Solid-State ^{13}C NMR Evidence for a Large Deviation from Linearity of the Fe-C-O Unit in the CO Complex with Myoglobin

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Abstract: The application of high-resolution solid-state ^{13}C NMR spectroscopy to investigate the bonding between carbon monoxide and myoglobin is explored. Selective pulse sequences (non-quaternary suppression and SELDOM) significantly reduce the problem of ^{13}CO peaks overlapping with those arising from the natural ^{13}C abundance myoglobin molecule. This enables the ^{13}CO spinning sideband manifold to be measured and, hence, the principal components of the ^{13}CO chemical shift tensor to be obtained. Results were obtained on two samples of myoglobin: one a dry powder and the other carefully prepared needle-like crystals containing water of crystallization. The spectra show that there is a large increase in the asymmetry of the ^{13}C shielding tensor in ^{13}CO-myoglobin compared to heme model compounds containing close to linear Fe-C-O moieties. FTIR measurements of both myoglobin samples show that the major ν_{CO} stretching frequency is due to the A_3 conformer. It can be concluded that in this particular CO-myoglobin substate there must be substantial deviation from linearity of the Fe-C-O unit, probably due to a significant polar interaction with the distal histidine.

Introduction

Knowledge of the electronic distribution and bond angles of the Fe-CO and Fe-O_2 moieties is fundamental to the understanding of reversible binding of diatomic molecules to hemoproteins.[1-4] In order to find a correlation between variation in ligand affinity and local structural changes, hemoproteins and model compounds have been investigated by several experimental methods.[1-13] X-ray and neutron diffraction measurements of crystalline hemoproteins have revealed that the O atom of bound CO deviates significantly from the normal to the heme plane through the iron atom. The deviation has in some cases been modeled as a bending of the Fe-C-O unit at the C atom and/or as a tilt at the Fe atom (Table 1). The Fe-C-O angle is, however, difficult to determine accurately, especially as there is a tendency for disorder in the CO positions.

Table 1. Fe-C-O Geometries of Hemoprotein CO Complexes

compd	method	geometry[a]	ref
sperm whale Mb	X-ray	bent (θ = 140° and 120°)	14
sperm whale Mb	neutron	tilted (φ = 24°)	15
Mb	neutron	bent (θ = 143° and 130°) and tilted (φ = 16°)	16
sperm whale			
(wild-type) Mb	X-ray	bent (θ = 167° or 169° [b])	17
(Gly 64) Mb	X-ray	bent (θ = 162° or 159° [b])	17
(Leu 64) Mb	X-ray	bent (θ = 166° or 156° [b])	17
(Gln 64) Mb	X-ray	bent (θ = 159° or 176° [b])	17
(Gly 64/Ala 68) Mb	X-ray	bent (θ = 168°)	17
sperm whale Mb	EXAFS	bent (θ = 127°)	18
Mb	XANES	bent (θ = 150°)	19
human HbA	X-ray	α sub-unit:bent (θ = 175°) and tilted (φ = 3°)	20
		β sub-unit:bent (θ = 171°) and tilted (φ = 6°)	
cowtown Hb	X-ray	α sub-unit:bent (θ = 169°) and tilted (φ = 3°)	20
		β sub-unit:bent (θ = 176°) and tilted (φ = 9°)	

[a] θ = Fe-C-O angle; φ = angle of the Fe-C bond from the heme normal. [b] The second θ angle quoted is that obtained when the pyrrole N-Fe-C angle and the Fe-C-O angle were weakly restrained to be 90° and 180°, respectively.

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(13) Abbreviations used: Mb, myoglobin; Hb, hemoglobin; 1-MeIm, 1-methylimidazole; hybrid-C_{12}, 5,15-[2,2′-(dodecanediamido)diphenyl]-α,α-10,20-bis(*o*-(pivaloylamino)phenyl)porphyrin; TpivPP, α,α,α,α-*meso*-tetrakis(*o*-pivalamidophenyl)porphinato; C_2Cap, 5,10,15,20-[pyromellitoyltetrakis-[(*o*-oxypropoxy)phenyl]]porphyrin; PBC_{14}, porphyrin bridged with a C_{14} polymethylene "handle" in a cross configuration. Structures of model porphyrins are shown in Figure 1.

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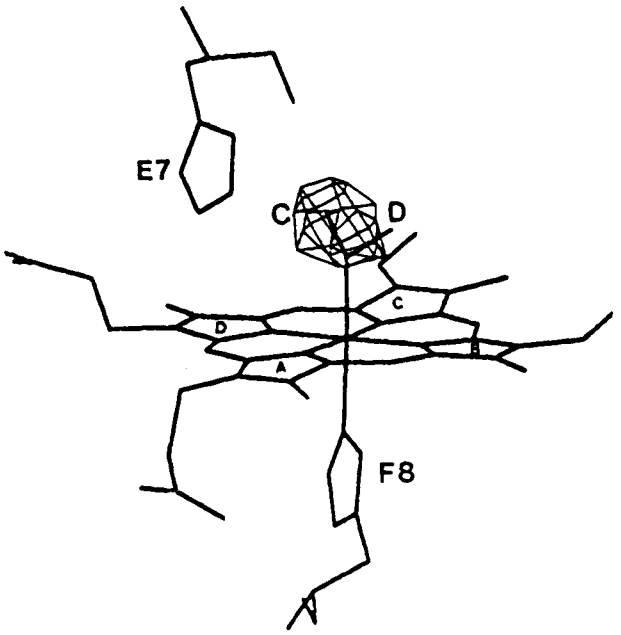

Figure 1. Schematic picture of the electron density of CO in sperm whale CO−myoglobin as derived from X-ray diffraction. C and D refer to the final two suggested conformations of the CO (ref 14).

In diffraction studies on the adduct of CO with myoglobin (MbCO), this disorder has been analyzed in terms of at least two highly bent conformers[14,16] (Figure 1). This observation has led to the notion that the distal histidine residue, His 64, may inhibit CO binding by forcing the Fe−C−O geometry to be nonlinear and that the extent of this inhibition is proportional to the deviation of the Fe−C−O unit from linearity.[21] Further, Collman and co-workers[21b] have suggested that the steric hindrance seen in heme proteins may play a functional role in lowering the CO affinity, thereby raising the threshold for CO poisoning. In contrast to this, comparison of the carbonyl structures of distal histidine mutants of sperm whale myoglobin shows that the Fe−C−O moiety does not deviate significantly from linearity and is independent of the identity of the amino acid at position 64.[17] EXAFS and XANES data on MbCO have been interpreted in terms of a highly bent structure.[18,19] On the other hand, vibrational data were found to be inconsistent with a large degree of Fe−C−O bending.[22]

NMR of solutions has also been extensively utilized in studying bonding to myoglobins, hemoglobins, and peroxidases.[23−27] For instance, one bond $J(^{13}C-^{57}Fe)$ coupling constant of sperm whale MbCO has been found to be essentially identical to that of the unhindered model compound *N*-methylimidazole protoporphyrin Fe(II), supporting the view that the carbonyl is not tilted with respect to the heme plane in solution.[23] NMR analysis has also established a ligand tilt of ca. 15° for the cyanide adduct of metHb as well as in the His (E7) Val mutant;[24] it was suggested that this deviation might present an upper limit for MbCO since the Fe(III)−CN^- unit is expected to have less back-bonding than the Fe(II)−CO moiety and thus is more likely to be bent.

We believe that significant new structural information can be obtained for these important biological systems using solid-state ^{13}C NMR spectroscopy in conjunction with the techniques of cross-polarization (CP) and magic-angle spinning (MAS). We have recently demonstrated the use of slow-spinning ^{13}C CP/MAS NMR spectroscopy in determining the principal components of the shielding tensor of ^{13}CO bonded to heme model compounds[28] and shown that the shielding tensor provides a sensitive probe of local Fe−C−O geometry. This contrasts with solution NMR spectra which can only provide the isotropic chemical shift. The chemical shift tensor is characterized by three independent principal components: δ_{11}, δ_{22}, and δ_{33}. The chemical shift tensor is often expressed in terms of anisotropy, $\Delta\sigma$, defined as $0.5(\delta_{11} + \delta_{22}) - \delta_{33}$, and asymmetry parameter, η, defined as $(\delta_{22} - \delta_{11})/(\delta_{33} - \delta_{iso})$, where δ_{iso} is the isotropic chemical shift, $(\delta_{11} + \delta_{22} + \delta_{33})/3$. It should be pointed out that the ^{13}C chemical shift tensor is affected only by local factors affecting the shielding at the ^{13}C site such as magnetic anisotropy and electronic influences. Thus, any deviation from axial symmetry, as would be the case for a nonlinear Fe−C−O entity, will manifest itself as a non-zero value for the asymmetry parameter, η.

Here we report ^{13}C CP/MAS solid-state NMR spectra of ^{13}CO bonded to two samples of horse myoglobin: one a dry powder, the other consisting of needle-like crystals containing water of crystallization. By the use of suitable selective pulse sequences (nonquaternary suppression and SELDOM), excellent spectra of the ^{13}CO spinning sideband manifold are obtained despite the large background signal arising from the natural ^{13}C abundance myoglobin molecule. These provide the first experimental evidence for a highly nonaxially symmetric shielding tensor for the ^{13}CO adduct of horse myoglobin. A previous study in the literature used ^{13}C NMR to compare crystalline and solution states of carbonyl hemoglobin A[29] but only measured the isotropic resonance position. We also performed FTIR measurements in order to establish the major conformers present in the MbCO adducts.

Materials and Methods

Reduced myoglobin was prepared by addition of a small excess of sodium dithionite to an oxidized form (Sigma) dissolved in 0.1 M tris buffer, pH 7.9, in an argon glovebox to minimize exposure to O_2. The solution was then loaded onto a gel filtration column (trisacryl GF 0,5, LKB) and eluted with the same buffer. The fraction of desired compound was then lyophilized to give a powder sample. A portion of this powder (*ca.* 400 mg) was dissolved in 2 M ammonium sulfate (2 mL) and crystallized by slow evaporation of the solvent (over approximately 4 days) to give needle-like crystals which were over 1 cm long. The two separate samples of the original reduced lyophilized

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powder and the crystals were introduced into MAS rotors. The powder sample was connected to a vacuum pump and evacuated at room temperature for 24 h (pressure of 10^{-4} Torr). ^{13}CO (99% enriched) under atmospheric pressure was then introduced to the samples and MbCO was obtained by the diffusion of the ^{13}CO ligand. In the case of the powder sample, there was a color change over the course of several hours during the uptake of ^{13}CO, while no obvious change was observed in the color of the crystals even after 48 h of exposure to ^{13}CO. Tight-fitting caps were placed on the rotors and the samples kept in a ^{13}CO atmosphere in sealed glass tubes prior to the NMR experiments. Some experiments were also performed on a sample of myoglobin powder loaded with ^{13}CO sealed in a glass ampule under reduced pressure in a fashion analogous to that used in our previous NMR measurements on heme model compounds.[28]

The NMR spectra were obtained on a Bruker MSL-300 using the techniques of cross-polarization (CP), magic-angle spinning (MAS), and high-power proton decoupling.[30,31] Nitrogen was used as the spinning gas to guarantee no exchange between ^{13}CO within the sample and O_2. However, later experiments using air as the spinning gas showed negligible loss of ^{13}CO from the samples over the course of several hours. Use was made of the nonquaternary suppression technique (NQS)[32] which removes signals from those ^{13}C nuclei with strong dipolar coupling to hydrogen nuclei. This has the effect of suppressing signals from CH and CH_2 groups from the spectrum; CH_3 groups are still normally observed due to rapid methyl rotation in the solid state reducing the dipolar coupling. The SELDOM technique (selectivity by destruction of magnetization)[33] was also found to be particularly useful for providing selectivity without distorting the spinning sideband manifold. In this technique, selected magnetization (e.g., from the isotropic ^{13}CO resonance and its associated spinning sidebands) is stored along the axis of the external magnetic field while there is a significant decay in the magnetization from other spins due to T_2 relaxation. The cycle is repeated for a number of loops. With a sufficient number of loops, excellent selectivity in solid-state NMR experiments can be achieved without distortion of the spinning sideband pattern. Good selectivity is, however, gained at the expense of a considerable loss in sensitivity, and a compromise between selectivity and sensitivity may have to be made. The principal components of the chemical shift tensor were obtained by simulation of the spinning sideband patterns in the NMR spectra based on the method of Herzfeld and Berger[34] adapted for least-squares fitting and extended to simulate sidebands up to ±8.[35] The program allows simultaneous fitting of spectra obtained at more than one spinning speed. Given the known fact that analysis of spinning sideband patterns close to axial symmetry can be prone to quite large errors,[35] it was found that simultaneous fitting of spectra obtained at three different spinning speeds was particularly important in reducing the uncertainty in resulting chemical shift components.

Results and Discussion

The use of routine solid-state NMR techniques on $Mb^{13}CO$ gives barely detectable ^{13}CO signals as they are buried under the natural ^{13}C abundance peaks from the large myoglobin molecule (molecular weight *ca.* 18 500). In order to circumvent this problem, we applied both the nonquaternary suppression (NQS) technique[32] and the selective excitation SELDOM pulse sequence.[33] First of all, however, it was necessary to check their viability on heme model compounds (Figure 2).

Figure 3a shows the conventional CP/MAS NMR spectrum of the heme model compound $Fe(^{13}CO)PBC_{14}$(1-MeIm).[28,36] The

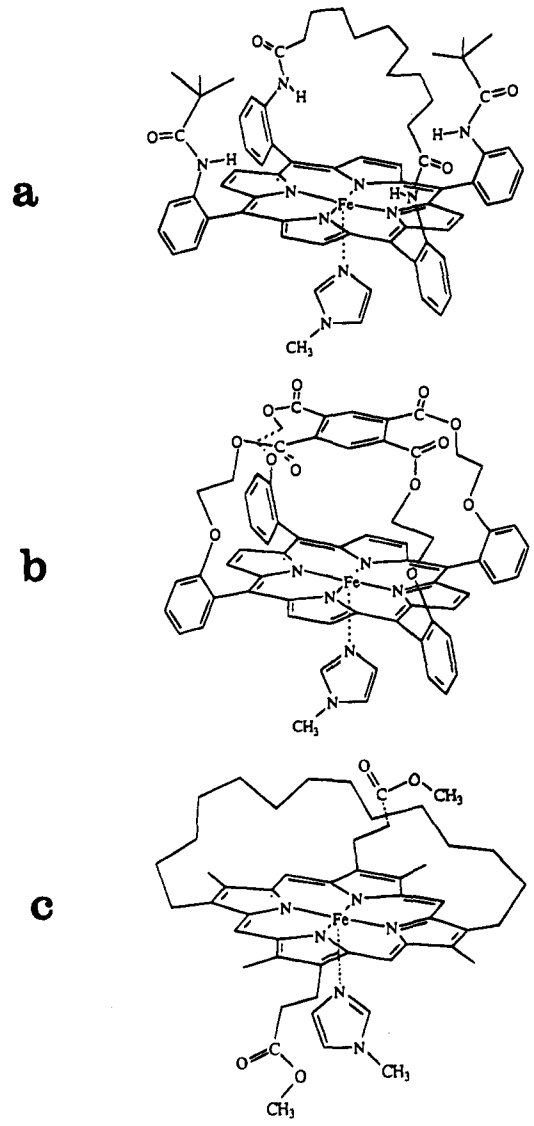

Figure 2. Schematic structures of (a) Fe(CO)(hybrid-C_{12})(1-MeIm), (b) Fe(CO)(C_2Cap)(1-MeIm), and (c) Fe(CO)PBC_{14}(1-MeIm).

spinning sidebands from the ^{13}CO environment are easily visible, though there are some background signals from the porphyrin skeleton. Figure 3b shows the spectrum recorded using the SELDOM pulse sequence. Excellent background signal suppression is obtained using the parameters employed, and the spinning sideband intensity pattern is identical to that using only CP/MAS or using CP/MAS together with NQS.

Figure 4a shows the conventional CP/MAS NMR spectrum of ^{13}CO in myoglobin, while Figure 4b shows the spectrum acquired using the NQS technique. The spinning sideband pattern is visible in the latter, and the intensities may be simulated to obtain the principal components of the chemical shift tensor. However, there is still the possibility of overlapping peaks affecting the measured intensities. Spectra were recorded with NQS at several different spinning speeds in an attempt to reduce this problem. The SELDOM pulse sequence offers another approach to reducing the protein background signal without distorting the ^{13}CO spinning sideband pattern. When used in combination with NQS, good suppression is achieved on the two myoglobin samples (see Figure 5) using parameters similar to those employed in Figure 3b. There are still some

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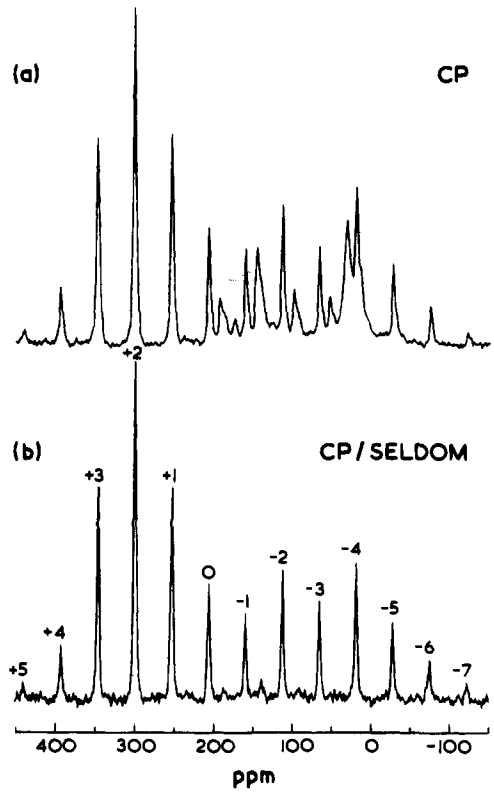

Figure 3. ^{13}C CP/MAS NMR spectra of Fe(^{13}CO)PBC$_{14}$(1-MeIm) (recorded in a 7.0 T field using a 3 ms contact time, 1 s recycle delay, and 3538 Hz spinning speed, processed using 80 Hz line broadening): (a) conventional spectrum (12 000 scans) and (b) spectrum obtained using the SELDOM pulse sequence (12 SELDOM loops with a 250 μs dephasing time, 12 000 scans). The numbers above the peaks indicate the sideband order.

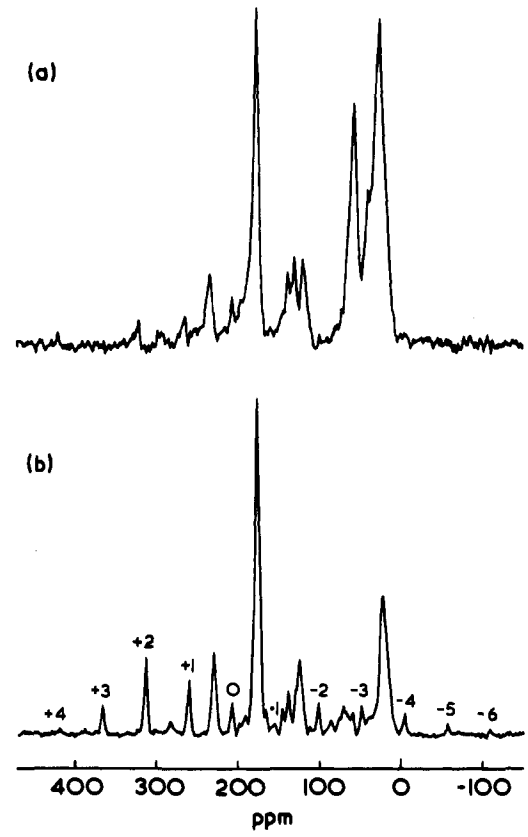

Figure 4. ^{13}C CP/MAS NMR spectra of ^{13}CO bonded to the myoglobin powder sample (7.0 T field, 3 ms contact time, 0.5 s recycle delay, processed using 100 Hz line broadening): (a) conventional spectrum (4326 Hz spinning speed, 13 000 scans) and (b) spectrum obtained using the NQS pulse sequence (3980 Hz spinning speed, 40 μs interrupted decoupling, 50 000 scans).

residual signals from the myoglobin molecule (notably from the methyl groups at 14 ppm and the peptide carbonyl groups at 176 ppm[29]), but further reduction of these could only be achieved at a greater loss in sensitivity. The spectra show only a single isotropic ^{13}CO peak in contrast to some diffraction structure determinations which suggest that there are at least two Fe−C−O geometries present with different bond angles[14,16] (Table 1). This may be due to the different ^{13}CO environments having the same isotropic chemical shift (within 1 ppm), to rapid exchange on the NMR time scale between the different geometries, or alternatively to only one conformer being present. The possibility of multiple conformations will be discussed further below in relation to our IR results.

The results of spinning sideband analysis on the spectra obtained on the myoglobin powder and crystals are shown in Table 2. It is found that the chemical shift tensor is very similar for ^{13}CO in the dry myoglobin powder and in the crystals containing water of crystallization, suggesting that water molecules are not directly involved at the active site. This is in agreement with a recent high-resolution X-ray diffraction study which shows the absence of water from the heme pocket in a cooperative dimeric hemoglobin carbonyl complex;[37] alternatively, neutron diffraction studies of MbCO have detected a water molecule in the heme pocket but located close to the histidine N$_\delta$ atom pointing away from the CO site.[16] Table 2 also reports minor changes to the principal components of the chemical shift tensor for the heme model compounds discussed in ref 28 due to an improved refinement procedure. It is worth noting that the shielding anisotropies we observe for Mb^{13}CO are significantly larger than those obtained indirectly from solution-state spectra by variable-field T_1 relaxation time measurements on adult hemoglobin ($\Delta\sigma \approx 198$ ppm)[38] but comparable to the result of a recent relaxation time study on sperm whale myoglobin ($\Delta\sigma \approx 500$ ppm).[39] Both relaxation time studies assumed an axially symmetric shielding tensor. Compared to sterically hindered heme models, there is a significant shift to high frequency of δ_{11} and a shift to low frequency of δ_{22}, while δ_{33} is relatively unchanged. Thus the isotropic chemical shift is essentially the same as that measured in model compounds such as Fe(CO)(hybrid-C$_{12}$)(1-MeIm). The X-ray structure of the latter shows that the Fe−C−O group is both linear and normal to the mean porphyrin plane[40] and that all contacts beween the terminal oxygen atom and the polymethylene chain are longer than 4 Å. The significant anisotropy of the shielding tensor observed therefore for both samples of Mb^{13}CO indicates that there must be a significant difference in local geometry. It is possible that molecular asymmetry or crystal packing could result in an asymmetric disposition of groups in the vicinity of the Fe−C−O moiety, which would affect the shielding values through magnetic anisotropy.[41,42] However, the distance of histidine E7 from the carbon under investigation is large (the distance between the N$_\epsilon$ atom of the

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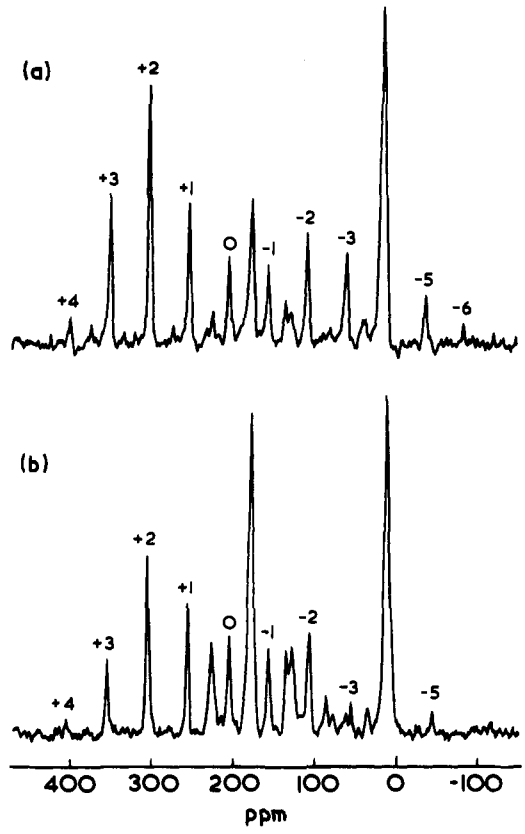

Figure 5. ^{13}C CP/MAS NMR spectra recorded with SELDOM and NQS (7.0 T field, 3 ms contact time, 0.5 s recycle delay, 12 SELDOM loops with 250 μs dephasing time, 40 μs interrupted decoupling, processed using 150 Hz line broadening): (a) ^{13}CO bonded to the myoglobin powder sample (3640 Hz spinning speed, 110 000 scans) and (b) ^{13}CO bonded to the myoglobin crystals (3758 Hz spinning speed, 110 000 scans).

distal histidine and the carbon of the Fe−C−O moiety is approximately 3.1 Å[16]). Further, the deviation of the carbon atom from the heme normal due to tilting can only be small (the greatest reported deviation of the carbon atom from the heme normal is less than 0.6 Å[16]). Thus magnetic anisotropy effects due to molecular asymmetry and/or Fe−C−O tilting are highly unlikely to account for changes in the δ values to the extent observed here. Hence we believe that the major effect on the shielding anisotropy is due to the bending of the Fe−C−O unit.

At present it is premature to attempt to quantify the expected correlation between the shielding asymmetry parameter and the bond angle, as there have only been a few X-ray structures of encumbered porphyrin compounds reported[43,44] and only a few measurements of shielding tensor components.[28] We are currently collaborating on *ab initio* theoretical calculations of shielding tensors in these and related systems. However, calculated anisotropy values are greatly affected by factors such as exact bond lengths, electron correlation, and vibrational corrections, as well as the bond angle which is the topic of interest, and the agreement between calculation and theory is not always good for even comparatively simple systems.[45] However, the fact that the model compound Fe(CO)(C_2Cap)-(1-MeIm), which has Fe−C−O bond angles determined by single crystal X-ray diffraction of 175.9° and 172.9°,[44] shows an asymmetry parameter of only 0.12 compared to 0.25 and 0.28 for the Mb^{13}CO samples strongly suggests that the deviation from linearity of the Fe−C−O unit in MbCO must be much larger than 7°.

A possible resolution of the puzzle posed by the different Fe−C−O geometries derived from different experiments may lie in the existence of multiple substates of MbCO. Thus IR spectra of MbCO have been found to contain several overlapping ν_{CO} bands[46−49] whose intensities vary with temperature,[48,50] pressure,[51] and solution conditions (pH and ionic strength)[46,48,52,53] and among crystals prepared in different ways.[47] Four ν_{CO} IR components have been studied extensively by Frauenfelder and co-workers,[54,55] who assigned them to different conformational substates of the proteins, labeled A_0−A_3, in order of decreasing frequency. The substates exhibit different kinetic parameters for CO photodissociation and recombination and thus may have different inclinations of the CO ligand relative to the heme normal. Spiro and co-workers,[56] in a recent comprehensive analysis of X-ray and neutron diffraction data and resonance Raman and IR spectra of heme proteins and models, suggested that the A_0 substate results from protonation of the distal imidazole which is displaced from the heme pocket, leaving the bound CO in an unhindered environment. The A_0 substate ($\nu_{CO} \sim 1966$ cm^{-1}) is not significantly populated near neutrality and is not a significant contributor to the X-ray or neutron crystal structures, which were determined at pH values above 5.7. Spiro and co-workers[56] suggested that $A_{1,2}$ ($\nu_{CO} \sim 1946$ cm^{-1}) and A_3 ($\nu_{CO} \sim 1933$ cm^{-1}) substates are associated with the two alternative tautomers of the distal imidazole, with the proton on N_ϵ and N_δ, respectively. The $A_{1,2}$ vibrational frequencies result from a positive polar interaction with the adjacent $N_\epsilon H$ group. The decreased A_3 IR frequency (~1933 cm^{-1}) is then a possible consequence of a weak interaction of the N_ϵ lone pair with the CO π^* orbital, an interaction originally suggested by Caughey and co-workers.[47,57] By inducing some sp^2 character at the carbonyl C atom, such an interaction would also induce a significant Fe−C−O bending. Such a hypothesis is supported from neutron diffraction structure analysis[16] which showed that there was no hydrogen bond between CO and the distal histidine residue and that the N_ϵ atom is not deuterated. Apparently the imidazole proton resides on N_δ. This means that the lone pair, rather than a hydrogen bond donor, faces the bound CO.

The IR spectrum in Nujol of a sample of dry powder MbCO, which was prepared under conditions identical to those used

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Table 2. Principal Components of the Chemical Shift Tensor of ^{13}CO Heme Compounds[a]

compd	δ_{iso}	δ_{11}	δ_{22}	δ_{33}	$\Delta\sigma$	η	Fe−C−O angle
CO−myoglobin (crystals)	205.5	394 ± 16	311 ± 12	−88 ± 7	440	0.28 ± 0.07	see Table 1
CO−myoglobin (dry powder)	205.5	390 ± 22	317 ± 18	−91 ± 5	445	0.25 ± 0.10	see Table 1
Fe(CO)(hybrid-C_{12})(1-MeIm)	205.0	358 ± 20	350 ± 19	−92 ± 2	446	0.03 ± 0.09	
		354 ± 1	354 ± 1	−92 ± 1	446	0.00[b]	180° (ref 40)
Fe(CO)(TpivPP)(1-MeIm)	205.0	360 ± 10	352 ± 10	−97 ± 3	453	0.03 ± 0.05	
		356 ± 2	356 ± 2	−97 ± 2	453	0.00[b]	unknown
Fe(CO)(C_2Cap)(1-MeIm)	202.5	368 ± 14	331 ± 11	−92 ± 5	442	0.12 ± 0.06	175.9° and 172.9° (ref 44)
Fe(CO)PBC_{14}(1-MeIm)	205.0	384 ± 5	324 ± 3	−93 ± 2	447	0.20 ± 0.02	unknown

[a] Chemical shifts, δ, are given in ppm relative to TMS. The values for the model compounds are improved refinements of the spectra reported in ref 28. The estimated uncertainties represent 95% confidence limits. [b] Refinement constrained so that $\eta = 0$.

for the NMR experiments in the solid (CO was used instead of ^{13}CO), reveals a major ν_{CO} component at 1934.7 cm^{-1} with a weak shoulder at 1942.5 cm^{-1} and a very weak band at 1959 cm^{-1}. This demonstrates that the A_3 substate is highly predominant[56] in the powder MbCO sample studied and that it is in this form that the NMR measurements showing a nonaxially symmetric chemical shift tensor are observed. FTIR spectra were also obtained on the crystalline sample after exposure to natural abundance CO. These were recorded on distinct individual crystals using a Nicolet IRμs scanning infrared microprobe. This method avoids any possible change in conformation with pressure that might have ensued in the preparation of pressed pellets with KBr and so ensures that the conformation is the same as that on which the ^{13}C NMR spectra were performed. The spectra show two main bands attributable to ν_{CO} at 1965.9 and 1924.5 cm^{-1}. The first is indicative of the presence of the A_0 substate (which is believed to have a close to linear Fe−C−O unit), while the second is indicative of the A_3 substate.[56] A band was also observed at 1878.6 cm^{-1} which is of uncertain origin. The detection of only a single ^{13}CO environment in the NMR studies, while more than one CO environment in the MbCO crystals is detected by FTIR, is probably due to the environments having the same isotropic chemical shift (within 1 ppm) or to rapid exchange on the NMR time scale between the different geometries. The asymmetry in the ^{13}C chemical shift tensor observed for the $Mb^{13}CO$ crystals, together with the FTIR spectra, suggests that in the crystals, as was found for the powder sample, the A_3 substate is the major conformation. This is in agreement with the suggestion that the A_3 substate is favored in crystals prepared at pH values above 5.7.[16,56,58]

Conclusions

This is the first application of a selective solid-state NMR pulse sequence to a complicated biological system of importance. Slow-spinning ^{13}C CP/MAS NMR spectra reveal information not available by solution NMR methods and enable comparisons with other structural methods such as FTIR and X-ray diffraction to be made. The spectra of $Mb^{13}CO$ enable the principal components of the ^{13}CO chemical shift tensor to be obtained, and these reveal a large asymmetry, thus showing that there must be a substantial deviation from linearity of the Fe−C−O unit in the A_3 substate in the CO complex with myoglobin. Solid-state NMR spectroscopy should prove useful in characterizing other conformational states of ^{13}CO in heme proteins and related biomolecules prepared under a variety of conditions.

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