# CO and $O_2$ Complexes of Soybean Leghemoglobins: pH Effects upon Infrared and Visible Spectra. Comparisons with CO and $O_2$ Complexes of Myoglobin and Hemoglobin†

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ABSTRACT: The effects of pH upon infrared spectra [CO stretching frequency ($\nu_{CO}$) region] and visible spectra of the CO complexes of soybean leghemoglobins *a*, $c_1$, and $c_2$, sperm whale myoglobin, and human hemoglobin A are reported. The $\nu_{CO}$ for leghemoglobin–CO complexes was 1947.5 $cm^{-1}$ at neutral pH. At acid pH myoglobin– and hemoglobin–CO complexes developed $\nu_{CO}$ bands at 1966–1968 $cm^{-1}$, whereas leghemoglobin–CO complexes developed $\nu_{CO}$ bands at ~1957 $cm^{-1}$. All $pK_{app}^{CO}$ values determined by pH-dependent variation of $\nu_{CO}$ fell in the range 4.0–4.6. The $pK_{app}^{CO}$ values determined from visible spectra were consistent with $\nu_{CO}$-determined values except for that of myoglobin–CO (visible $pK_{app}^{CO}$ = 5.8). The $pK_{app}^{CO}$ values in the 4.0–4.6 range appear to be p*K* values of the distal histidines, while the visible $pK_{app}^{CO}$ of myoglobin–CO appears to be the p*K* of a group other than the distal and proximal histidines. The data are consistent with a model in which protonation of the distal histidine permits protein-free heme FeCO geometry in leghemoglobin–CO complexes but not in myoglobin– or hemoglobin–CO complexes. Thus the heme pockets of leghemoglobins appear to be more flexible than the heme pockets of myoglobin and hemoglobin. The effects of pH upon visible spectra of the $O_2$ complexes of soybean leghemoglobins *a*, $c_1$, and $c_2$, sperm whale myoglobin, and human hemoglobin A also are reported. $pK_{app}^{O_2}$ values of ~5.5 (leghemoglobins) and 4.4 (hemoglobin) are probably the p*K* values of the distal histidines. Comparisons of $pK_{app}^{O_2}$ values with $pK_{app}^{CO}$ values indicate a more flexible heme pocket in leghemoglobins than in hemoglobin. The $O_2$ complex of leghemoglobin $c_2$ differed significantly from the $O_2$ complexes of leghemoglobins *a* and $c_1$ in visible spectra and titration behavior. These differences might be associated with the small structural differences in the region between the E and F helixes of the leghemoglobins.

Leghemoglobins are monomeric hemeproteins associated with $N_2$ fixation by rhizobium bacteroids in legume root nodule symbioses. Leghemoglobins appear to facilitate $O_2$ diffusion to bacteroidal surfaces and to regulate the free $O_2$ concentration in fluids surrounding bacteroids (Appleby, 1974). Under normal conditions the biologically important forms of leghemoglobins are their deoxy and oxy forms.

Leghemoglobin–CO complexes appear to be attractive models for leghemoglobin–$O_2$ complexes. Both $O_2$ and CO bind at the iron(II) site of the heme. Soybean leghemoglobin *a* exhibits high affinities for both $O_2$ and CO (Appleby, 1962; Imamura et al., 1972; Wittenberg et al., 1972) and unusually rapid binding of both $O_2$ and CO (Imamura et al., 1972; Wittenberg et al., 1972). But unlike $O_2$, CO is incapable of oxidizing the heme. Thus not only does CO appear to be a mimic of $O_2$ but also CO offers the experimental advantage of not causing autoxidation, a significant side reaction in leghemoglobin oxygenation studies (Appleby, 1962). There are, however, at least slight differences between the structures of $O_2$ and CO complexes of leghemoglobin *a*, as $^1$H NMR[1] spectra are not identical (Wright & Appleby, 1977).

Recent $^1$H NMR studies on the CO complex of soybean leghemoglobin *a* demonstrated pH-dependent motion of the distal histidine with respect to the heme (Johnson et al., 1978). Interactions between the distal histidine and bound CO are evident from IR measurements of the carbon–oxygen stretching frequencies ($\nu_{CO}$ values) of abnormal human hemoglobins (Caughey et al., 1969). Close proximity of the distal histidine and bound CO has been shown crystallographically in myoglobin–CO (Norvell et al., 1975) and hemoglobin–CO (Heidner et al., 1976). Interactions between the distal histidine and bound $O_2$ or CO may be important in regulating affinities and kinetics of binding and release of these ligands. We have followed up the $^1$H NMR studies by monitoring effects at the CO ligand (by IR $\nu_{CO}$ measurements) and effects at the heme (by measurements of visible spectra) as the distal histidine of leghemoglobin *a* CO moves with respect to the heme. We here report pH effects upon $\nu_{CO}$ values and visible spectra of leghemoglobin *a*–CO. We also compare the effects of pH upon visible spectra of the leghemoglobin *a*–CO complex with the effects of pH upon visible spectra of the leghemoglobin *a*–$O_2$ complex. A portion of the CO complex data has been presented in preliminary form (Fuchsman & Appleby, 1978).

Soybean leghemoglobin consists of several major components, of which leghemoglobin *a* is one (Appleby et al., 1975). Since it is possible that leghemoglobin heterogeneity has a biological function (Fuchsman et al., 1976), we also describe the pH dependence of the visible spectra of CO and $O_2$ complexes of soybean leghemoglobins $c_1$ and $c_2$.[2]

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[1] Abbreviations used: $^1$H NMR, proton magnetic resonance; NMR, nuclear magnetic resonance; IR, infrared; $\nu_{CO}$, carbon–oxygen stretching frequency; EDTA, ethylenediaminetetraacetate; Mes, 2-(*N*-morpholino)ethanesulfonate; Mops, 3-(*N*-morpholino)propanesulfonate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; pH*, pH meter reading in $D_2O$; Lb, leghemoglobin; Mb, myoglobin; Hb, hemoglobin; $pK_{app}^{CO}$, apparent p*K* for CO complex; $pK_{app}^{O_2}$, apparent p*K* for $O_2$ complex; $pK_{app}^{deoxy}$, apparent p*K* for deoxy compound. Throughout this paper we use $pK_{app}$ for experimentally determined $pK_a$ values, and we use p*K* for the theoretical $pK_a$ values of single functional groups.

[2] After the completion of this work, we showed that leghemoglobin $c_2$ is a mixture of two separable hemeproteins (W. H. Fuchsman and C. A. Appleby, unpublished experiments), consistent with the amino acid sequence (Hurrell & Leach, 1977) which showed microheterogeneity (Glu and Asp) of one amino acid residue remote from the heme. The heterogeneity of leghemoglobin $c_2$ does not affect the interpretations or conclusions in this paper.

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Crystallographic data on the CO complexes of myoglobin (sperm whale) and hemoglobin (horse) are available (Norvell et al., 1975; Heidner et al., 1976), whereas comparable data on leghemoglobins are not available. In order to relate our leghemoglobin–CO and leghemoglobin–$O_2$ results to known structures, we examined and here report pH effects upon $\nu_{CO}$ values and visible spectra of sperm whale myoglobin–CO and human hemoglobin A–CO, as well as pH effects upon visible spectra of myoglobin–$O_2$ and hemoglobin–$O_2$. Reports of $\nu_{CO}$ values for myoglobin–CO and human hemoglobin–CO at low pH (O'Toole, 1972; Caughey et al., 1973; Yoshikawa et al., 1977) and the results of pH titrations of sperm whale myoglobin–CO which were monitored by visible spectra (Hayashi et al., 1976) were published before this work was begun.

## Experimental Section

### *Materials*

Analytical grade reagents were used. All glassware and other items in contact with hemeprotein samples were washed with doubly glass-distilled water. Doubly glass-distilled water was used in the preparation of solutions.

*Leghemoglobins.* Soybean metleghemoglobins *a*, $c_1$, and $c_2$ were prepared as previously described (Appleby et al., 1975) and were stored in liquid nitrogen as ~10 mM stock solutions in 10 mM potassium phosphate, pH 7. When needed, portions were thawed and stored in ice for several days during use.

*Myoglobin.* Sperm whale metmyoglobin ("salt free") was purchased from Calbiochem (batch 601472). It was dissolved in 10 mM potassium phosphate, pH 7, centrifuged to remove the precipitate, and stored in ice as an ~10 mM stock solution.

*Hemoglobin.* Oxyhemoglobin A (slightly contaminated with methemoglobin A) was prepared from human blood by the method of Brown et al. (1976), which more adequately removes erythrocyte stroma from whole hemoglobin than does the method (Geraci et al., 1969) used in previous IR studies on hemoglobin. Oxyhemoglobin was stored in ice as an ~10 mM stock solution (concentration calculated for monomer) in 10 mM potassium phosphate, pH 7.

*Oxyhemeproteins.* Methemeproteins (leghemoglobins *a*, $c_1$, $c_2$, and myoglobin) and oxyhemeprotein contaminated with small amounts of methemeprotein (hemoglobin) were reduced with $Na_2S_2O_4$. The $Na_2S_2O_4$ was removed and the deoxyhemeproteins were equilibrated with $O_2$ by passage through a short column of Sephadex G-25 equilibrated with air-saturated buffer (10 mM potassium phosphate, pH 7, containing 1.0 mM EDTA). The resultant oxyhemeproteins exhibited no evidence of methemeprotein contamination (i.e., no visible absorption band or shoulder at ~630 nm). Samples were stored in ice and were used within 8 h of their preparation.

Hemeprotein concentrations were determined by the pyridine hemochrome method. Molar absorptivities of Ohlsson & Paul (1976) were used.

*Choice of Buffer for Titrations.* A mixed buffer was chosen to provide good buffer capacity within the desired pH range (pH 3.5–8.0). Buffers which were likely to cause specific ion interactions with hemeproteins (such as acetate, phosphate, and Tris) were not used, although dilute phosphate buffer was used to prepare and stabilize hemeprotein stock solutions. EDTA was included in the buffer to retard metal ion promoted oxyhemeprotein autoxidation.

### *Methods*

*Infrared Measurements on CO Complexes.* IR measurements of percent transmission in the region 1900–2000 $cm^{-1}$ were carried out on hemeprotein–CO complexes by use of a Perkin-Elmer 225 instrument under conditions of 4-$cm^{-1}$ resolution. Samples were contained in demountable cells with $CaF_2$ windows, ~0.05-mm Teflon spacers, and specially machined brass backplates through which water (1–2 °C at bath) was circulated. The instrument was flushed with dry air. Spectra were calibrated with gaseous DCl bands. Hemeprotein samples, when examined without reference beam compensation, always exhibited >10% transmission. Spectra of hemeprotein–CO sample solutions were recorded with equimolar methemeprotein (leghemoglobins *a*, $c_1$, $c_2$, and myoglobin) or oxyhemeprotein (hemoglobin) solutions in the reference beam.

Percent transmission scans were manually converted to absorbance vs. frequency plots. Base-line corrections were applied to absorbance plots; absorbance base lines were assumed to be linear. Visible spectra of all samples were examined with a Cary 14 spectrophotometer before and after IR spectra were recorded. The absorbances of the $\beta$ bands in the visible spectra, which were nearly pH independent, were used to correct for small IR cell path length and/or hemeprotein concentration variations.

*Infrared-Monitored Titrations of CO Complexes.* All steps were performed in an ice bath. Three microliters of a 0.1 M EDTA solution was added to a sample tube, followed by 300 $\mu$L of 10 mM methemeprotein (leghemoglobins *a*, $c_1$, $c_2$, and myoglobin) or oxyhemeprotein (hemoglobin) stock solution. The solutions were mixed and equilibrated with CO by gentle CO bubbling, which continued throughout the subsequent steps. Approximately 2 mg of $Na_2S_2O_4$ was added, followed after an equilibration period by 66 $\mu$L of a mixed buffer of desired pH (0.5 M citrate, 0.5 M Mes, 0.5 M Mops). The pH was measured and then the sample was injected into a CO-purged infrared sample cell.

*Visible-Monitored Titrations of CO Complexes.* All steps were performed in an ice bath. A 600 $\mu$M stock solution of methemeprotein (leghemoglobins *a*, $c_1$, $c_2$, and myoglobin) or oxyhemeprotein (hemoglobin) in 10 mM potassium phosphate, pH 7, was equilibrated with CO by gentle bubbling and reduced by addition of ~2 mg of $Na_2S_2O_4$. Stock solutions of a mixed buffer (1.0 mM EDTA, 0.11 M citrate, 0.11 M Mes, 0.11 M Mops) were also equilibrated with CO. To 1.80 mL of buffer was added 0.20 mL of hemeprotein solution; 1.0 mL of the resultant solution was used to rinse the cuvette (1.0 cm, semimicro), and the remainder was added to the cuvette. The cuvette was sealed under CO and placed in the thermostated sample compartment (2 °C) of a Cary 14 spectrophotometer, and after a short, fixed delay time (1 or 2 min, depending on the experiment) the visible spectrum was recorded. Sample pH values were measured at 2 °C after the visible spectra had been recorded.

*Infrared Reversibility Test on the CO Complex of Leghemoglobin a.* Because the contents of IR cells could not be removed quantitatively without exposure to air, we instead adjusted the pH of a large sample down and back up and measured $\nu_{CO}$ on aliquots with appropriate pH values. All steps were performed in an ice bath; the sample was kept under CO throughout. To 15 $\mu$L of 0.1 M EDTA was added 1.5 mL of 11.8 mM metleghemoglobin *a* stock solution in 10 mM potassium phosphate, pH 7. The mixture was magnetically stirred and equilibrated with CO with gentle bubbling. Approximately 2 mg of $Na_2S_2O_4$ was added, the pH measured, an aliquot removed, and $\nu_{CO}$ measured, after which the pH of the remainder was adjusted down by slow addition of 0.5 M HCl from a micrometer syringe. An aliquot was removed and $\nu_{CO}$ determined, after which the pH of the remainder was adjusted back to the original value by slow addition of 0.5 M

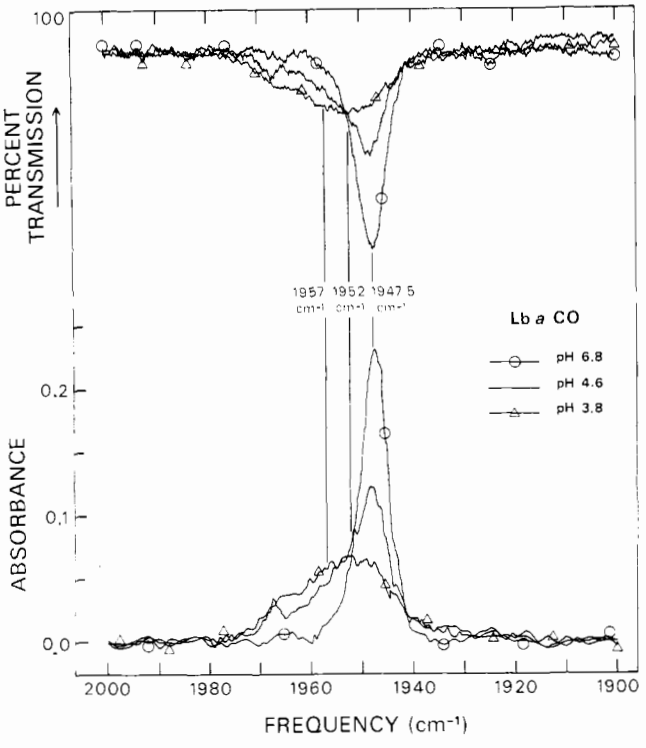


FIGURE 1: Percent transmission scans and corrected absorbance plots of $\nu_{CO}$ region of IR spectra of soybean leghemoglobin *a*–CO at pH 6.8 (○), pH 4.6 (—), and pH 3.8 (△). Base lines of percent transmission scans have been brought into coincidence by slight vertical movement of entire scans. Conditions: ~2 °C, 9.5 mM leghemoglobin *a*–CO, 1.0 mM EDTA, 0.10 M citrate, 0.10 M Mes, 0.10 M Mops.

KOH from a micrometer syringe. An aliquot was used for the final $\nu_{CO}$ determination.

*Visible Reversibility Tests on CO Complexes.* After IR spectra were recorded, visible spectra were recorded without removal of the samples from IR cells. Then an aliquot of each sample was removed and diluted with CO-saturated pH 7.5 buffer (0.1 M citrate, 0.1 M Mes, 0.1 M Mops), and the visible spectrum was measured again.

*Visible-Monitored Titrations of $O_2$ Complexes.* All steps were performed in an ice bath. To 1.80 mL of mixed buffer (1.0 mM EDTA, 0.11 M citrate, 0.11 M Mes, 0.11 M Mops) of appropriate pH was added 0.20 mL of a 600 μM oxyhemeprotein solution in 10 mM potassium phosphate, pH 7, containing 1.0 mM EDTA; 1.0 mL of the resultant solution was used to rinse the cuvette (1.0 cm, semimicro), and the remainder was added to the cuvette, which was placed in the thermostated sample compartment (2 °C) of a Cary 14 spectrophotometer. After a short, timed delay (1 or 2 min, depending on the experiment), the visible spectrum was recorded. Spectra which did not remain constant for ~10 min usually were discarded in the subsequent data analysis. Sample pH values were measured at 2 °C after visible spectra had been recorded.

## Results

*Infrared Measurements on CO Complexes.* Carbon–oxygen stretching frequencies ($\nu_{CO}$ values) and their associated half-bandwidths ($\Delta\nu_{1/2}$ values) of the neutral-pH forms and the low-pH forms of soybean leghemoglobin *a*–CO, soybean leghemoglobin $c_1$–CO, soybean leghemoglobin $c_2$–CO, sperm whale myoglobin–CO, and human hemoglobin A–CO are listed in Table I. The main $\nu_{CO}$ band of myoglobin–CO was assumed to be symmetric to allow $\Delta\nu_{1/2}$ determination. The $\nu_{CO}$ band of the low-pH form of leghemoglobin *a*–CO overlapped the $\nu_{CO}$ band of the neutral-pH form when both were

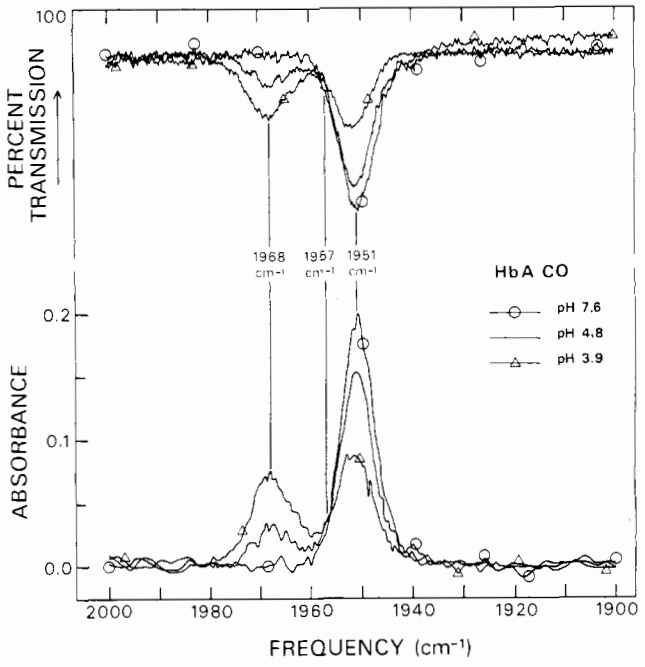


FIGURE 2: Percent transmission scans and corrected absorbance plots of $\nu_{CO}$ region of IR spectra of human hemoglobin A–CO at pH 7.6 (○), pH 4.8 (—), and pH 3.9 (△). Base lines of percent transmission scans have been brought into coincidence by slight vertical movement of entire scans. Conditions: ~2 °C, 8.9 mM hemoglobin–CO, 1.0 mM EDTA, 0.10 M citrate, 0.10 M Mes, 0.10 M Mops.

present (Figure 1). Even at the lowest pH to which leghemoglobin *a*–CO samples could be adjusted without precipitation, accurate values of $\nu_{CO}$ and $\Delta\nu_{1/2}$ for the low-pH form were unobtainable. Table I contains our best approximations, which were obtained from a pH 3.5 sample not shown in Figure 1. In contrast, the $\nu_{CO}$ bands of the low-pH forms of hemoglobin–CO (Figure 2) and myoglobin–CO were distinctly separated from the $\nu_{CO}$ bands of the neutral-pH forms, and accurate measurements were possible.

For unambiguous determination of the presence or absence of isosbestic points and for quantitative measurements of pH effects on $\nu_{CO}$ bands, careful conversion of the original percent transmission vs. frequency scans to absorbance vs. frequency plots was necessary. Figures 1 and 2 compare selected percent transmission scans (adjusted to approximately constant base lines and thus slightly distorted in relation to one another) with replots on an absorbance scale. The replots have been corrected for base-line position and slope and for small variations in optical path length and/or hemeprotein concentrations.

Isosbestic points were noted at 1952 $cm^{-1}$ for leghemoglobin *a*–CO (Figure 1) and at 1957 $cm^{-1}$ for hemoglobin–CO (Figure 2). Myoglobin–CO exhibited more complicated behavior, with a fast reaction resulting in a shift in $\nu_{CO}$ from 1944 to 1945 $cm^{-1}$ as the pH was lowered from ~7 to ~6, another fast reaction resulting in appearance of a 1966 $cm^{-1}$ $\nu_{CO}$ band at pH <6, and evidence of slow changes occurring at low pH. Our data had too much scatter to permit successful analysis of the small changes in myoglobin–CO $\nu_{CO}$ values; therefore, our analysis was limited to the reaction which resulted in formation of a 1966 $cm^{-1}$ $\nu_{CO}$ band.

The pH-dependent change in $\nu_{CO}$ of leghemoglobin *a*–CO was completely reversible when the pH was lowered from pH 6.3 to 4.4 and then returned to pH 6.3 (Figure 3).

Quantitative analysis of the pH-dependent behavior of $\nu_{CO}$ bands was carried out on corrected absorbance values taken from smoothed percent transmission scans. We used the frequencies of maximal absorption of the neutral-pH forms

Table I: Infrared Data for CO Complexes of Soybean Leghemoglobins, Sperm Whale Myoglobin, Human Hemoglobin A, and Other Hemeproteins[a]

| hemeprotein | neutral pH $\nu_{CO}$ ($cm^{-1}$) | neutral pH $\Delta\nu_{1/2}$ ($cm^{-1}$) %T | neutral pH $\Delta\nu_{1/2}$ ($cm^{-1}$) A | neutral pH no. of values averaged | low pH $\nu_{CO}$ ($cm^{-1}$) | low pH $\Delta\nu_{1/2}$ ($cm^{-1}$) %T | low pH $\Delta\nu_{1/2}$ ($cm^{-1}$) A | low pH no. of values averaged |
|---|---|---|---|---|---|---|---|---|
| soybean leghemoglobin *a* | | | | | | | | |
| this work | 1947.6 ± 0.1 | 7 | 6 | 6 | ~1957 | >10 | | 1 |
| literature[b] | 1948 | | 7 | | | | | |
| soybean leghemoglobin $c_1$ | | | | | | | | |
| this work | 1947.6 | 7 | | 1 | *c* | | | |
| soybean leghemoglobin $c_2$ | | | | | | | | |
| this work | 1947.6 | 8 | | 1 | *c* | | | |
| sperm whale myoglobin | | | | | | | | |
| this work | 1943.8 ± 0.4 | 10 | 9 | 3 | 1965.8 ± 0.1 | 9 | 9 | 2 |
| | ~1933 sh | | | | | | | |
| literature[d] | 1944 | | 12[e] | | 1966 | | ~20 | |
| | 1932 sh | | | | | | | |
| human hemoglobin A | | | | | | | | |
| this work | 1950.7 | 8 | 7 | 1 | 1968.4 | 11 | 10 | 1 |
| literature[f] | 1951 | 8 | | | 1966 | | ~20 | |
| *Chironomus thummi thummi* hemoglobins | | | | | | | | |
| literature[g] I | 1963 | 8 | | | | | | |
| III | 1964 | 8 | | | | | | |
| IV | 1964 | 8 | | | | | | |
| dimeric | 1962 | 8 | | | | | | |
| bovine cytochrome *c* oxidase | | | | | | | | |
| literature | 1963.5[h] | | 5.5[h] | | | | | |
| | | | 3.5[i] | | | | | |
| horseradish peroxidases | | | | | | | | |
| literature[j] A2 | 1938 | | 11 | | | | | |
| | 1925 | | | | | | | |
| | 1906 | | 19 | | | | | |
| C | 1933 | | ~12 | | | | | |
| | 1929 | | | | | | | |
| | 1905 | | 17 | | | | | |

[a] Abbreviations: $\nu_{CO}$, carbon-oxygen stretching; $\Delta\nu_{1/2}$, half-bandwidth; %T, from percent transmission vs. frequency scan; A, from absorbance vs. frequency plot or scan; sh, shoulder. In this work neutral pH means the range pH 6.6–7.6; low pH means 3.5 (leghemoglobin *a*), pH 4.8–5.0 (myoglobin), or pH 3.9 (hemoglobin A). Variation, when shown, is standard deviation. Data from this work were collected under conditions of ~2 °C, 1.0 mM EDTA, 0.10 M citrate, 0.10 M Mes, 0.10 M Mops, ~10 mM hemeprotein (hemoglobin concentration calculated for the monomer). [b] Unpublished experiments of C. A. Appleby, J. C. Maxwell, and W. S. Caughey, quoted in Maxwell & Caughey (1978). [c] At intermediate pH values, $\nu_{CO}$ region spectra were consistent with low-pH $\nu_{CO}$ values similar to that of leghemoglobin *a*–CO. [d] Data of McCoy & Caughey (1971) and O'Toole (1972). [e] Data of Maxwell & Caughey (1978). The 8-$cm^{-1}$ $\Delta\nu_{1/2}$ value quoted for myoglobin–CO by Barlow et al. (1976) appears to be a typographical error, as it is not reported in the reference cited (McCoy & Caughey, 1971). [f] Data of Alben & Caughey (1968) for neutral pH and Yoshikawa et al. (1977) for low pH. [g] Data of Wollmer et al. (1977). [h] Data of Volpe et al. (1975) and Yoshikawa et al. (1977). [i] Data of Alben (1978). [j] Data of Barlow et al. (1976).

of leghemoglobin *a*–CO and hemoglobin–CO and a near maximal absorption frequency of the neutral-pH form of myoglobin–CO chosen to minimize the effects of the shift in $\nu_{CO}$ from 1944 to 1945 $cm^{-1}$. To obtain apparent p*K* values we used the familiar equation (derived from the Henderson–Hasselbalch equation)

$$\log \frac{A_{obsd} - A_{acid\ limit}}{A_{basic\ limit} - A_{obsd}} = n\text{pH} - n\text{p}K_{app} \qquad (1)$$

where $A_{obsd}$ is the observed (and corrected) absorbance, $A_{acid\ limit}$ is the expected absorbance when the hemeprotein–CO is fully in the low-pH form, $A_{basic\ limit}$ is the absorbance when the hemeprotein–CO is fully in the neutral-pH form, and $n = 1$ for a single reaction involving a single protonation. $A_{basic\ limit}$ values were the averages of observed (and corrected) absorbance values at neutral pH. Because $\nu_{CO}$ bands from the two forms were clearly separated in hemoglobin–CO and myoglobin–CO spectra, $A_{acid\ limit}$ values of 0.00 were used. Because of band overlap in leghemoglobin *a*–CO spectra, its $A_{acid\ limit}$ value was obtained by maximizing the least-squares coefficient of determination ($r^2$) values of the best fit lines of $\log [(A_{obsd} - A_{acid\ limit})/(A_{basic\ limit} - A_{obsd})]$ vs. pH plots as a function of the variable $A_{acid\ limit}$. Titration curves were constructed from 10 intermediate $\nu_{CO}$ absorbance values for leghemoglobin *a*–CO, five intermediate values for hemoglobin–CO, and six intermediate values for myoglobin–CO (Figure 4). Table II lists the $pK_{app}^{CO}$ and *n* values which resulted.

The IR $\nu_{CO}$ spectrum of myoglobin–CO at neutral pH consists of a main band and a lower frequency shoulder (Table I). We observed pH insensitivity of the relative absorbances of the main band and shoulder in this spectrum (Figure 5).

*Visible Measurements on CO and $O_2$ Complexes.* Visible-monitored titrations were analyzed in the same manner as IR-monitored titrations, except that absorbance differences ($\Delta A$ values) instead of absorbances were used. The pairs of wavelengths used for absorbance differences were selected to maximize the magnitudes of pH-induced changes while minimizing the use of data from steeply sloped portions of the visible spectra. $\Delta A_{basic\ limit}$ values were obtained from visible data at neutral pH. $\Delta A_{acid\ limit}$ values were determined by maximization of least-squares $r^2$ values as described above for leghemoglobin *a*–CO IR data. Titration curves (Figure 6) were constructed from 13 intermediate $\Delta A$ ($A_{561.5nm} - A_{537.5nm}$) values for leghemoglobin *a*–CO, 14 intermediate $\Delta A$ ($A_{574nm} - A_{540nm}$) values for leghemoglobin *a*–$O_2$, 14 intermediate $\Delta A$ ($A_{561.5nm} - A_{537.5nm}$) values for leghemoglobin $c_1$–CO, 13 intermediate $\Delta A$ ($A_{574nm} - A_{541nm}$) values for leghemoglobin

Table II: Experimentally Determined Constants for pH Titrations of CO and $O_2$ Complexes of Soybean Leghemoglobins, Sperm Whale Myoglobin, and Human Hemoglobin[a]

| hemeprotein | method | $pK_{app}^{CO}$ | $n^{CO}$ | $pK_{app}^{O_2}$ | $n^{O_2}$ |
|---|---|---|---|---|---|
| soybean leghemoglobin *a* | | | | | |
| this work | IR | 4.5 | 0.7 | | |
| | vis | 4.3 | 0.7 | 5.5 | 1.0 |
| literature[b] | $^1$H NMR | 4.1 | 0.8 | | |
| soybean leghemoglobin $c_1$ | | | | | |
| this work | vis | 4.3 | 0.7 | 5.6 | 0.9 |
| soybean leghemoglobin $c_2$ | | | | | |
| this work | vis | 4.3 | 0.8 | 5.2 | 0.7 |
| sperm whale myoglobin | | | | | |
| this work | IR | 4.6 | 0.9 | | |
| | vis | 5.8 | 1.0 | 5.7 | 0.8 |
| literature[c] | vis | 5.7 | | | |
| human hemoglobin A | | | | | |
| this work | IR | 4.0 | 0.8 | | |
| | vis | 4.1 | 1.2 | 4.4 | 1.1 |

[a] Abbreviation used: vis, visible spectroscopy. Data from this work were collected under conditions of ~2 °C, 1.0 mM EDTA, 0.10 M citrate, 0.10 M Mes, 0.10 M Mops, ~10 mM hemeprotein (IR) or 60 μM hemeprotein (vis) (hemoglobin concentration calculated as the monomer). [b] Data of Johnson et al. (1978). [c] Data of Hayashi et al. (1976).

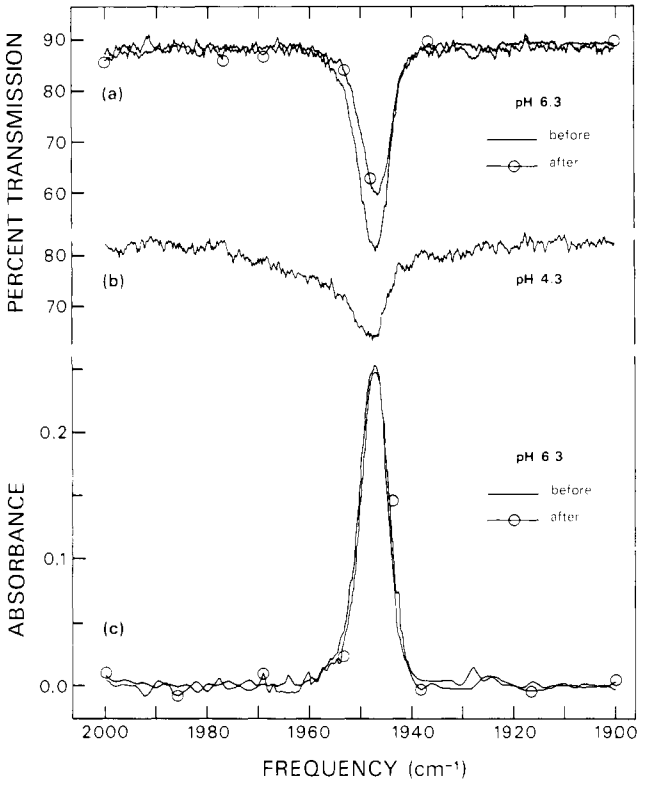


FIGURE 3: Percent transmission scans and corrected absorbance plots of $\nu_{CO}$ region of IR spectra of leghemoglobin *a*–CO in reversibility test. Percent transmission scans have not been adjusted or corrected in any way. (a) Percent transmission scans of leghemoglobin *a*–CO at pH 6.3 before addition of acid (—) and after acid was added and the product returned to pH 6.3 addition of base (O). (b) Percent transmission scan of leghemoglobin *a*–CO at pH 4.3, the product of acid addition to pH 6.3 sample. (c) Corrected absorbance plot of leghemoglobin *a*–CO at pH 6.3 before addition of acid (—) and after acid was added and the product returned to pH 6.3 by addition of base (O). Dilution effects evident in (a) have been corrected in (c).

$c_1$–$O_2$, 11 intermediate $\Delta A$ ($A_{562.5nm} - A_{538nm}$) values for leghemoglobin $c_2$–CO, 13 intermediate $\Delta A$ ($A_{575.5nm} - A_{542.5nm}$) values for leghemoglobin $c_2$–$O_2$, eight intermediate $\Delta A$ ($A_{568.5nm} - A_{538.5nm}$) values for hemoglobin–CO, seven intermediate $\Delta A$ ($A_{576nm} - A_{541nm}$) values for hemoglobin–$O_2$, five intermediate $\Delta A$ ($A_{583nm} - A_{569nm}$) values for myoglobin–CO, and eight intermediate $\Delta A$ ($A_{581nm} - A_{543nm}$) values for myoglobin–$O_2$. Table II contains the visible $pK_{app}^{CO}$, $pK_{app}^{O_2}$, and *n* values. Figures 7 and 8 show the magnitude and nature of pH-dependent changes in visible spectra of hemeprotein CO and $O_2$ complexes. Tables III and IV contain visible spectral data for neutral and acidic solutions of CO and $O_2$ complexes of hemeproteins. Visible changes of CO complexes were reversible; reversibility of visible changes of $O_2$ complexes was not tested.

Table III: Peak Wavelength and Relative Absorbances in Visible Spectra of CO Complexes of Soybean Leghemoglobin, Sperm Whale Myoglobin, and Human Hemoglobin[a]

| | | $\lambda_{max}$ (nm) | | |
|---|---|---|---|---|
| hemeprotein | pH | α band | β band | $A_\alpha/A_\beta$ |
| soybean leghemoglobin *a* | 7.7 | 561.5 | 537.0 | 1.027 |
| | 4.5 | 563.0 | 537.0 | 0.996 |
| soybean leghemoglobin $c_1$ | 7.6 | 561.5 | 537.0 | 1.013 |
| | 4.5 | 563.0 | 537.5 | 0.974 |
| soybean leghemoglobin $c_2$ | 7.6 | 562.5 | 538.0 | 1.018 |
| | 4.5 | 564.0 | 538.0 | 0.996 |
| sperm whale myoglobin | 7.6 | 578.5 | 541.0 | 0.891 |
| | 5.5 | 576.0 | 540.0 | 0.866 |
| human hemoglobin A | 7.7 | 568.0 | 537.5 | 1.018 |
| | 4.0 | 567.5 | 538.0 | 0.980 |

[a] Acid pH data is for pH near $pK_{app}^{CO}$. Conditions: 2 °C, 1.0 mM EDTA, 0.10 M citrate, 0.10 M Mes, 0.10 M Mops, 60 μM hemeprotein–CO (hemoglobin concentration calculated as the monomer). $\lambda_{max}$ values were determined to the nearest 0.5 nm.

Table IV: Peak Wavelengths and Relative Absorbances in Visible Spectra of $O_2$ Complexes of Soybean Leghemoglobins, Sperm Whale Myoglobin, and Human Hemoglobin[a]

| | | $\lambda_{max}$ (nm) | | |
|---|---|---|---|---|
| hemeprotein | pH | α band | β band | $A_\alpha/A_\beta$ |
| soybean leghemoglobin *a* | 7.8 | 574.0 | 540.5 | 1.086 |
| | 5.4 | 574.5 | 540.5 | 1.021 |
| soybean leghemoglobin $c_1$ | 7.8 | 574.0 | 540.0 | 1.068 |
| | 5.4 | 574.5 | 540.5 | 1.000 |
| soybean leghemoglobin $c_2$ | 7.8 | 575.5 | 541.5 | 1.048 |
| | 5.4 | 576.0 | 541.5 | 0.978 |
| sperm whale myoglobin | 7.8 | 581.0 | 543.0 | 1.077 |
| | 5.6 | 579.5 | 543.0 | 1.064 |
| human hemoglobin A | 7.8 | 576.0 | 541.5 | 1.080 |
| | 4.3 | 575.5 | 540.5 | 1.059 |

[a] Acid-pH data are for pH near $pK_{app}^{O_2}$. Conditions: 2 °C, 1.0 mM EDTA, 0.10 M citrate, 0.10 M Mes, 0.10 M Mops, 60 μM hemeprotein–$O_2$ (hemoglobin concentration calculated as the monomer). $\lambda_{max}$ values were determined to the nearest 0.5 nm.

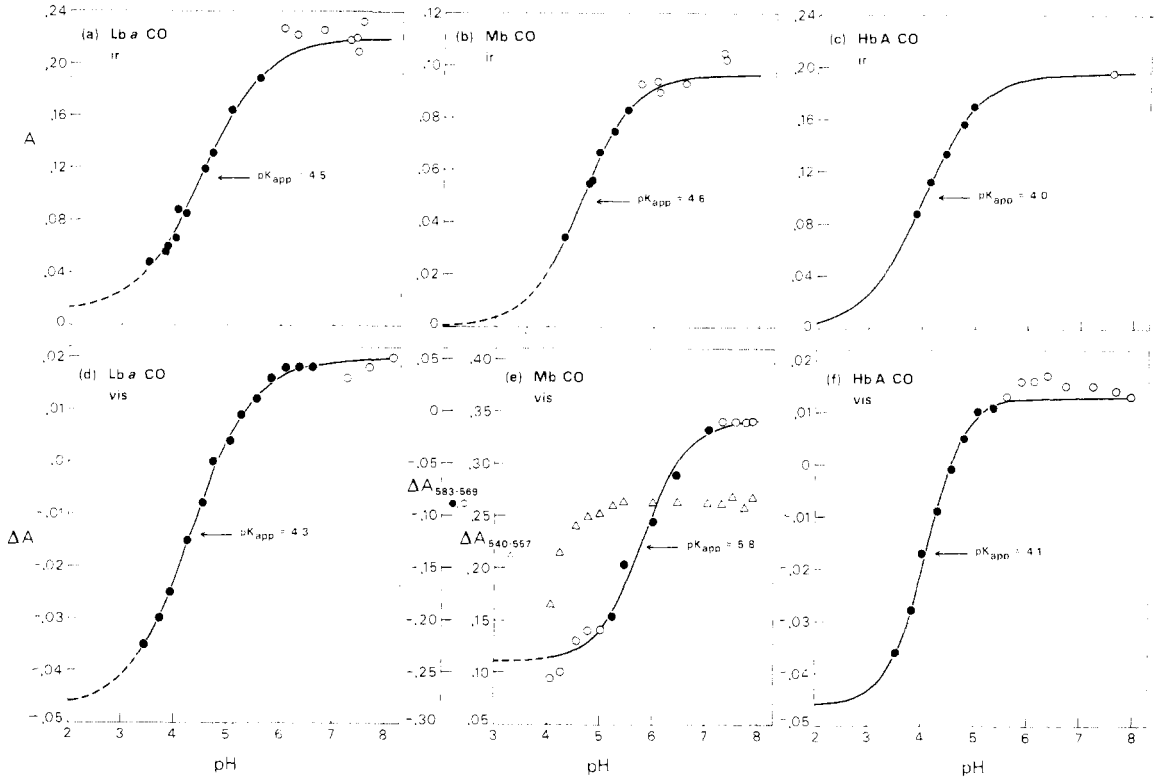


FIGURE 4: Experimental and theoretical pH titration curves of CO complexes of soybean leghemoglobin *a*, sperm whale myoglobin, and human hemoglobin A. Abbreviation used: vis, data from visible spectra. Data points are shown by circles; filled circles denote the data points used to determine $pK_{app}$, $n$, and acid-limit values. Theoretical curves are shown by continuous lines in regions of data and by dashed lines in acid-pH regions where irreversible denaturation occurs. (a) IR absorbances (corrected) at 1947 $cm^{-1}$ for leghemoglobin *a*–CO; theoretical curve for $pK = 4.5$, $n = 0.71$, $A_{acid\ limit} = 0.010$, $A_{basic\ limit} = 0.220$. (b) IR absorbances (corrected) at 1945 $cm^{-1}$ for myoglobin–CO; theoretical curve for $pK = 4.6$, $n = 0.88$, $A_{acid\ limit} = 0.000$, $A_{basic\ limit} = 0.097$. (c) IR absorbances (corrected) at 1951 $cm^{-1}$ for hemoglobin A–CO; theoretical curve for $pK = 4.0$, $n = 0.81$, $A_{acid\ limit} = 0.000$, $A_{basic\ limit} = 0.196$. (d) Visible absorbance differences ($A_{561.5nm} - A_{537.5nm}$) for leghemoglobin *a*–CO; theoretical curve for $pK = 4.3$, $n = 0.74$, $\Delta A_{acid\ limit} = -0.047$, $\Delta A_{basic\ limit} = 0.020$. (e) Visible absorbance differences [$A_{583nm} - A_{569nm}$, (O) and $A_{540nm} - A_{557nm}$ (Δ)] for myoglobin–CO; fit of $A_{583nm} - A_{569nm}$ data to theoretical curve for $pK = 5.8$, $n = 1.02$, $\Delta A_{acid\ limit} = -0.240$, $\Delta A_{basic\ limit} = -0.010$. (f) Visible absorbance differences ($A_{568.5nm} - A_{538.5nm}$) for hemoglobin A–CO; theoretical curve for $pK = 4.1$, $n = 1.20$, $\Delta A_{acid\ limit} = -0.046$, $\Delta A_{basic\ limit} = 0.013$.

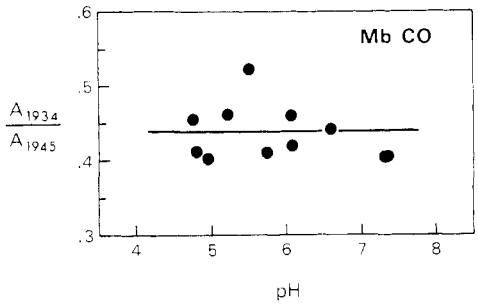


FIGURE 5: Effect of pH on IR absorbance ratios of $\nu_{CO}$ shoulder (1934 $cm^{-1}$) to main band (1945 $cm^{-1}$) in samples of sperm whale myoglobin–CO. The horizontal line indicates the average absorbance ratio.

## Discussion

*Nature of the pH-Dependent Reaction.* The reactions we have detected by pH variation were reversible. Hayashi et al. (1976) had previously demonstrated the reversibility of the visible-monitored, pH-dependent reaction of myoglobin–CO. The pH-dependent $^1H$ NMR changes in leghemoglobin *a*–CO observed by Johnson et al. (1978) resulted from a rapid equilibrium reaction, since only single distal histidine C-2 ($C_\epsilon$) proton resonances with average chemical shifts were detected. We have explicitly demonstrated reversibility of visible-monitored changes of leghemoglobin *a*–CO, hemoglobin–CO, and myoglobin–CO and of the IR-monitored, pH-dependent change in leghemoglobin *a*–CO. In addition, the absence of all-or-nothing pH-dependent changes in CO and $O_2$ complexes is indirect but strong evidence that all the reactions are reversible. We can ascribe the observed changes to the general reaction

$$\text{low-pH form} \rightleftharpoons \text{neutral-pH form} + H^+ \qquad (2)$$

Formation of the low-pH forms of CO complexes did not appear to involve protein unfolding. Caughey and co-workers (O'Toole, 1972; Caughey et al., 1973; Yoshikawa et al., 1977) have attributed the broad 1966-$cm^{-1}$ $\nu_{CO}$ bands obtained for hemoglobin–CO and myoglobin–CO at pH 3 to denatured hemeprotein–CO complexes. Although our $\nu_{CO}$ values for reversibly formed low-pH forms of hemoglobin–CO and myoglobin–CO are in good agreement with the $\nu_{CO}$ values reported by Caughey and co-workers (Table I), we have observed significantly narrower bands. We conclude that reaction 2 does involve conformational change but does not involve protein unfolding. We suspect that the half-bandwidths (but not the $\nu_{CO}$ values) of low-pH forms previously reported at pH 3 have resulted from significant protein unfolding in a reaction occurring at pH values below those which we used to observe reaction 2.

$^1H$ NMR spectra of leghemoglobin *a*–CO at pH* near 4 were also consistent with conformational change without protein unfolding, since the sharp resonances characteristic of reversibly denatured (i.e., unfolded) proteins were absent (R. N. Johnson, J. H. Bradbury, and C. A. Appleby, unpublished experiments).

At pH <5, myoglobin–CO and –$O_2$ complexes showed time-dependent (slow) changes which probably were due to irreversible denaturation. At pH <4, hemoglobin–$O_2$ also

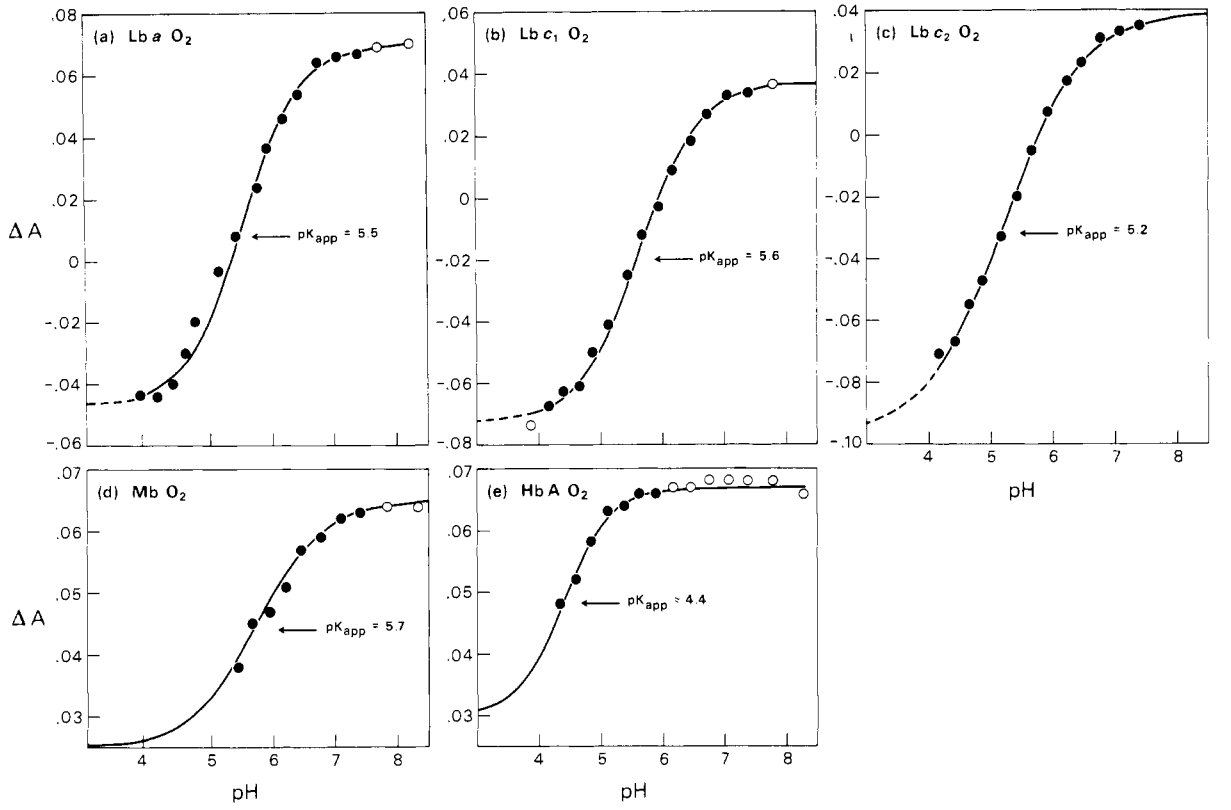


FIGURE 6: Experimental and theoretical pH titration curves of $O_2$ complexes of soybean leghemoglobins, sperm whale myoglobin, and human hemoglobin A. Data points are shown by circles; filled circles denote the data points used to determine $pK_{app}$, $n$, and acid-limit values for absorbance differences. Theoretical curves are shown by continuous lines in regions of data and by dashed lines in acid-pH regions where irreversible denaturation occurs. (a) Visible absorbance differences ($A_{574nm} - A_{540nm}$) for leghemoglobin $a$–$O_2$; theoretical curve for $pK = 5.5$, $n = 0.95$, $\Delta A_{acid\ limit} = 0.047$, $\Delta A_{basic\ limit} = 0.070$. (b) Visible absorbance differences ($A_{574nm} - A_{541nm}$) for leghemoglobin $c_1$–$O_2$; theoretical curve for $pK = 5.6$, $n = 0.90$, $\Delta A_{acid\ limit} = -0.052$, $\Delta A_{basic\ limit} = 0.057$. (c) Visible absorbance differences ($A_{575.5nm} - A_{542.5nm}$) for leghemoglobin $c_2$–$O_2$; theoretical curve for $pK = 5.2$, $n = 0.68$, $\Delta A_{acid\ limit} = -0.097$, $\Delta A_{basic\ limit} = 0.040$. (d) Visible absorbance differences ($A_{581nm} - A_{543nm}$) for sperm whale myoglobin–$O_2$; theoretical curve for $pK = 5.7$, $n = 0.80$, $\Delta A_{acid\ limit} = 0.025$, $\Delta A_{basic\ limit} = 0.065$. (e) Visible absorbance differences ($A_{576nm} - A_{541nm}$) for human hemoglobin A–$O_2$; theoretical curve for $pK = 4.4$, $n = 1.14$, $\Delta A_{acid\ limit} = 0.030$, $\Delta A_{basic\ limit} = 0.067$.

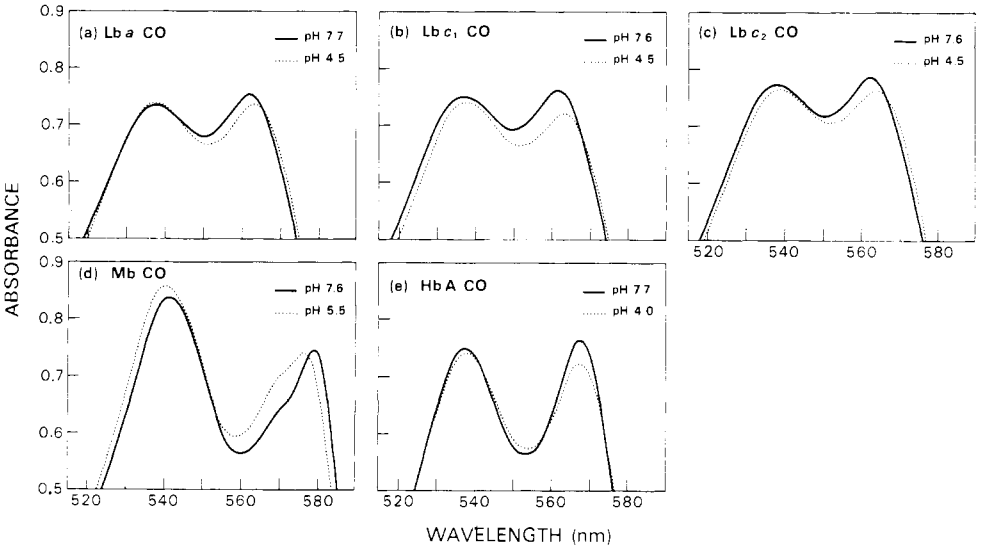


FIGURE 7: Visible absorbance scans of hemeprotein–CO complexes. (a) Soybean leghemoglobin $a$–CO, pH 7.7 (—) and pH 4.5 (···). (b) Soybean leghemoglobin $c_1$–CO, pH 7.6 (—) and pH 4.5 (···). (c) Soybean leghemoglobin $c_2$–CO, pH 7.6 (—) and pH 4.5 (···). (d) Sperm whale myoglobin–CO, pH 7.6 (—) and pH 5.5 (···). (e) Human hemoglobin A–CO, pH 7.7 (—) and pH 4.0 (···). Conditions: 2 °C, 1.0 mM EDTA, 0.10 M citrate, 0.10 M Mes, 0.10 M Mops, 60 $\mu$M hemeprotein–CO (hemoglobin concentration calculated as the monomer).

showed time-dependent changes. The time-dependent changes occurred at pH values below those used for $pK_{app}$ determinations.

It is possible that the low-pH forms of CO–hemeproteins might be formed under biological conditions. IR spectra of hemoglobin A–CO in situ [Figure 4A of Alben & Caughey (1968) and Figure 1C of Maxwell et al. (1974)] suggest marginally detectable levels of the low-pH form of hemoglobin–CO, and IR spectra of certain animal and abnormal human hemoglobins exhibit weak but significant $\nu_{CO}$ bands due to low-pH forms even at neutral pH (Caughey et al., 1978).

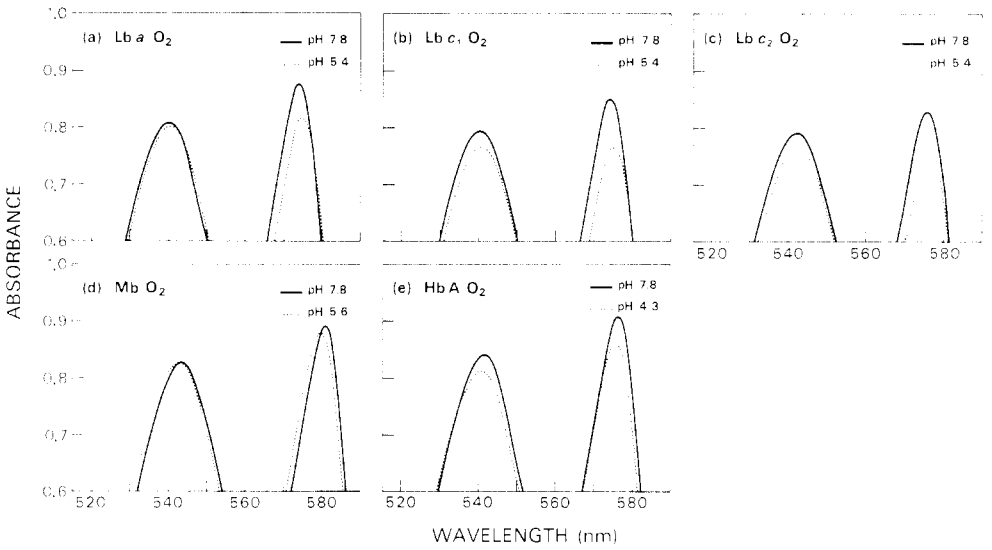


FIGURE 8: Visible absorbance scans of hemeprotein–$O_2$ complexes. (a) Soybean leghemoglobin $a$–$O_2$, pH 7.8 (—) and pH 5.4 (···). (b) Soybean leghemoglobin $c_1$–$O_2$, pH 7.8 (—) and pH 5.4 (···). (c) Soybean leghemolobin $c_2$–$O_2$, pH 7.8 (—) and pH 5.4 (···). (d) Sperm whale myoglobin–$O_2$, pH 7.8 (—) and pH 5.6 (···). (e) Human hemoglobin A–$O_2$, pH 7.8 (—) and pH 4.3 (···). Conditions: 2 °C, 1.0 mM EDTA, 0.10 M citrate, 0.10 M Mes, 0.10 M Mops, 60 $\mu$M hemeprotein–$O_2$ (hemoglobin concentration calculated as the monomer).

*Comparison of Leghemoglobin–CO with Other Hemeprotein–CO Complexes.* The $\nu_{CO}$ values of the neutral-pH forms of leghemoglobin $a$–CO, leghemoglobin $c_1$–CO, and leghemoglobin $c_2$–CO are similar to the $\nu_{CO}$ values of the neutral-pH forms of myoglobin–CO and hemoglobin–CO but distinctly different from the $\nu_{CO}$ values at neutral pH of bovine cytochrome oxidase–CO, horseradish peroxidase A– and C2–CO complexes, and chironomus hemoglobin–CO complexes (Table I). The $\nu_{CO}$ value of leghemoglobin $a$–CO does not fit the proposed (Barlow et al., 1976) linear relationship between neutral-pH $\nu_{CO}$ and reduction potential. When the reduction potential for leghemoglobin $a$ (Henderson & Appleby, 1972) is used, the $\nu_{CO}$–reduction potential relationship predicts a $\nu_{CO}$ value for leghemoglobin $a$–CO (1956 $cm^{-1}$) that is almost 10 $cm^{-1}$ higher than the observed value.

In exhibiting a single, sharp $\nu_{CO}$ band at neutral pH, which implies a highly ordered and/or nonpolar environment for bound CO and a single mode of CO binding, leghemoglobin $a$–CO, leghemoglobin $c_1$–CO, and leghemoglobin $c_2$–CO resemble hemoglobin–CO, cytochrome oxidase–CO, and chironomus hemoglobin–CO complexes but not myoglobin–CO (band with shoulder) or peroxidase–CO complexes [three broader bands, two of which are in pH-dependent equilibrium with one another (Barlow et al., 1976)].

The low-pH forms of hemoglobin–CO and myoglobin–CO resemble each other in $\nu_{CO}$ but differ from the low-pH form of leghemoglobin $a$–CO (Table I). The 1957-$cm^{-1}$ $\nu_{CO}$ of the low-pH form of leghemoglobin $a$–CO resembles the 1958-$cm^{-1}$ $\nu_{CO}$ of the neutral-pH form of the abnormal $\beta$ chains of human hemoglobin Zürich (Caughey et al., 1969), in which the distal histidines are replaced by arginine residues which are not accommodated within the heme pocket (Perutz & Lehmann, 1968; Tucker et al., 1978). This similarity is particularly striking because of the known movement of distal histidine away from the heme in formation of the low-pH form of leghemoglobin $a$–CO (Johnson et al., 1978).

In exhibiting correlation between IR and visible behavior with changing pH, leghemoglobin $a$–CO and hemoglobin–CO resemble each other, and both appear to differ from myoglobin–CO (Table II). However, use of the wavelength pair 540 and 557 nm for absorbance differences showed a visible change in myoglobin–CO which may have a $pK_{app}^{CO}$ near 4 (Figure 4); instability of visible spectra of myoglobin–CO at pH <5 made detailed analysis impossible.

*Assignment of $pK_{app}^{CO}$ Values.* Our IR $pK_{app}^{CO}$ values appear to be associated with protonation–deprotonation of distal histidines. Of the experimental results summarized in Table II, the $^1H$ NMR experiments of Johnson et al. (1978) provide the most detailed molecular information. The simplest explanation of the $^1H$ NMR data is that decreases in pH* result in movement of the distal histidine of leghemoglobin $a$–CO away from the heme with simultaneous protonation of the distal histidine's imidazole ring (presumably protonation of $N_\epsilon$, the atom which at neutral pH is probably in van der Waals contact with bound CO). Because the $pK_{app}^{CO}$ value obtained by Johnson et al. is within reasonable agreement with all of the $pK_{app}^{CO}$ values obtained by IR-monitored titrations and with the $pK_{app}^{CO}$ values obtained by visible-monitored titrations of leghemoglobin $a$–CO and hemoglobin–CO, it is likely that in leghemoglobin $a$–CO, myoglobin–CO, and hemoglobin–CO the distal histidine side chain has a p$K$ of 4.0–4.6, and its protonation results in the change of the neutral-pH form $\nu_{CO}$ band to the low-pH form $\nu_{CO}$ band. In all cases visible changes accompany changes in $\nu_{CO}$, but in the case of myoglobin–CO a prominent visible change with $pK_{app}$ = 5.7 tends to obscure another visible change associated with change in $\nu_{CO}$.

We attribute the visible $pK_{app}^{CO}$ of myoglobin to conformational changes associated with protonation–deprotonation of a group other than the distal histidine. The $pK_{app}$ = 5.7 visible change of myoglobin–CO was attributed to the distal histidine p$K$ by Hayashi et al. (1976), but the same investigators demonstrated that the $pK_{app}$ = 5.7 visible change of myoglobin was independent of the presence or absence of CO. Since the distal histidine $N_\epsilon$ is very close to bound CO (Norvell et al., 1975), it is unlikely that the distal histidine p$K$ is independent of bound CO. It is also difficult to reconcile the $pK_{app}^{CO}$ of 4.6 for $\nu_{CO}$ change with the visible $pK_{app}$ of 5.7, unless the $pK_{app}$ = 5.7 visible change is caused by protonation of something other than the distal histidine in myoglobin–CO (and in deoxymyoglobin). $^1H$ NMR titrations of myoglobin–CO demonstrated three histidines with p$K$ < 6 (Thompson et al., 1971). Histidine $^{13}C$ NMR titrations of horse cyanometmyoglobins (Wilbur & Allerhand, 1977) demonstrated

three histidines titrating with p$K$ values between 5.3 and 5.5, one of which titrated with a p$K$ of 5.7 in horse metmyoglobin. It is also possible that the $pK_{app}$ = 5.7 visible change results from titration of a high-p$K$ carboxyl group, perhaps one which is hydrogen bonded in its protonated form or one located in a region of high negative charge density. Consistent with our suggestion that the p$K$ of the *distal* histidine of myoglobin–CO is 4.6, an approximate p$K$ assigned tentatively to the distal histidine in horse cyanometmyoglobin was 4.4 (Wilbur & Allerhand, 1977).

The slight shift in $\nu_{CO}$ of the neutral-pH form of myoglobin–CO between pH 7 and 6 might be associated with the p$K$ = 5.7 visible change. A slight pH-induced shift in $\nu_{CO}$ for trout hemoglobin IV–CO also occurs in the same pH range (Ascoli et al., 1978).

We found no evidence to support three possible alternatives to our assignment of the $pK_{app}^{CO}$ values of 4.0–4.6 to the distal histidines of leghemoglobin–, myoglobin–, and hemoglobin–CO complexes. The alternatives are to associate $pK_{app}^{CO}$ values with the p$K$ values of the proximal histidines, the p$K$ values of other (but not distal) histidines, or the p$K$ values of carboxyl groups either on the heme or on amino acid side chains. It is unlikely that protonation of the proximal histidine, which would break the nitrogen–iron(II) bond, would cause such small visible changes as we observed (Figure 7). It also appears unlikely that protonation of a remote histidine or a remote protein carboxyl group would cause as profound changes in $\nu_{CO}$ as we observed (Table I and Figures 1 and 2). In fact, soybean leghemoglobins have no remote histidines (Ellfolk & Sievers, 1971, 1974; Appleby et al., 1975; Hurrell & Leach, 1977).

*A Model Consistent with $\nu_{CO}$ Changes.* The crystallographic studies of Norvell et al. (1975) and Heidner et al. (1976), the visible studies of Hayashi et al. (1976), and the $^1$H NMR studies of Johnson et al. (1978), together with the results reported here, permit development of a working model for pH-dependent interactions between the distal histidine and bound CO in leghemoglobin *a–CO*, hemoglobin–CO, and myoglobin–CO. (On the basis of visible data, leghemoglobin $c_1$–CO and leghemoglobin $c_2$–CO resemble leghemoglobin *a*–CO.) In developing the model described here we have assumed that similarities exist among leghemoglobin *a*–CO, hemoglobin–CO, and myoglobin–CO except when experimental evidence indicated the contrary. The structures of the heme pockets of crystalline hemoglobin–CO (Heidner et al., 1976) and crystalline myoglobin–CO (Norvell et al., 1975) have been partially described. Although leghemoglobin *a*–CO has not yet been crystallized, lupin metleghemoglobin has been shown to have a similar heme pocket to the heme pockets of metmyoglobin and methemoglobin (Vainshtein et al., 1977). Therefore, we assumed that soybean leghemoglobin *a*–CO has a heme pocket similar to those of hemoglobin–CO and myoglobin–CO. As will become evident, significant differences in heme pocket behavior are also inescapable.

Accounting rigorously for the direction and magnitude of $\nu_{CO}$ shifts is not possible because a wide variety of effects can cause increases or decreases in $\nu_{CO}$ of hemeprotein-bound CO. However, for the working model we propose, two factors affecting $\nu_{CO}$ appear preeminent: geometric effects and effects due to electron donor–acceptor bonding.

The first effect is geometric. The preferred FeCO geometry of heme–CO complexes, in which a linear FeCO group is perpendicular to the heme plane [unpublished experiments of J. P. Collman, R. R. Gagne, T. R. Halbert, J. L. Hoard, C. A. Reed, and A. A. Sayler, quoted in Hoard (1975); Peng & Ibers, 1976], is distorted by steric pressures in the heme pockets of hemeproteins (Huber et al., 1970; Padlan & Love, 1974; Norvell et al., 1975; Heidner et al., 1976). Distortion takes the form of bending the FeCO group to give an FeCO angle of <180° and/or forcing the FeC axis to be less than perpendicular to the heme plane. The latter distortion appears to occur in hemoglobin CO (Heidner et al., 1976) and may occur widely (Peng & Ibers, 1976). Geometric effects on $\nu_{CO}$ are caused by variation in d–$\pi$ back-bonding between iron(II) and bound CO; decreased back-bonding results in increased $\nu_{CO}$. Although an early proposal (which successfully predicted nonlinear FeCO geometry in hemeprotein–CO complexes) tried to show that $\nu_{CO,linear} > \nu_{CO,bent}$ (Caughey, 1970), it is more likely that back-bonding between iron(II) and CO decreases whenever the FeCO group is nonlinear or not perpendicular to the heme plane. Thus $\nu_{CO,linear,perpendicular} < \nu_{CO,bent}$ (e.g., Goedken et al., 1976), and $\nu_{CO,linear,perpendicular} < \nu_{CO,linear,nonperpendicular}$ (Figure 9a).

The second effect is due to an electron donor–acceptor interaction between the unprotonated $N_\epsilon$ of the distal histidine (electron donor) and the CO carbon (electron acceptor) as proposed by Maxwell & Caughey (1976) and discussed by Satterlee et al. (1978) and Tucker et al. (1978). Such an electron donor–acceptor bond (Figure 9b) would decrease $\nu_{CO}$ by giving more double bond character to the CO carbon–oxygen bond. Protonation of $N_\epsilon$ would eliminate electron donor–acceptor bonding and thereby would increase $\nu_{CO}$.

Figure 10 shows our working model. There is demonstrably no hydrogen bonding between the distal histidine and bound CO in myoglobin–CO at pH 5.7 (Norvell et al., 1975) or hemoglobin–CO at presumably neutral pH (Heidner et al., 1976), where the distal histidines are extremely close to the hemes. Because the distal histidine is extremely close to the heme in leghemoglobin *a*–CO at neutral pH* (Johnson et al., 1978), we have assumed no histidine–CO hydrogen bonding in leghemoglobin *a*–CO at neutral pH. Instead we assume that the donor–acceptor proposal for the distal histidine–CO interaction (Maxwell & Caughey, 1976) is correct for all three CO complexes at neutral pH (Figure 10a). Since low pH form $\nu_{CO}$ values are similar for myoglobin–CO and hemoglobin–CO but not leghemoglobin *a*–CO (Table I), we suggest that protonation of the distal histidine $N_\epsilon$ results in two types of structure. In the case of myoglobin–CO and hemoglobin–CO, the protonated distal histidine moves a small distance, but the steric strain forcing a distorted FeCO geometry remains (Figure 10b). A higher $\nu_{CO}$ results from the loss of electron donor–acceptor bonding. Also, in the case of leghemoglobin *a*–CO, protonation of the distal histidine $N_\epsilon$ results in loss of electron donor–acceptor bonding. But in leghemoglobin *a*–CO the much greater movement of the protonated distal histidine away from the heme permits a near linear and perpendicular FeCO geometry (Figure 10c). The change in FeCO geometry partially compensates for loss of electron donor–acceptor binding, making the $\nu_{CO}$ value of the leghemoglobin *a*–CO low-pH form appreciably less than the $\nu_{CO}$ values of the myoglobin–CO and hemoglobin–CO low-pH forms.

The observed p$K$ values appear inconsistent with hydrogen bonding in the acid-pH forms of leghemoglobins, myoglobin, and hemoglobin: because hydrogen bonding would increase p$K$, it is unlikely that hydrogen-bonded imidazolium rings would exhibit p$K$ < 5 when histidine imidazolium rings normally exhibit p$K$ > 6.

Our frequent finding that the $n$ values resulting from titration analyses (Table II) were less than unity may mean that a more complicated set of reactions occurs, but as yet we have

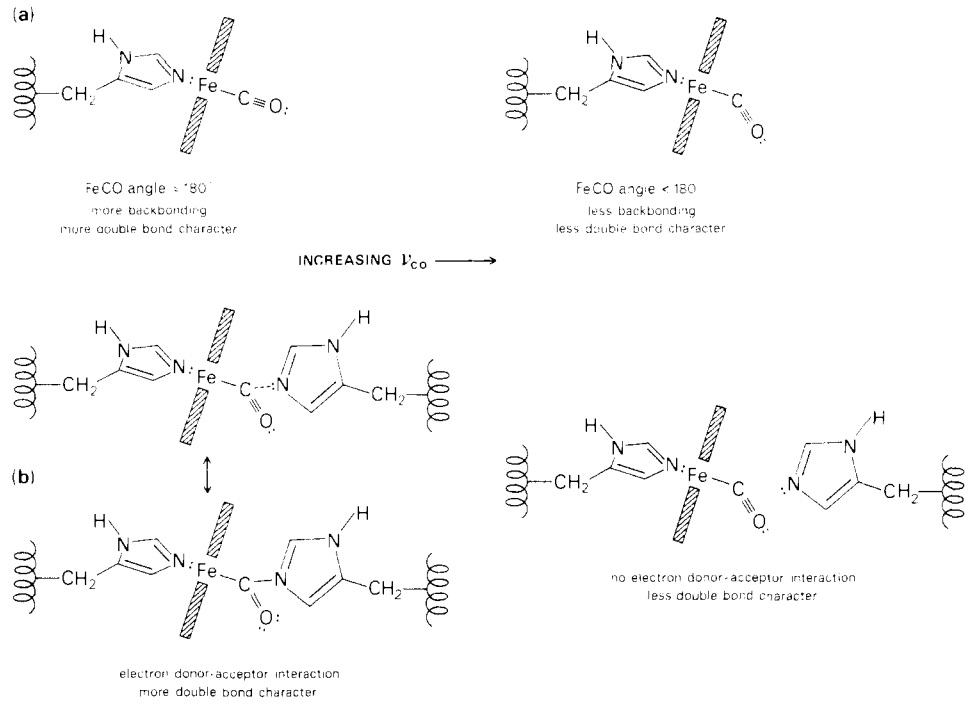


FIGURE 9: Effects upon $\nu_{CO}$ values of hemeprotein–CO complexes. (a) Effect of FeCO geometry (only bending distortion shown). (b) Effects of electron donor–acceptor bond involving the carbon of CO.

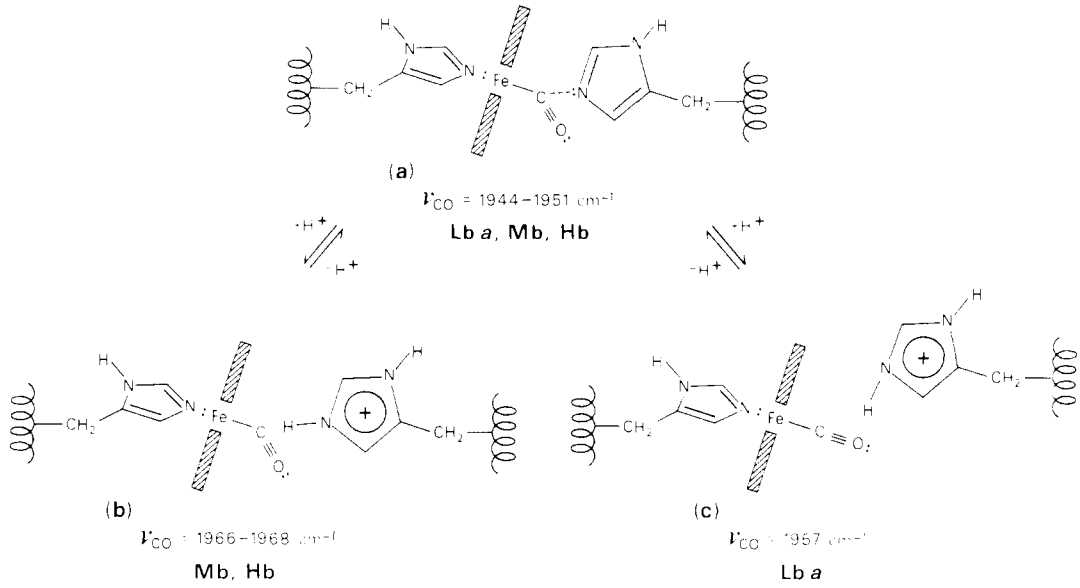


FIGURE 10: Model which fits pH-dependent behavior of CO complexes of soybean leghemoglobins *a*, $c_1$, and $c_2$, sperm whale myoglobin, and human hemoglobin A. Bent FeCO geometry is shown in neutral-pH forms and in low-pH forms of myoglobin and hemoglobin because it is more readily perceived than other geometric distortions.

found no compelling evidence that requires a more complicated model. An interesting possibility is that simultaneous protonations of heme carboxylate groups and distal histidines both contribute to the observed spectral changes.

A recent suggestion that the FeCO group is not bent but is linear in solutions of myoglobin–CO at pH 7.6 (LaMar et al., 1978) conflicts with our explanation of the differences between the low-pH forms of myoglobin–CO and leghemoglobin *a*–CO. The suggestion was based upon the assumption that the $^{13}C$–$^{57}Fe$ NMR coupling constant [$J$($^{13}C$–$^{57}Fe$)] is sensitive to FeCO geometry in heme–CO and hemeprotein–CO complexes. The IR evidence that there are two modes of CO bonding in myoglobin–CO in solution (McCoy & Caughey, 1971) and the possibilities of more than one type of distortion of the FeCO group from linearity and perpendicularity complicate interpretations of $J$($^{13}C$–$^{57}Fe$) data. It is likely that distal histidine–CO electron donor–acceptor bonding weakens the Fe–C bond (Tucker et al., 1978) and thus, in terms of $J$($^{13}C$–$^{57}Fe$) and visible spectra, would likely mimic the effects of FeCO distortion from linearity and perpendicularity. Indeed, the reported similarity in $J$($^{13}C$–$^{57}Fe$) between myoglobin–CO at pH 7.6 and *N*-methylimidazole protoheme–CO in dimethylformamide *containing a large excess of N-methylimidazole* (cf. Figure 1 of LaMar et al., 1978) suggests the interesting possibility that in the model system there is electron donor–acceptor bonding between bound CO and the unmethylated nitrogen of *N*-methylimidazole. Such bonding could explain why $\nu_{CO}$ for *N*-methylimidazole protoheme diester–CO *in N-methylimidazole as solvent* [1954 cm$^{-1}$ (Maxwell & Caughey,

1976)] is closer to the $\nu_{CO}$ values of hemoglobin–CO, myoglobin–CO, and leghemoglobin–CO than is $\nu_{CO}$ for the same protoheme diester–CO complex *in the absence of excess N-methylimidazole* [1959–1980 $cm^{-1}$ in halogenated hydrocarbon solvents (Maxwell & Caughey, 1976)].

According to our model the $\nu_{CO}$ value of the neutral-pH form of the $\beta$ chain of hemoglobin Zürich [1958 $cm^{-1}$ (Caughey et al., 1969)] might result from a near linear and perpendicular FeCO geometry permitted by steric consequences of the distal arginine's absence from the heme pocket. Unfortunately, recent crystallographic studies on hemoglobin Zürich–CO (Tucker et al., 1978) were unable to determine the FeCO geometry of the abnormal $\beta$ chain with sufficient precision to test our prediction.

Although there is still insufficient information to permit inclusion in our model of two binding modes of CO to myoglobin, our IR data provide information about the meaning of the $\nu_{CO}$ band and shoulder in IR spectra of myoglobin–CO. The presence of a shoulder is evidence for two types of binding of CO to myoglobin (McCoy & Caughey, 1971). The absence of a pH effect on the shoulder to peak absorbance ratio (Figure 5) and the presence of only one $^{13}C$ peak in $^{13}C$ NMR spectra of myoglobin–$^{13}CO$ (Moon et al., 1977) indicate that the two forms are in rapid, pH-independent equilibrium with one another. Our observed absence of a pH effect upon the shoulder to peak absorbance ratio in the acid–pH region (Figure 5) appears to be in conflict with the report of a significant pH effect (McCoy & Caughey, 1971). The earlier positive report was based upon manual determinations of the shoulder to peak integrated absorbance ratios for four widely spaced pH values (3, 5, 9, and 12). Although we have not investigated the alkaline end of the pH range, the reported shoulder to peak integrated absorbance ratio of 0.40 at pH 12 (McCoy & Caughey, 1971) is within reasonable agreement with our average absorbance ratio of 0.44. The shoulder to peak integrated absorbance ratio of 0.67 reported for pH 3 (McCoy & Caughey, 1971) may be due to the inherent difficulty in manually resolving the shoulder from the peak, to the slow changes which occur at low pH, and to the extremely low absorbances involved at pH <4.

*Assignment of $pK_{app}^{O_2}$ Values.* The $pK_{app}^{O_2}$ values for leghemoglobins and hemoglobin are probably due to p$K$ values for distal histidines. The $pK_{app}^{O_2}$ values for leghemoglobins $a$, $c_1$, and $c_2$ were between 5 and 6 (Table II) and are therefore likely to be due to histidine p$K$ values, but the visible changes were smaller (Figure 8) than would be expected if the *proximal* histidines were protonated. Since soybean leghemoglobins contain no remote residues of histidine (Ellfolk & Sievers, 1971, 1974; Appleby et al., 1975; Hurrell & Leach, 1977), $pK_{app}^{O_2}$ values for leghemoglobins $a$, $c_1$, and $c_2$ are likely due to the p$K$ values of the *distal* histidines. However, we cannot rule out the possibility that $pK_{app}^{O_2}$ values are due to the p$K$ values of high-p$K$ carboxyl groups. The visible $pK_{app}^{O_2}$ value of hemoglobin is so close in value to hemoglobin's IR and visible $pK_{app}^{CO}$ values (Table II) that the assignment of $pK_{app}^{O_2}$ to the p$K$ of the distal histidine of hemoglobin $O_2$ is reasonable.

The visible $pK_{app}^{O_2}$ of myoglobin is more difficult to interpret. The similarities of visible $pK_{app}^{O_2}$ (Table II), visible $pK_{app}^{CO}$ (Table II), and visible $pK_{app}^{deoxy}$ [5.6 (Hayashi et al., 1976)] values of myoglobin indicate that the presence or absence of a sixth ligand has no effect upon the myoglobin visible $pK_{app}$. Therefore, it seems likely that the visible $pK_{app}^{O_2}$, like the visible $pK_{app}^{CO}$, is associated with the p$K$ of a group other than the distal and proximal histidines in myoglobin–$O_2$. Since the $pK_{app}^{O_2}$ for myoglobin is similar in value to the $pK_{app}^{O_2}$ values for leghemoglobins (Table II), it also is possible that in myoglobin–$O_2$ the distal histidine and other group(s) titrate simultaneously. Because the titrations of different groups would be expected to have different effects upon the visible spectrum of the heme, we cannot eliminate the possibility of simultaneous titration by examining the experimental value of $n$. However, we suspect that the distal histidine p$K$ of myoglobin–$O_2$, like that of hemoglobin–$O_2$, is in fact <5 but experimentally inaccessible because of degradation at pH <5.

*Sixth-Ligand Effects on Leghemoglobins.* The soybean leghemoglobins are strikingly different from hemoglobin and myoglobin in that their $pK_{app}^{O_2}$ values are unambiguously and significantly different from their $pK_{app}^{CO}$ values (Table II).

Differences between the $pK_{app}^{O_2}$ and $pK_{app}^{CO}$ values of leghemoglobins can be explained in terms of the preferred geometries for $O_2$ and CO binding to hemes. In free heme–CO complexes, the FeCO angle is 180° and the FeCO axis is perpendicular to the heme plane [unpublished experiments of J. P Collman, R. R. Gagne, T. R. Halbert, J. L. Hoard, C. A. Reed, and A. A. Sayler, quoted in Hoard (1975); Peng & Ibers, 1976], whereas in free heme–$O_2$ complexes, the FeOO group is nonlinear (Collman et al., 1975). Since the heme pocket evolved for $O_2$ binding, steric constraints inside the heme pocket distort the geometry of the FeCO group in hemeprotein–CO complexes (Huber et al., 1970; Padlan & Love, 1974; Norvell et al., 1975; Heidner et al., 1976), whereas in hemeprotein–$O_2$ complexes it is likely that the FeOO geometry is nearly the same as in free heme–$O_2$ complexes. The resulting disturbance of the heme pocket by bound CO but not by bound $O_2$ could well lead to differences in various properties [e.g., Wright & Appleby (1977)], including pH-dependent behavior.

The observation that $pK_{app}^{O_2} \neq pK_{app}^{CO}$ could be explained either in terms of a flexible heme pocket or in terms of a rigid heme pocket, but $^1H$ NMR data on leghemoglobin $a$–CO (Johnson et al., 1978) favor a flexible heme pocket. Therefore, we propose that flexibility within the E helixes or at the EF junctions of leghemoglobins must be great enough to cause a change in the heme pocket when CO is replaced with $O_2$. This conclusion holds whether $pK_{app}^{O_2}$ values are due to distal histidines or high-p$K$ carboxyl groups. If our assignment of $pK_{app}^{O_2}$ values to the p$K$ values of distal histidines is correct, we can further conclude the following from the fact that $pK_{app}^{O_2} > pK_{app}^{CO}$: at neutral pH the distal histidines in $O_2$ complexes of leghemoglobins are not as close to $O_2$ as they are to CO in CO complexes.

*Biological Implications.* Our results permit comparisons of the active sites of soybean leghemoglobins with those of myoglobin and hemoglobin, comparisons among the active sites of soybean leghemoglobins $a$, $c_1$, and $c_2$, and evaluation of the biological significance of possible acid-pH effects upon CO and $O_2$ binding.

The heme pockets of soybean leghemoglobin CO complexes appear to be more flexible about the distal histidine than do the heme pockets of CO complexes of myoglobin and hemoglobin. Low-pH forms of soybean leghemoglobins exhibited $\nu_{CO}$ values which were appreciably lower in frequency than the $\nu_{CO}$ values of the low-pH forms of myoglobin and hemoglobin (Table I), and these differences are explicable by a model (Figure 10) in which the distal histidines of leghemoglobins are freer to move upon protonation than are the distal histidines of myoglobin and hemoglobin.

(a) Heme ring contacts on distal histidine side

| | E11 | G8 | CD1 |
|---|---|---|---|
| Hb A α | val 62 | leu 101 | phe 43 |
| Hb A β | val 67 | leu 106 | phe 42 |
| Mb | val 65 | ile 104 | phe 42 |
| Lb a | leu 65 | val 104 | phe 44 |
| Lb $c_1$ | leu 65 | val 104 | phe 44 |
| Lb $c_2$ | leu 65 | val 104 | phe 44 |

(b) E helices

| | |
|---|---|
| Hb A α | ser-ala-gln-val-lys-gly-his-gly-lys-lys-val-ala-asp-ala-leu-thr-asn-ala-val-ala |
| Hb A β | asn-pro-lys-val-lys-ala-his-gly-lys-lys-val-leu-gly-ala-phe-ser-asp-gly-leu-ala |
| Mb | ser-glu-asp-leu-lys-lys-his-gly-val-thr-val-leu-thr-ala-leu-gly-ala-ile-leu-lys |
| Lb a | asn-pro-lys-leu-thr-gly-his-ala-glu-lys-leu-phe-ala-leu-val-arg-asp-ser-ala-gly |
| Lb $c_1$ | asn-pro-lys-leu-thr-gly-his-ala-glu-lys-leu-phe-ala-leu-val-arg-asp-ser-ala-gly |
| Lb $c_2$ | asn-pro-lys-leu-thr-gly-his-ala-glu-lys-leu-phe-gly-leu-val-arg-asp-ser-ala-gly |

(c) E-F regions

| | |
|---|---|
| Hb A α | his-val-asp-asp-----met-pro-asn-ala |
| Hb A β | his-lys-asp-asn-----leu-lys-gly-thr |
| Mb | lys-lys-gly-his-----his-glu-ala-glu |
| Lb a | leu-lys-ala-ser-gly-thr-val-val-ala |
| Lb $c_1$ | leu-lys-ala-ser-gly-thr-val-val-ala |
| Lb $c_2$ | leu-lys-ala---------thr-val-val-ala |

FIGURE 11: Comparisons of portions of amino acid sequences of soybean leghemoglobins *a*, $c_1$, and $c_2$ with homologous portions of amino acid sequences of human hemoglobin A $\alpha$ and $\beta$ chains and sperm whale myoglobin. Alignment of hemoglobin, myoglobin, and leghemoglobin *a* sequences is that of Dayhoff (1972). Heme contacts on the distal histidine side are taken from Perutz et al. (1968).

Leghemoglobin–$O_2$ complexes appear to have more flexible heme pockets than does hemoglobin–$O_2$. Comparisons of $pK_{app}^{O_2}$ and $pK_{app}^{CO}$ values (Table II) indicate rigidity in the hemoglobin heme pocket ($pK_{app}^{O_2} \simeq pK_{app}^{CO}$) and flexibility in the heme pockets of leghemoglobins ($pK_{app}^{O_2} \neq pK_{app}^{CO}$).

We propose that deoxyleghemoglobins have more open pockets than deoxyhemoglobin, but that the leghemoglobin pockets close around bound $O_2$ to give oxygenated heme pockets similar to those of hemoglobin–$O_2$ and probably myoglobin–$O_2$. Leghemoglobins' unusually high rates of $O_2$ (and CO) binding, normal rates of $O_2$ (and CO) dissociation, and thus high affinities for $O_2$ (and CO) can be explained in molecular terms by our proposal. Our experimental results show that the requisite flexibility is present in leghemoglobins.

While the CO complexes of soybean leghemoglobins *a*, $c_1$, and $c_2$ differ only slightly from each other (Tables I–III), it is clear from $\lambda_{max}$ values (Table IV) and titration data (Table II) that leghemoglobin $c_2$–$O_2$ differs appreciably from leghemoglobin *a*–$O_2$ and leghemoglobin $c_1$–$O_2$. Alignment of the amino acid sequences of soybean leghemoglobin *a* [Sievers & Ellfolk (1971) as corrected by Sievers & Ellfolk (1974)], soybean leghemoglobin $c_1$ [partially determined by Nicola (1975)], and soybean leghemoglobin $c_2$ [Hurrell & Leach, 1977] with the amino acid sequences of human hemoglobin A $\alpha$ and $\beta$ chains and sperm whale myoglobin (Dayhoff, 1972) indicate that among leghemoglobins *a*, $c_1$, and $c_2$ there are no differences in the amino acid residues likely to make contact with the heme on the distal histidine side (Figure 11a). The E helixes of leghemoglobins *a*, $c_1$, and $c_2$ are almost identical, except for a glycine in leghemoglobin $c_2$ where leghemoglobins *a* and $c_1$ have alanine (Figure 11b). More significantly, in the EF region, which might control motion of the entire E helix (which contains the distal histidine), leghemoglobins *a* and $c_1$ contain two more amino acid residues than does leghemoglobin $c_2$ (Figure 11c).

The $O_2$ complexes of soybean leghemoglobins *a* and $c_1$ are significantly different from the $O_2$ complex of leghemoglobin $c_2$ in their responses to pH changes in the pH range 5.5–6.5 (a physiologically significant pH range in root nodules). The degree of protonation of the distal histidine is likely to be related to the affinity for $O_2$. Therefore, a differential pH effect upon $O_2$ affinities under physiological conditions is suggested, as is a different percent oxygenation of leghemoglobin $c_2$ compared with leghemoglobins *a* and $c_1$ under fixed conditions of pH. At pH 7.0 unseparated leghemoglobin *c* has about half the oxygen affinity of leghemoglobin *a* (Appleby, 1962). Since leghemoglobin *c* is composed of leghemoglobin $c_1$ as well as leghemoglobin $c_2$, and since pH 7.0 is sufficiently distant from the observed visible $pK_{app}^{O_2}$ values to partially mask differences in $O_2$ affinities, an $O_2$ affinity difference of >50% between leghemoglobins *a* and $c_2$ may exist under physiologically significant conditions. Experiments testing this possibility are in progress.

Acid Bohr effects in hemeproteins might be associated with distal histidine protonation. For example, our observed pH-dependent changes in hemoglobin–CO and $-O_2$ visible spectra at acid pH (Figures 7 and 8) might be associated with the acid Bohr effects on the CO and $O_2$ affinities of hemoglobin (cf. Figure 10 of Rifkind, 1973; Antonini et al., 1963). The $pK_{app}$ values in the 4.0–4.6 range are too low for biological significance, but slight, visible changes at pH >6 (Figures 4 and 6) offered tantalizing evidence that the biologically significant alkaline Bohr effects of hemoglobin–CO and $-O_2$ complexes also might be associated with pH-dependent visible changes. Experiments examining whether the pH-dependent visible changes in oxyleghemoglobins (Table II and Figure 8) are associated with Bohr effects are in progress.

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