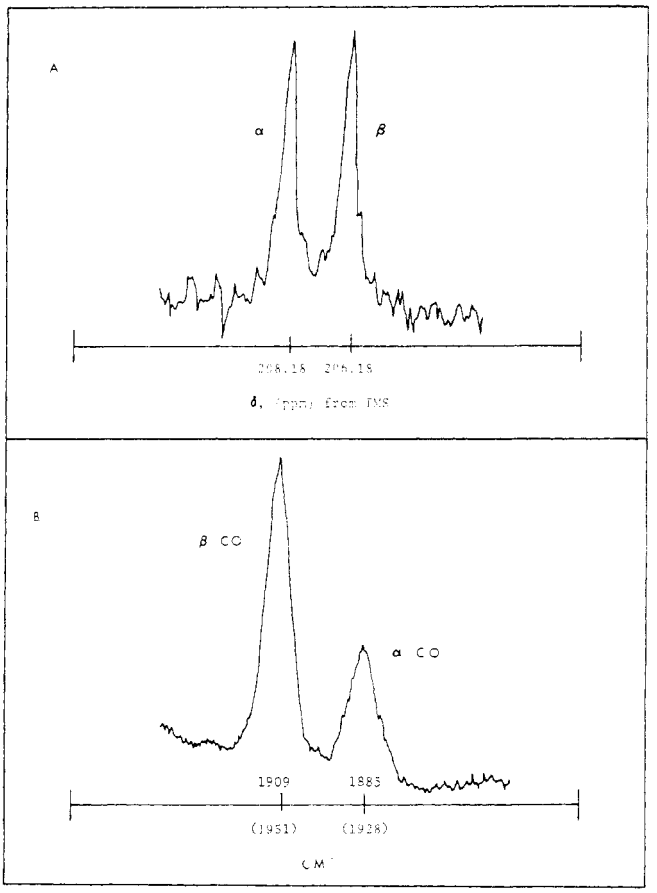

FIGURE 1: Representative ^{13}C NMR (A) and IR (B) spectra of rabbit Hb-^{13}CO. These spectra originate from a single sample of hemoglobin. The absorptions of equal intensity in the NMR spectrum indicate that both α and β subunits are ligated to the same extent. The infrared spectrum shows the carbon monoxide stretching frequencies of ^{13}CO bound to the α and β subunits. The corresponding frequencies for ^{12}CO are given in parentheses.

as for human isolated, PMB-labeled α-CO and β-CO chains. The values of the apparent integrated absorption intensity, B, presented in this table are approximately three times greater than those previously determined for heme proteins (Volpe et al., 1975). We reproducibly determine the values of B given in Table I regardless of variations in cell composition, pathlength, or protein concentration. At this time we can offer no specific rationalization for these discrepancies, although variations in sample preparation and instrumentation may account for them. For the purposes of this paper we note that the conclusions presented herein are based primarily on deviations in relative intensities and are not critically dependent upon the absolute value of B.

Rabbit hemoglobin is unusual not only in displaying two frequencies (1951 cm^{-1} and 1928 cm^{-1}) but also in that the total integrated intensities of these two rabbit absorptions is only three-quarters that of the total intensity from human Hb-CO. This difference lies entirely in the low intensity of the band at 1928 cm^{-1} (the band at 1951 cm^{-1} in rabbit Hb-CO seems normal having one-half the intensity of human Hb-CO, which represents the sum of α and β subunits with bound CO). It has previously been stated that individual rabbits display a marked variability in the relative intensities of these vibrational bands (Caughey et al., 1973). The six rabbits used in this study came from a colony of strain pure New Zealand white rabbits and no variability in the relative IR intensities of the CO bands was observed.

TABLE I: Stretching Frequencies and Apparent Integrated Absorption Intensities (B) of Heme Bound Carbon Monoxide in Human, Horse, and Rabbit Hemoglobins and in the Isolated, PMB-Labeled Subunits of Human Hb-CO.

Temp (°C)	Sample[a]	Heme concn. (mM)	B $\times 10^4$ M^{-1} cm^{-2})	ν^b (C-O) (cm^{-1})
29	Human	10.0	10.4	1951
29	Human βPMB chains	9.3	9.7	1951
29	Human αPMB chains	10.8	9.3	1951
29	Horse	4.4	9.9	1951
29	Rabbit	7.5	10.7(β)	1951
			5.1(α)	1928

[a] Hemoglobin samples constructed in inert atmosphere glove box. [b] Values reported are accurate and reproducible to ± 1 cm^{-1}.

Figure 1A shows the ^{13}C NMR spectra of ^{13}CO ligated rabbit hemoglobin, whose resonances have been assigned (Moon & Richards, 1974), and Figure 1B shows the infrared spectrum of rabbit Hb-^{13}CO prepared from a portion of the same sample. Specific assignments of these resonances are based on the observation that the smaller of the two peaks (1928 cm^{-1}) decreases preferentially when O_2 is added to the sample (caused by displacement of CO by O_2 preferentially from the α subunits (Moon & Richards, 1974)). Also, α chains of rabbit hemoglobin are known to be oxidized almost completely before oxidation of rabbit β chains (Moon & Richards, 1974; Matwiyoff et al., 1973). After partial oxidation of rabbit Hb followed by ligation with CO, the smaller absorption at 1928 cm^{-1} (1885 cm^{-1} with ^{13}CO) is absent. In the NMR, the lower field (α-^{13}CO) resonance is likewise absent after such oxidation.

Comparison of the infrared and NMR spectra in Figure 1 confirm that both subunits are equally ligated. The NMR spectrum shows that the ratio of α-^{13}CO to β-^{13}CO is 0.97 to 1.00 while, for the same sample, the ratios of infrared intensities for α-^{13}CO to β-^{13}CO are 0.47 (α) to 1.00 (β). To eliminate the possibility that the lower intensity for the α-CO absorption was due to a preferential dissociative loss of CO from the α-heme by photolysis in the IR beam while recording the spectra, a variety of neutral density filters and a red filter were independently interposed between the IR beam and the sample. In all cases the positions of the absorptions and their relative intensities were unchanged.

Figures 2 and 3 show, qualitatively, the effect on rabbit Hb-CO of exposure to gases which compete with the heme coordination site. Figure 2 shows the exchange of ^{12}CO for ^{13}CO; the half-life for this exchange is about 2 h. Figure 3 shows the exchange of rabbit Hb-^{12}CO with excess oxygen; only the α-ligand exchanges, with a half-life of approximately 2 h.

To confirm that the anomalous IR spectrum of rabbit hemoglobin was not the result of the manner in which the protein had been treated during isolation, the IR spectrum of Hb-CO in intact rabbit erythrocytes was obtained. The spectrum was found to be identical with that observed for purified rabbit Hb-CO.

For rabbit and human Hb-CO, the CO stretching frequencies and relative intensities are unaffected by changing solvent from H_2O to D_2O. There is no change in these frequencies even when deuterium exchange involves (i) removal of the heme group from tetrameric rabbit hemoglobin,

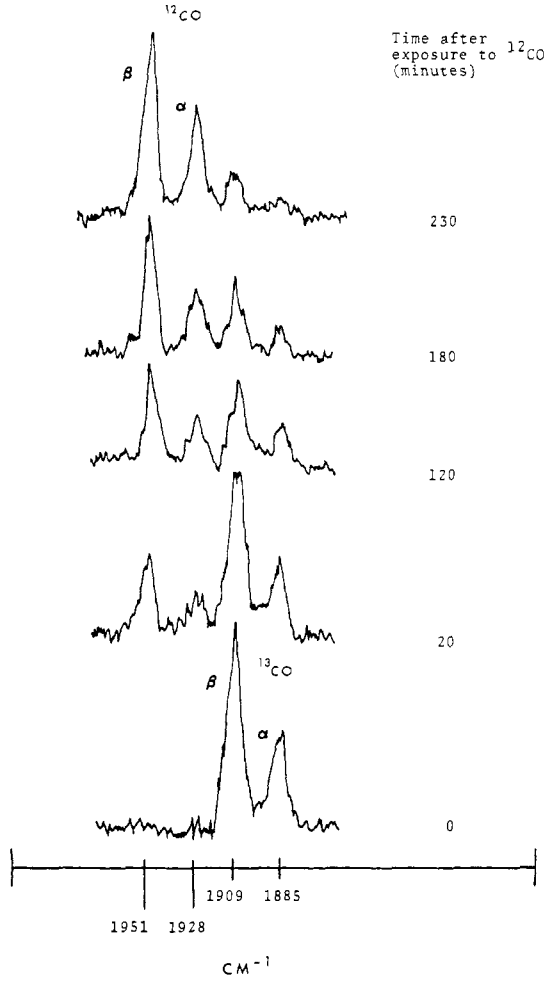

FIGURE 2: Spectra of rabbit Hb-^{13}CO in the presence of excess ^{12}CO gas. These spectra reveal the time course of CO exchange. Spectra were obtained from a sample of hemoglobin prepared in 50% D_2O, 0.10 M Tris buffer at pH 7.8.

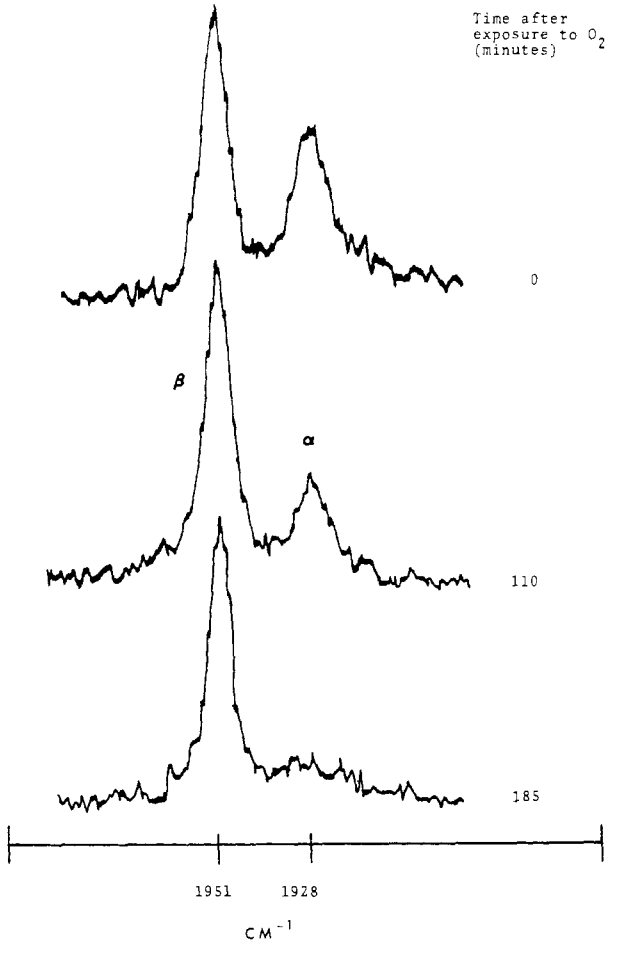

FIGURE 3: Spectra of rabbit Hb-^{12}CO in the presence of excess O_2 gas. These spectra show that only the α subunits exchange CO for O_2 over the time period that this reaction was followed. Experimental conditions were the same as described in Figure 2.

(ii) exchange of the apoprotein with D_2O for up to 48 h at pH 5.8, (iii) reconstitution with heme, and (iv) formation of Hb-CO.

Finally, we have observed no pH dependence of the CO stretching frequencies for rabbit Hb-CO in the range pH 6 to 9.

Discussion

Interactions of Bound Ligand. A ligand bound to hemoglobin is directly bonded to the iron atom of the heme (Antonini & Brunori, 1971; Perutz, 1976; Norvell et al., 1975; Huber et al., 1970; Heidner et al., 1976). In addition to this primary bond, the possibilities of other interactions between bound ligand and amino acid residues particularly on the distal side of the heme (that side to which the ligand is attached) have been extensively discussed (Caughey et al., 1969, 1973, 1975; Collman et al., 1976; Barlow et al., 1976). Calculations from the atomic coordinates in horse Hb-CO (Heidner et al., 1976) and from the Watson-Kendrew atomic coordinates in myoglobin (Antonini & Brunori, 1971; Watson & Kendrew, 1961) reveal that nitrogen $N_{\epsilon 2}$ of the imidazole ring of the distal histidine residue (E7) approaches well within the minimum van der Waals radius required for contact with heme ligands, providing, thereby, the potential for an interaction between histidine-E7 and the heme bound ligand. This type of interaction may be of considerable importance in the optimal functioning of these proteins for the transportation and storage of oxygen. The possibility of such an interaction is reinforced by the observed sensitivity of the CO stretching frequency to the nature of the amino acid residues at E7 in contrast to the relative insensitivity of this parameter to amino acid substitutions elsewhere in the globin molecule (Caughey et al., 1969; Caughey, 1970).

Possible interactions between a suitably oriented distal histidine and a heme bound ligand such as CO or O_2 include: (1) Hydrogen bonding with histidine E7 has been postulated (Stryer et al., 1964) and was originally thought to occur in metmyoglobin azide (Watson & Kendrew, 1961). Figure 4A depicts such a bond. (2) A dipole–dipole interaction between the molecular dipole moments of the distal residue and the heme ligand has been considered (Barlow et al., 1976). (3) Steric interference between bulky amino acid side chains in the heme pocket and ligand has been advanced (Collman et al., 1976) as the primary function of distal residues. (4) The possibility of a donor:acceptor interaction, involving donation of electrons from the distal histidine to the ligated CO (Figure 4B) has been postulated by Caughey (1970).

Hydrogen Bonding. If there were hydrogen bonding between histidine-E7 and CO, one could expect a change in the spectral characteristics of bound CO on exchange of deuterium for hydrogen. Such behavior would be analogous to the solvent dependence of carbonyl stretching frequencies (Hallam, 1963) and to the known shift of ν(CO) when deuterium is substituted for protium in a hydrogen bond (Venkata & Bai, 1973). One

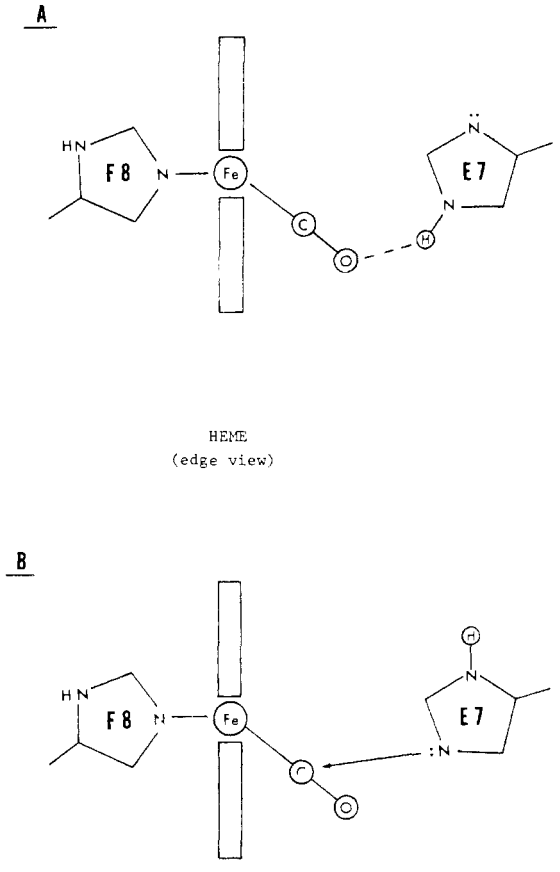

FIGURE 4: Two possible modes of interaction between the distal histidine (E7) and heme bound CO. (A) Hydrogen bonding and (B) nucleophilic interaction.

might also anticipate a pH dependence of ν(CO) if the proton involved in such a hydrogen bond could ionize, leading, therefore, to a pH dependence of ν(CO).

Indeed, such behavior has been observed in studies of carbonyl horseradish peroxidase (Barlow et al., 1976) where a pH-dependent shift in the CO stretching frequency of about 40 cm^{-1} was observed. In contrast with these results, there is no pH dependence for the azide stretching frequency in azidomethemoglobin (McCoy & Caughey, 1970) and metmyoglobins (McCoy & Caughey, 1971). Also, the vibrational frequencies of heme bound CO in myoglobin (McCoy & Caughey, 1971) and rabbit hemoglobin (vide supra) are independent of pH. Moreover, we have observed no change in the stretching frequency of bound CO even after exchange of deuterium for hydrogen in the rabbit apoprotein for some hours. Lastly, the chemical shift of heme bound ^{13}CO in various hemoglobins and myoglobins does not change over the pH range from 6.0 to 10.0. These results all argue against the presence of a hydrogen bond between histidine-E7 and ligand. Nor does the most precise structural information, a neutron diffraction study of myoglobin-CO (Norvell et al., 1975) support such a hydrogen bond.

Utilization of Infrared Intensity Data to Interpret the Spectrum of Rabbit Hb-CO. A basis for interpreting the infrared spectrum of rabbit Hb-CO in terms of specific interaction between heme bound CO and histidine-E7 arises from consideration of integrated peak intensities together with stretching frequencies. Previous work has relied upon CO stretching frequencies to describe the heme environment. However, the apparent integrated peak intensity can provide much useful information (Darensbourg, 1971, 1972a,b; Braterman, 1975, 1976; Brown & Darensbourg, 1967). The apparent integrated absorption intensity is defined by eq 1

$$B = 1/Cl \int \log(I_0/I) d\nu \quad (1)$$

where C = heme-CO concentration, l = sample path length, and the integral is taken over all frequencies of the absorption. For our purposes, the importance of this parameter is its relationship to the square of the dipole moment matrix element (eq 2) (Hornig & McKean, 1955; Barrow, 1975; Levine, 1975). This relationship shows that the intensity, B, increases with the square of the matrix element of the

$$B \propto |\langle m|d|n\rangle|^2 \quad (2)$$

operator d (the transition moment, which links vibrational states described by wave functions m and n). For vibrational transitions, the specific form of the operator is taken as a series expansion of the molecular dipole, expanded around the equilibrium molecular geometry; the terms of this expansion describe changes in the dipole moment of the molecule during the vibration which contribute to the transition moment. Thus the intensity of an infrared absorption is related to the change in dipole moment in the CO group.

Previous work has shown (Darensbourg, 1972) that both the intensities and frequencies of carbonyl stretching bands in the IR spectra of transition metal carbonyls are sensitive to the mode of metal ligand bonding. For example, σ-bonded carbonyls exhibited CO stretching frequencies greater than 2175 cm^{-1} accompanied by low intensities (less than 1×10^4 M^{-1} cm^{-2}) whereas π bonded carbonyls have frequencies lower than 2175 cm^{-1} and larger intensities (up to 73×10^4 M^{-1} cm^{-2}). The origins of this effect have been previously described (Darensbourg & Brown, 1968; Braterman, 1976; Abel et al., 1969; Bigorgne & Benliau, 1967). The intensities of infrared bands are generally thought to be due to orbital following which is a consequence of back bonding. One may qualitatively visualize this effect as a lowering in the CO ($2\pi^*$) orbital during stretching with a resultant increase in Fe-CO bonding. The consequent dynamic fluctuation in back bonding produces an oscillating electric dipole which in turn gives rise to the observed intensity enhancements.

The IR spectrum of CO bonded to the α subunit of rabbit hemoglobin is anomalous in two respects; compared with human α-CO and β-CO and rabbit β-CO, the frequency of rabbit α-CO is shifted 23 ± 2 cm^{-1} to lower frequencies and the intensity is lower by about a factor of two (Table I). One might consider that the lower frequency of the rabbit α-CO vibration arises as the result of increased Fe→CO bonding in the α subunit. However, such an increase in π bonding should result in an increased intensity for the absorption whereas in the present case the intensity is markedly reduced.

Three possible explanations for the simultaneous lowering of both frequency and intensity exist. We believe the first of these to be the most plausible.

1. *Nucleophilic Interaction.* This effect, first proposed by Caughey (1970), is illustrated in Figure 4B and involves donation of electron density from a nitrogen of the distal histidine to the carbon atom of the heme coordinated CO. Such an interaction seems plausible if one considers the probable ground state charge distribution within the Fe-CO unit. For a variety of metal carbonyls, significant amounts of positive charge have been calculated to be localized on the carbonyl carbon atoms (Fenske & DeKoch, 1970; Caulton & Fenske, 1968; Schreiner & Brown, 1968; Carroll & McGlynn, 1968). Indeed nucleophilic attack on such an electrophilic carbon has been observed in transition metal carbonyls (Darensbourg & Darensbourg, 1970), and in ferric porphyrin-bis(cyanide) complexes (La Mar, 1977).

Such nucleophilic donation to the carbon of the liganded CO group should: (i) lower the permanent dipole moment of the CO by decreasing the positive character of the carbonyl carbon with a concomittant decrease in the magnitude of the *change* of the dipole moment during vibration, resulting in a lowered intensity for the absorption; (ii) directly decrease the change in dipole moment during vibration by introducing an additional interaction potential field such that stretching of the FeCO unit will less appreciably lower the CO(π^*) orbital, which will thereby diminish the oscillating dipole moment by decreasing the orbital following; (iii) lower the stretching frequency as a result of the increased electron density in the CO(π^*) orbital, thereby weakening the CO bond.

2. *Bending in the Fe-CO Unit.* Distortion of the Fe-CO unit from a linear geometry could account for the differences in stretching frequencies (Caughey et al., 1973), although the quantitative relationship between Fe-CO angle and CO stretching frequency has yet to be established. However, as σ and π overlap between Fe and CO are maximal in a linear Fe-CO group, bending the CO group, in the absence of rehybridization of the carbonyl carbon, should decrease Fe-CO σ and π overlap with a resultant increase in ν(CO) and a decrease in the intensity of the absorption.

3. *Trans Effect.* Change in ν(CO) caused by some perturbation in the proximal histidine (F8), though possible, seems improbable as the explanation for these observations, essentially because only small changes in ν(CO) result from relatively radical changes in the trans ligand. For example, in model complexes of ferrous protoporphyrin (IX) dimethyl ester with CO as an axial ligand, changing the other, trans, axial ligand from a hydrazone to pyridine to 1-methylimidazole caused ν(CO) to vary by only 10 cm^{-1} (Caughey et al., 1973). Thus, the possible structural modifications available to histidine-F8 seem by themselves unlikely to be able to alter ν(CO) by the 23 $\pm$ 2 cm^{-1} observed for rabbit α-CO.

Steric Effects and Coordination in the Heme Pocket. Heme ligand binding sites in hemoglobins and myoglobins appear to have evolved specifically to accommodate nonlinear ligands such as O_2. Indeed, the suggestion has been made (Collman et al., 1976) that the primary function of the amino acids lining the distal side of the heme is to provide a sterically crowded environment, which would lower the carbon monoxide affinity by distortion of the preferred linear Fe-CO geometry; this distortion would lead to a correlation between lower ν(CO) with decreased CO affinity.

Our experiments on gas exchange of rabbit Hb-CO support such a correlation. Figure 2 shows that $t_{1/2}$ for exchange of rabbit Hb-^{13}CO with ^{12}CO is about 2 h and that the rate of this exchange is the same for both subunits. Thus the dissociation rates of CO from either the α or β subunit are essentially the same. In contrast, when the exchange is between CO and O_2 the α subunits demonstrated a markedly lower thermodynamic preference for CO relative to O_2 when compared with the β subunits. In human hemoglobin, the α and β subunits have approximately the same thermodynamic preferences for CO relative to O_2. One can interpret these results to indicate that the α subunit of rabbit hemoglobin in a completely ligated tetramer has an anomalously low affinity for CO which is reflected in the anomalously low ν(CO) for rabbit α(CO). Moreover, crystallographic data provide evidence for distorted Fe-CO units, further substantiating the view that the binding pocket on the distal face of the heme is constructed so as to require ligands to bind with a nonlinear geometry.

However, other interactions between ligand and distal residues, in addition to those of simple steric crowding, seem also to be important. For example, changes in ν(CO) result from substitutions of the distal histidine by arginine in hemoglobin Zurich ($\Delta\nu$CO $-$ 8 cm^{-1}) and by tyrosine in hemoglobin M Emory ($\Delta\nu$CO $-$ 18 cm^{-1}). Such changes suggest the importance of interactions between ligand and the E7 residue more specific than those caused by the sheer bulk of the side chain residues.

It is further suggested that the higher CO stretching frequencies in Hb Zurich and Hb M Emory are the result of a decrease in electron donation to the CO caused by replacement of the distal histidine. To support this we call attention to the fact that Perutz & Lehmann (1968) have shown that arginine-E7, in the mutated subunit of Hb Zurich, cannot be physically accommodated within the heme pocket. Moreover, the charged guanidinium moiety of this amino acid would surely seek an aqueous environment. For these reasons the arginine-E7 is most likely to be exposed to the outside solution in an external position, removing any interaction with ligated CO. It is also likely that tyrosine-E7 in the mutant subunits of Hb M Emory would seek an aqueous environment due to its polarity and ionizability.

The view that higher CO frequencies correlate with a decrease in nucleophilic donation from residue E7 is further corroborated by infrared studies of separate single chain hemoglobins from the species *C. thummi thummi* and *G. dibranchiata*. *C. thummi thummi* hemoglobin-CO displays a stretching frequency of 1964 cm^{-1} (Wollmer et al., 1977). It is known from crystallographic data that the histidine-E7 is not in its customary position inside the heme pocket, but lies in an external position, effectively removed from any interaction with the ligated CO. *G. dibranchiata* hemoglobin-CO has a stretching frequency of 1970 cm^{-1} (Satterlee et al., 1978) and does not have a distal histidine at position E7.

Cause of Anomalous Behavior of Rabbit α Chains. Rabbit hemoglobin contains 39 substitutions compared with human hemoglobin; 25 of these appear in the α chain (von Ehrenstein, 1966; Braunitzer et al., 1966, 1968; Best et al., 1968). Most of these substitutions are between amino acids of similar size and they are probably of relatively minor functional importance. Two substitutions in the CD region seem to be of special significance (Moon & Richards, 1974); Phe for Leu (CD6) and Thr for Ser (CD7) are unique to rabbit α chains. Because of the substitution at CD6, the rabbit α chain has three phenylalanines in the C/D region (CD1,4,6) which might sufficiently modify the tertiary structure around the distal face of the heme to allow the enhanced interaction between the distal histidine and the CO ligand. Such an explanation would predict that the strength of this interaction should be inversely related to the affinity for CO and to the infrared stretching frequency of bound CO; this interaction should therefore be in the order rabbit α > human, horse α,β; rabbit β > *C. thummi thummi* (external distal histidine). The observed IR frequencies 1928 cm^{-1} (rabbit/α), 1951 cm^{-1} (human, horse/α,β, rabbit/β) and 1964 cm^{-1} (*C. thummi thummi*) (Wollmer et al., 1977) accord with this expectation.

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