Proc. Natl. Acad. Sci. USA
Vol. 78, No. 5, pp. 2903–2907, May 1981
Biochemistry

Dynamic protein structures: Infrared evidence for four discrete rapidly interconverting conformers at the carbon monoxide binding site of bovine heart myoglobin

(heme protein/infrared spectroscopy)

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Communicated by Marshall Fixman, February 13, 1981

ABSTRACT **Infrared spectra for the carbon monoxide complex with myoglobin isolated as the oxygenyl species from bovine heart muscle were carefully examined in the C—O stretch region as either the pH or the temperature was varied. Deconvolutions of these spectra into bands of Gaussian shape suggest the presence of four bands near 1938(I), 1944(II), 1954(III), and 1965(IV) cm^{-1} with halfband widths of about 18, 9, 9, and 10 cm^{-1}, respectively. The relative intensities of the four bands varied with changes in pH or temperature. ^{13}C NMR spectra and other evidence indicate that the four C—O stretch bands arise from four discrete rapidly interconverting conformers: CI, CII, CIII, and CIV. Under conditions of physiological pH and temperature, the relative stabilities are CI ≈ CII ≫ CIII ≈ CIV. The ΔH and ΔS values for conformer interconversions are estimated to range from −8 to 34 kJ/mol and −27 to 87 $J \cdot mol^{-1} K^{-1}$, respectively; therefore the structures of the conformers may be expected to vary significantly. These findings provide evidence for a highly flexible, dynamic structure at the ligand-binding site of bovine myoglobin, even when ligands are bound.**

The early x-ray crystallographic structures reported by Kendrew *et al.* (1) for sperm whale myoglobin and by Perutz *et al.* (2) for hemoglobins increased dramatically our ability to perceive protein structures. However, these structures have been frequently regarded as indicative of a far more rigid structure than is possible if a ligand such as O_2 is to gain entry to, or exit from, the binding site at heme iron within the globin. Also inconsistent with only one structure are the multiple C—O stretch bands for myoglobin carbonyls first reported by McCoy and Caughey in 1971 (3). Nevertheless, a subsequent neutron diffraction study for sperm whale myoglobin carbonyl indicated only one location for CO (4). Multiple C—O stretch bands also became apparent during a wide survey of human and animal hemoglobins (5, 6); changes in pH and temperature as well as globin structure altered these bands (7, 8). Because crystallization was considered as a possible reason for only one location for CO in the structures from crystallographic studies (9, 10), infrared spectra for crystals of the carbonyls for Hb A and Hb Zurich were examined and found to be similar to solution spectra (11). Spectra for sperm whale Mb carbonyl in solution and in a crystal can differ; multiple C—O bands are present in both cases (7, 12).

Here we report the detailed measurements of spectra in the C—O stretch region for bovine heart MbCO, the deconvolution of these spectra into individual bands, and the effects of changes in temperature and pH. We conclude, on the basis of our findings, that four C—O bands are present and arise from four discrete rapidly interconverting conformers of a highly flexible, structurally dynamic protein.

EXPERIMENTAL

Oxygenated myoglobin (MbO_2) was isolated from bovine heart muscle and purified by the procedures of Yamazaki *et al.* (13) and Gotoh and Shikama (14). The purified MbO_2 was found to be homogeneous on the basis of both polyacrylamide gel electrophoresis and gel permeation chromatography by using a Toyo Soda 3000 SW apparatus (15). The purified MbO_2 was concentrated to ≈3 mM by adsorption on DEAE-cellulose followed by elution with 0.1 M Tris·HCl at pH 8.5. All preparative steps were carried out at 4°C unless described otherwise. MbO_2 was converted to MbCO by passing CO gas over the surface of a stirred solution. Further concentration of MbCO was achieved in an Amicon ultrafiltration apparatus under CO. To change the medium for MbCO to 0.1 M potassium phosphate of appropriate pH, dialysis was carried out for 4 hr with semimicro dialysis tubing (dry thickness, 0.04 mm; Spectrum Medical Industries, Los Angeles). Transfer of the sample into an infrared cell with CaF_2 windows (sample path length, 0.10 mm) was done via a gas-tight syringe under a CO atmosphere. The sample in the infrared cell was shown to be free from discernible amounts of metMb by visible spectral measurements with a Cary 17 spectrophotometer. The MbCO concentration was determined on the basis of an extinction coefficient of 14.9 $mM^{-1} \cdot cm^{-1}$ at 542 nm, the wavelength of one of the isosbestic points associated with a pH-dependent change in the visible spectrum (unpublished data).

Infrared spectra were recorded on a Perkin–Elmer model 180 infrared spectrophotometer interfaced with a Tektronix graphic computer model 4051. Seven to 12 single scans between 2000 and 1900 cm^{-1} with a resolution of 4 to 2.6 cm^{-1} were accumulated and averaged by the computer. The averaged spectrum was stored for further manipulation of spectral data. Temperatures of both sample and reference cells were controlled; the temperature of the medium was monitored by a copper/constantan thermocouple inserted in a cell window. The reference cell, well matched to the sample cell, contained the same buffer as the sample and its temperature was kept within 1°C of the sample cell as required to get a flat baseline in the infrared spectrum. Other details of spectral measurements have been described (8, 16, 17). After the spectra were measured, the pH of the sample in the cell was measured with a combination microelectrode (Microelectrodes, Londonderry, NH).

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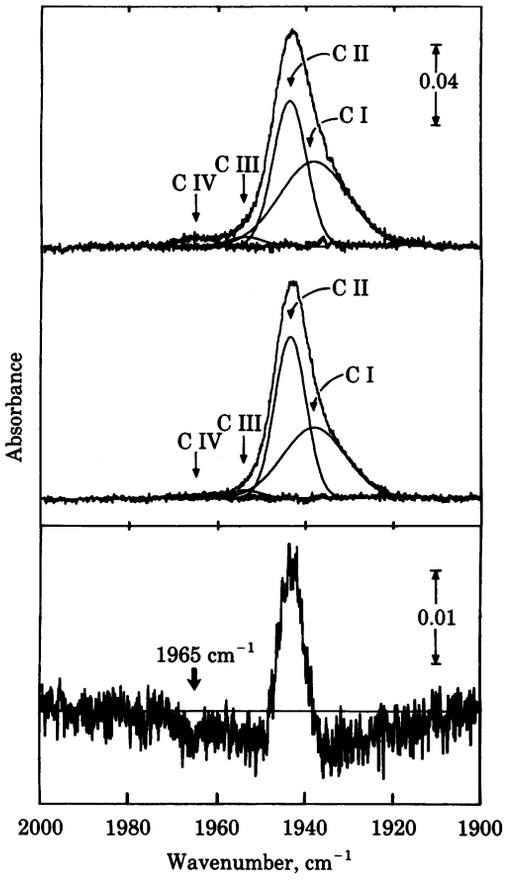

FIG. 1. Infrared spectra of CO bound as a ligand to Mb: Effects of temperature. Spectrum of 4.6 mM MbCO in 0.1 M potassium phosphate buffer at pH 7.75 (20°C) was measured relative to the buffer. Spectra represent a computer average of 7–12 accumulated single scans. (*Top*) Spectrum of MbCO at 35°C. (*Middle*) Spectrum of MbCO at 8.0°C. (*Bottom*) Computer-generated difference spectrum between the spectra at 8.0 and 35°C. Curves for deconvoluted bands are computed Gaussian curves with parameters (frequency, bandwidths, maximal absorption) as indicated in Table 1. A residual spectrum (i.e., the difference between the observed spectrum and the sum of the Gaussian curves) is shown to demonstrate the success of the curve-fitting procedure.

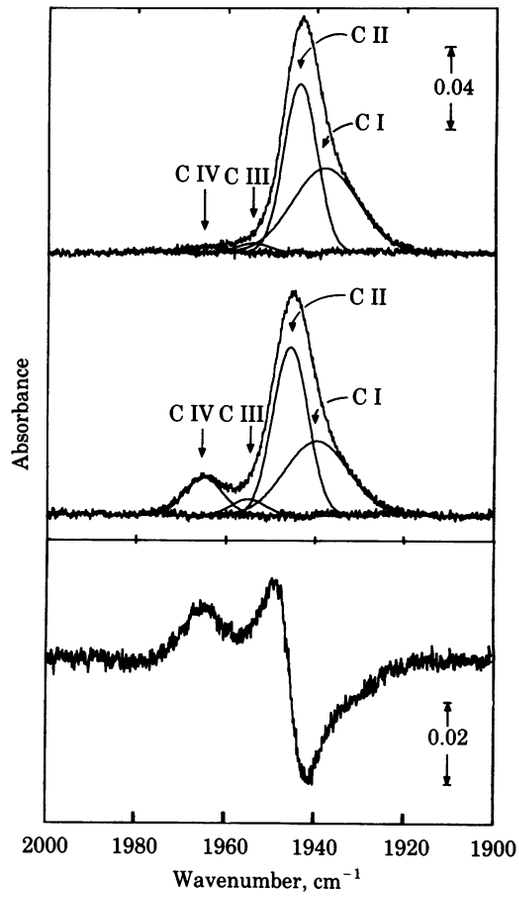

FIG. 2. Infrared spectra of MbCO: Effects of pH. Spectra represent a computer average of 10 accumulated single scans at 20°C. (*Top*) Spectrum of 4.6 mM MbCO in 0.1 M potassium phosphate buffer at pH 7.75 (20°C) with only the buffer in the reference cell. (*Middle*) Spectrum of 4.4 mM MbCO in the same buffer as above but at pH 5.18 (20°C). (*Bottom*) Computer-generated difference spectrum between the spectra at pH 5.18 and 7.75 after the pH 7.8 spectrum had been normalized to that expected if the concentration were the same as in the solution giving the pH 5.2 spectrum.

RESULTS

Temperature Effects on C—O Stretch Bands. Infrared spectra in the C—O stretch region for bovine MbCO at pH 7.8 for three different temperatures (8, 20, and 35°C) are shown in Figs. 1 and 2. When the solution was cooled from 35°C to 8°C, the large asymmetric band with a maximum at 1944 cm^{-1} became stronger and narrower whereas the minor band at 1965 cm^{-1} became less intense. The temperature difference spectrum (8°C minus 35°C) of Fig. 1 contains an intense positive symmetric band at 1944 cm^{-1}, a broad negative band between ≈1960 and ≈1920 cm^{-1}, and a small negative band at 1965 cm^{-1}. These spectra provide clear evidence for the presence of at least three bands and for changes in spectrum with a change in temperature.

The nature of the multiple C—O stretch bands was explored by curve-fitting procedures in which both Lorentzian and Gaussian shapes were assumed. We used a program that allowed the computer to generate component spectra and to plot a difference spectrum for the observed spectrum minus the sum of the generated component spectra. The degree to which this difference spectrum corresponded to a flat baseline showed us the closeness of fit between the computed spectrum and the recorded spectrum. Good agreement was not obtained between the observed spectrum and the sum of three or four bands assumed

Table 1. Infrared C—O band parameters for conformers I–IV

		CI		CII		CIII		CIV	
Temp., °C	pH	ν_{CO} ($\Delta\nu^1/_2$), cm^{-1}	$A \times 10^2$	ν_{CO} ($\Delta\nu^1/_2$), cm^{-1}	$A \times 10^2$	ν_{CO} ($\Delta\nu^1/_2$), cm^{-1}	$A \times 10^2$	ν_{CO} ($\Delta\nu^1/_2$), cm^{-1}	$A \times 10^2$
8	7.8	1938 (17)	4.0	1943.4(8.4)	9.1	1953.6(8.0)	0.46	1965(10)	0.15
20	5.2	1939.5(17)	3.1	1945.5(9.3)	7.0	1955 (9.0)	0.70	1965(10)	1.60
20	7.8	1938(17.5)	4.1	1943.6(8.8)	8.2	1954.2(8.5)	0.46	1965(10)	0.30
35	7.8	1938(18.5)	4.2	1943.5(9.0)	7.2	1953.5(9.0)	0.48	1965(10)	0.45

Table 2. Integrated C—O band areas for conformers I–IV

Temp., °C	pH	Total area*	% of total band area			
			CI	CII	CIII	CIV
8	7.8	1.59	45.4	51.1	2.5	1.0
20	5.2	1.57†	37.6	46.5	4.5	11.4
20	7.8	1.60	47.6	47.8	2.6	2.0
35	7.8	1.61	51.3	42.8	2.9	3.0

* The area of each conformer was calculated as: area = $1.064 \times \Delta\nu_{1/2} \times$ maximum absorbance.
† The value was normalized to the spectrum at pH 7.8.

to have Lorentzian shapes; large deviations appeared in the "wings" unless an unreasonably large number (≈10) of bands was used. However, with Gaussian shapes, close agreement between the sum of four bands and the observed spectrum was obtained in each case—i.e., the flat difference spectra of Figs. 1 and 2. The four bands from low to high wavenumber are designated CI, CII, CIII, and CIV. The ν_{CO} and $\Delta\nu_{1/2}$ values for these bands are given in Table 1 and the relative integrated band areas are given in Table 2. A change in temperature affected frequencies and bandwidths only subtly but altered the relative intensities markedly.

Independent direct support for the computer-derived shapes of bands CI, CII, and CIV is found in temperature difference spectra (Fig. 1). The intensities of CI, CIII, and CIV increase as the temperature increases whereas CII loses intensity; the overall integrated intensity (i.e., the sum of the areas for CI, CII, CIII, and CIV) remains essentially constant as shown in Table 2. These shifts due to temperature changes are completely reversible and are found in Tris as well as phosphate buffer.

Effects of Changes in pH on C—O Stretch Bands. Infrared spectra remained essentially unchanged from pH 7.3 to pH 11. However, below pH 7.3, reversible pH-dependent changes occurred. A decrease in pH from 7.8 to 5.2 at 20°C resulted in a blue shift of 2 cm^{-1} for the large asymmetric band and an intensification of the 1965 cm^{-1} band (Fig. 2). The pH 5.2 minus pH 7.8 difference spectrum exhibits a minimum at 1942 cm^{-1} and maxima at 1949 and 1965 cm^{-1}. The curve-fitting procedure

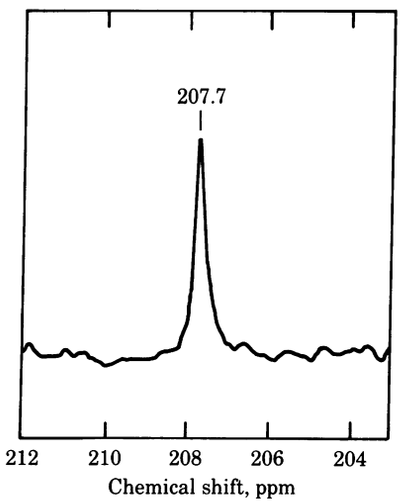

FIG. 3. ^{13}C NMR spectrum of CO (90% ^{13}C) bound to myoglobin from beef heart muscle. The spectrum of 5 mM MbCO in 50 mM Tris·HCl buffer at pH 8.5 was obtained by using a JEOL-FX-100 Fourier transform spectrometer operating at 25.05 MHz and 4°C; 9620 scans were accumulated. The results are shown as shift from tetramethylsilane.

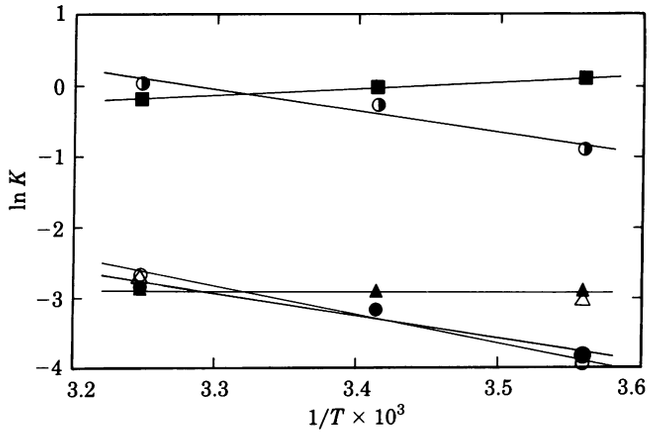

FIG. 4. Plots of equilibrium constants (ln K) among four conformers against the inverse of temperature in K ($1/T$): ●, Equilibrium between the CI and CIV conformers; here the equilibrium constant represents the ratio of the relative band area of CI to that of CIV (K = CIV/CI). ○, Equilibrium between CII and CIV. ◑, Equilibrium between CIII and CIV. ▲, Equilibrium between CI and CIII. △, Equilibrium between CII and CIII. ■, Equilibrium between CI and CII.

for the pH 5.2 spectrum also gave four bands with parameters as listed in Table 1. As found for changes in temperature, a change in pH does not alter ν_{CO} or $\Delta\nu_{1/2}$ greatly but can cause a marked redistribution among band intensities (Table 2). When either the temperature was increased or the pH was decreased, the intensity of CII decreased whereas the intensities of CIII and CIV increased. However, with CI, raising the temperature increased intensity but lowering the pH decreased intensity. Whichever method of perturbation was used, the total integrated areas remained almost constant. The data of Table 2 show that a change in temperature of 27°C is approximately as effective as a change in pH of 1.6 pH units in terms of altering the distribution of intensity among the four computed bands.

^{13}C NMR Spectrum. A solution of Mb^{13}CO produced a ^{13}C NMR spectrum with a single resonance due to the carbonyl carbon at 207.7 ppm from tetramethylsilane (Fig. 3). This result compares with values of 207.9, 207.7, and 207.8 ppm from tetramethylsilane for the single ^{13}CO bands reported by Moon *et al.* (18) for carbonyl Mbs from sperm whale, dolphin, and horse, respectively. Thus, only a single resonance is seen in carbonyl ^{13}C NMR spectra whereas four bands are evident in the infrared C—O stretch region. As discussed below, the single resonance is reasonably explained by the four conformers undergoing interconversions at rates greater than the ^{13}C NMR time scale.

ΔH and ΔS Values for Conformer Interconversions. Rough calculations of thermodynamic parameters (ΔH, ΔS) for con-

Table 3. Estimated enthalpy and entropy differences for interconversion between MbCO conformers in 0.1 M potassium phosphate at pH 7.8

Transition	$\Delta H°$, kJ/mol	$\Delta S°$, $J \cdot mol^{-1} \cdot K^{-1}$
CI → CII	−8.0 ± 0.8	−27 ± 3
CI → CIII	0.8 ± 0.1	−21 ± 1
CI → CIV	26 ± 6	60 ± 20
CII → CIII	8.8 ± 0.9	6.1 ± 3.0
CII → CIV	34 ± 5	87 ± 16
CIII → CIV	25 ± 6	81 ± 21

$\Delta H°$ and $\Delta S°$ values are based upon analyses as illustrated in Fig. 4. Uncertainties are based on the SD of the regression coefficient (i.e., slope and intercept) associated with a simple linear regression analysis.

former interconversions at pH 7.8 may be made from the relative band area data in Table 2. Because the total integrated areas remain essentially constant, relative areas can be considered to be measures of relative concentrations. Plots of equilibrium constants among the four conformers versus the reciprocal of temperature are shown in Fig. 4. Values of $\Delta H°$ and $\Delta S°$ are calculated from the intercepts at $1/T = 0$ and the slopes of these plots (Table 3).

DISCUSSION

The infrared spectra for bovine Mb carbonyl under different pH and temperature conditions reveal multiple C—O stretch bands. The high reproducibility and accuracy of the infrared spectra obtained made it possible to apply curve-fitting techniques for the resolution of component bands. The most satisfactory fitting with a minimum number of bands was achieved by utilizing four bands of Gaussian shape with the frequency, width, and intensity parameters listed in Tables 1 and 2. The multiple bands are found to be interconvertible and thus are not reasonably ascribed to protein heterogeneity, a view supported by the homogeneity demonstrated electrophoretically and chromatographically. This feature of multiple bands is shared by all other Mbs studied thus far. Three bands were evident in less-refined spectra for sperm whale Mb carbonyl both in solutions and in crystals (7, 12).

We chose graphic procedures and Gaussian band shapes for curve fitting. Digital programming, in which linear or nonlinear least-squares methods are used, is often preferred because of greater precision in a mathematical sense compared with the graphic approach (19) but errors from badly chosen initial band parameters, an inability to detect small bands that are overlapped by large bands, and the excessive time required for computing are frequently encountered disadvantages of the digital method (20–23). The analog method or graphical procedure of curve fitting is applicable for the resolution of moderately overlapping bands and gives the user the ability to recognize small bands among overlapping large bands, to establish input data rapidly, and to be in complete control of the process in order to develop a model that makes sense in terms of other evidence (23). However, because the graphic method cannot easily consider a large number of parameters at the same time, the method is not preferred for a very complicated spectrum or an analytical function that involves many parameters. Vandeginste and Galen (20) discussed the sources of error associated with curve fitting and emphasized the importance of knowing the number of bands and the position of the baseline before curve fitting is attempted. It therefore is most helpful that at least three bands are easily estimated from the spectra, especially the temperature difference spectra. Furthermore, with the MbCO system the position of the baseline (a flat baseline) can be estimated without difficulty by keeping the reference and sample cells under identical conditions while the spectrum is recorded. As many have pointed out, if the results obtained by curve fitting are to have physical meaning, it is important that independent evidence in support of those results be obtained. Therefore, the shifts in spectrum found with changes in temperature or pH provide critical evidence for the number and the nature of the bands involved.

The choice of the Gaussian function to describe the shape of the C—O stretch bands was made for the practical reason that more self-consistent, more satisfactory curve fitting was obtained than was the case if Lorentzian (or Cauchy) functions were used. Ramsay (24) represented the true shape of a single absorption band ($\Delta\nu_{1/2} \approx 10\ cm^{-1}$) of a carbonyl in a liquid by a Lorentzian function. Seshadri and Jones (25) viewed the evidence that, although collisions between molecules in liquid give rise to a Lorentzian shape, other effects such as solvent–solute interactions and the existence of multiple conformations produce a Gaussian shape. They also noted that instrumental problems generally modify the band shape toward the Gaussian function. In this regard, in the work reported here, spectra obtained at four different resolutions (3.8, 3.0, 2.4, and 1.5 cm^{-1} at 1944 cm^{-1}) gave identical curve-fitting results. We conclude that the individual bands are due to different conformers of MbCO and are of the Gaussian form because of interactions of the C—O vibrator with its immediate environment provided by protein or medium.

We conclude from ^{13}C NMR evidence that at least the two major conformers (CI and CII), and probably all four conformers, can interconvert more rapidly than the NMR time scale ($\approx 10^{-4}$ sec). For example, a ^{13}C NMR spectrum for bovine Mb^{13}CO under neutral conditions at 4°C revealed only a single resonance at 207.7 ppm. Under similar conditions at 20°C, HbCO A gave resonances for α and β subunits at 206.4 and 206.0 ppm and Hb Zurich CO gave resonances at 206.5 and 205.5 ppm (8). Because the C—O stretch (ν_{CO}) for the α and β subunits of Hb A differ in the major bands by only $\approx 2\ cm^{-1}$ and for Hb Zurich, by $\approx 8\ cm^{-1}$ whereas bands I and II for the MbCO differ by $\approx 6\ cm^{-1}$, ^{13}C NMR resonances for CI and CII may be expected to be resolvable unless these conformers are interconverting too rapidly. Further support for the resolvability of slowly interconverting conformers is the fact that Mb is only ≈25% the molecular weight of the Hb tetramer and therefore, on the basis of weight alone, should give better resolved NMR spectra. For these reasons, we consider a reasonable explanation of the infrared and ^{13}C NMR results is that rates of interconversions among conformers CI, CII, CIII, and CIV are less than the infrared time scale ($\approx 10^{-13}$ sec) but greater than the NMR time scale.

The reasonable assumption that CI, CII, CIII, and CIV are in an equilibrium that is perturbed by a change in temperature permits the estimation of ΔH and ΔS values for the interconversions between these conformers at pH 7.8 (Fig. 3; Table 3). To go from CI to CII is favorable in terms of ΔH but not of ΔS. Transition of CI ($\Delta\nu_{1/2} = 17.5\ cm^{-1}$ at 20°C) to either CII ($\Delta\nu_{1/2} = 8.8\ cm^{-1}$) or CIII ($\Delta\nu_{1/2} = 8.5\ cm^{-1}$) is accompanied by both a negative ΔS and a decrease in bandwidth. We suggest that these changes in ΔS and $\Delta\nu_{1/2}$ are related in the sense that a greater $\Delta\nu_{1/2}$ indicates a less uniform environment about the CO; in other words, there may be more "wiggle" in the amino acid residues or solvent molecules that are adjacent to the ligand when the bandwidth is greater (17, 26). However, the CI-to-CIV transition is favorable in terms of ΔS despite a decrease in $\Delta\nu_{1/2}$. That the CI, CII, or CIII transition to CIV is highly unfavorable in terms of ΔH and a favorable ΔS suggests the involvement of a considerable change in structure away from the more stable structures to form CIV. However, because the $\Delta\nu_{1/2}$ is decreased in the case of the CI-to-CIV change, the large positive ΔS associated with conformation change in the protein can be considered to outweigh the decrease in ΔS associated with a smaller $\Delta\nu_{1/2}$. When ΔH is plotted against ΔS, the value of ΔH is proportional to ΔS, $\Delta H = 0.31\,\Delta S + 4.75$, with the square of the coefficient of linear correlation = 0.95. Such a relationship between ΔH and ΔS suggests that the transitions between conformers are controlled by similar factors, presumably the dynamic flexibility of protein about the CO ligand. X-ray studies over a wide temperature range by Frauenfelder *et al.* (27) provided evidence for protein flexibility in sperm whale Mb but whether the areas of flexibility involved in the x-ray and CO infrared data are related is by no means clear. Furthermore, Case and Karplus (28) considered theoretical aspects of the

movement in protein structure required for ligand entry and exit.

The distribution among four, and only four, conformers is dependent on pH as well as temperature. The effects of a change in pH from 5.2 to 7.8 at 20°C (shown in Fig. 2 and Tables 1 and 2) are fully reversible. Similar effects have been observed over a much wider range of pH and will be discussed in detail elsewhere. Bands have been reported near 1970 cm^{-1} ($\Delta\nu_{1/2} \approx 20$ cm^{-1}) for MbCO and HbCO denatured by exposure to extremes in pH (7, 29, 30). These results raise the possibility that CIV should be considered to be a denatured form. However, this does not appear to be the case because $\Delta\nu_{1/2}$ for CIV is less than one-half the value for the denatured products and CIV is interconvertible with CI, CII, and CIII. Also, the spectral features presented were always found in each of many different Mb preparations. We conclude that CIV is a true conformer of the native Mb. We have also reported a band at $\approx$1968 cm^{-1} similar to CIV for hemoglobins as well as for sperm whale MbCO (6, 7, 12). Fuchsman and Appleby (31) reported the appearance of a band at 1966 cm^{-1} ($\Delta\nu_{1/2} = 9$ cm^{-1}) from sperm whale MbCO when the pH was decreased. They ascribed this band to a protonated form of MbCO with pK = 4.6. We find CIV to intensify without discernible changes in ν and $\Delta\nu_{1/2}$ as the pH is decreased and, if we assume CIV to be due to the product of the association of one proton with one MbCO molecule, the protonation would obey pK < 5. The 1966 cm^{-1} band of Fuchsman and Appleby and CIV thus share similar characteristics. However, we observed CIV even at pH 7.8 and its intensity was increased by raising the temperature to an extent that is accompanied by a change in pH of only $\approx$0.1 unit. Furthermore, the change in temperature yielded essentially the same spectral parameters as did the change in pH. Thus, the same conformer appears to be obtained by changes in either pH or temperature. Because CIV was increased by increasing the temperature at a pH higher than the reported pK for the 1966 cm^{-1} band of the sperm whale protein by $\approx$3 pH units, we must conclude that the presence of CIV does not in fact result directly from protonation of MbCO.

In conclusion, these data provide strong evidence for four, but only four, conformers at the ligand-binding site of bovine MbCO. These conformations appear to be able to interconvert rapidly under physiological conditions and to differ significantly in structure as indicated by the wide range of ν_{CO} and $\Delta\nu_{1/2}$ values found for the four conformers. It is apparent from the x-ray and neutron diffraction crystallographic data for Hbs and Mbs, including CO complexes (4, 9, 10, 32, 33), that the crystal structures obtained would not provide adequate pathways for ligand entry and departure from the site of binding at heme iron without an "opening up" of globin structure (34, 35). The findings reported here provide evidence of a flexibility—a dynamic nature—in the protein structure which may be required to permit ligands to enter or leave the heme site.

This work was supported by Grant HL-15980 from the National Institutes of Health.

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