Solid-State Oxygen-17 Nuclear Magnetic Resonance Spectroscopic Studies of [$^{17}O_2$] Picket Fence Porphyrin, Myoglobin, and Hemoglobin

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Abstract: We have studied a model compound for oxyhemoglobin and oxymyoglobin, the iron–dioxygen complex of "picket fence porphyrin" (5,10,15,20-tetrakis(α,α,α,α-*O*-pivalamidophenyl)porphyrinato)iron (II) ((1-MeIm)O_2), as well as oxymyoglobin and oxyhemoglobin themselves, by using ^{17}O solid-state nuclear magnetic resonance spectroscopy. For the model picket fence porphyrin, the principal components of the chemical shift tensors for both bridging and terminal oxygens in the Fe–O_2 unit have been determined, and the isotropic chemical shifts occur at 1200–1600 and 2000 ppm, respectively, somewhat deshielded from the ~1750 and ~2500 ppm values found by Gerothanassis et al. in solution (*J. Am. Chem. Soc.* **1989**, *111*, 7006–7012). The anisotropies of the shift tensors are very large for both oxygens ($\Delta\delta$ = ~2200 ppm for the bridging oxygen and $\Delta\delta$ = 3350 ppm for the terminal oxygen, at 77 K). From partial averaging of the shift tensors at room temperature, due to fast axial rotation of the dioxygen ligand, an Fe–O–O bond angle of ~140° has been derived for the model system. Temperature dependence studies indicate essentially no change in the isotropic chemical shift of the terminal oxygen down to 4.2 K, while there is an apparent low-frequency shift of the bridging oxygen on cooling to 77 K, possibly due to the freezing in of one conformational substate. Spectra of oxymyoglobin and oxyhemoglobin, at 77 K, are very similar to those of the model compound at low temperature. Our results indicate that the ^{17}O nuclear quadrupole coupling constants must be relatively small for both oxygens (≲5 MHz) in all systems, much smaller than the 8.5- and 20-MHz values found for ozone, suggesting extensive π-delocalization in the Fe–O–O fragment. Our results are also consistent with an overwhelmingly spin paired configuration, both in the model system, and in oxyhemoglobin and oxymyoglobin themselves.

Introduction

The topic of the interaction between O_2 and a variety of metalloproteins (and model systems) has been the subject of continuous interest and lively debate for over half a century. Major emphasis has been placed on elucidating the nature of iron–dioxygen bonding in the oxygen carriers oxyhemoglobin (HbO_2) and oxymyoglobin (MbO_2), and although X-ray results on MbO_2[1] and HbO_2,[2] and on model compounds,[3] support Pauling's bent end-on structure[4] over Griffith's triangular, sideways structures,[5] the detailed electronic structure of the Fe–O_2 moiety still remains somewhat controversial, and a number of formulations have been postulated to best describe the Fe–O_2 unit in oxyhemoglobin.[6-9]

Pauling[4,6] first proposed a totally spin paired Fe(II) [S = 0] O_2 [S = 0] configuration, based on the observation of diamagnetism in oxyhemoglobin. Weiss[7] then suggested the *met*-superoxide formulation (Fe(III)·O_2^-) in which the low-spin ferric iron has its unpaired electron antiferromagnetically coupled to that of the coordinated superoxide oxygen. A number of results from experimental[10-15] and theoretical[8,9,16-19] studies have been used to support both models. In some theoretical calculations, Goddard and Olafson[8] postulated an ozone-like Fe–O–O fragment with Fe(II) [S = 1] O_2 [S = 1] bonding, while Case et al.[9] proposed an Fe–O_2 unit consisting of a mixture of Fe(II) [S = 0] O_2 [S = 0] and Fe(II) [S = 1] O_2 [S = 1] states. Our belief is that NMR spectroscopy can, *in principle*, provide useful data with which to test the various bonding models, since a quantum-chemical model should be capable of predicting (or fitting) *all* of the observables. Oxygen-17 NMR spectroscopy appears to be promising in this respect, since it is a direct probe of metal–oxygen interaction in such systems, but to date, all attempts at observation of ^{17}O NMR resonances of MbO_2 or HbO_2 have failed.[20-22] Similar early failures to observe ^{17}O NMR from the peroxidic "model" Vaskas' and Pt dioxygen complexes, attributed either to exchange broadening or to long correlation times, were overcome by us previously by use of spin-echo methods,[23] and we demonstrated that neither ligand exchange nor large line widths but purely instrumental (sensitivity) considerations were the cause of the early difficulties. More recently, Gerothanassis et al.[24-25] have used spin-echo methods to observe the ^{17}O NMR of several oxy picket fence porphyrins, in solution, but to date, no spectra

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of oxyhemoglobin or oxymyoglobin have been reported. We believe that the observation of high-resolution ^{17}O NMR spectra of oxymyoglobin and oxyhemoglobin in solution may be quite difficult, since some time ago[26,27] we noted that while the ^{13}C NMR resonances of the hemes in carbonmonoxymyoglobin and carbonmonoxyhemoglobin in solution could be readily detected, the corresponding features in the ^{13}C NMR spectra of MbO_2 and HbO_2 were never observed, even after extensive manipulations of buffer type, pH, pO_2, and temperature were carried out. One possibility is that fast O_2 exchange between deoxy Mb and MbO_2 (or Hb and HbO_2) occurred, broadening the heme ^{13}C resonance. A second possibility is that there was some residual paramagnetism delocalized onto the heme, in the oxy species.

One way to help clarify this type of problem is to obtain ^{17}O NMR spectra of oxy picket fence porphyrin, oxymyoglobin, and oxyhemoglobin *in the solid state*. Unlike the situation with solution-state NMR, solid-state interactions are likely to be overwhelmingly inhomogeneous, and spin-echo techniques are expected to be effective in causing echo formation. In solution, long correlation times, when combined with even moderate e^2qQ/h values, cause very extensive homogeneous broadening, and conventional solution NMR methods are rendered ineffective. Moreover, in solution, it certainly seems plausible that O_2 exchange rates will be larger than in the solid state, and of course in the solid state a far larger range of temperatures (in our case, down to ~3 K) are accessible. Thus, problems characteristic of the solution state, exchange and lifetime broadening, are greatly ameliorated in the solid state—although again, of course, a new class of "solid vs solution" questions can be asked, but can unfortunately only be fully answered when solution spectra of MbO_2 and HbO_2 are in fact seen.

In any case, we believe that our observation of the ^{17}O NMR spectra of oxy picket fence porphyrin, oxymyoglobin, and oxyhemoglobin represent an important advance in this area, since the chemical shift, observed in solution NMR as a scalar quantity due to motional averaging, is actually a tensor, and is related to the complete three-dimensional electron distribution about the nucleus. As discussed below in detail, our results (based on some 650 individual spectra and 300 computer simulations) strongly suggest that the observed line shapes in each system are overwhelmingly dominated by the anisotropic chemical shift interaction, with quadrupolar contributions to the line shape being rather small. We place an upper limit on e^2qQ/h of ~5 MHz, a value much smaller than that found for either oxygen in ozone. Thus, at present, solid-state techniques appear to have considerable advantages over solution-state techniques for investigating the heme proteins themselves. We discuss below then our observation, assignment, and preliminary analysis of the ^{17}O NMR spectra of oxy picket fence porphyrin, oxymyoglobin, and oxyhemoglobin, concentrating on the static and dynamic aspects of the model system.

Experimental Section

Chemical Aspects. The dioxygen complex of "picket fence porphyrin", (5,10,15,20-tetrakis($\alpha,\alpha,\alpha,\alpha$-*O*-pivalamidophenyl)porphyrinato)iron(II), was prepared using a method described previously,[3] from free $\alpha,\alpha,\alpha,\alpha$-$T_{piv}PP$, purchased from Mid-Century Chemicals (Posen, IL). For the preparation of a powdered sample of Fe($\alpha,\alpha,\alpha,\alpha$-$T_{piv}PP$)(1-MeIm)-$^{17}O_2$, the $^{16}O_2$ complex of the picket fence porphyrin was deoxygenated in the solid state by evacuating for several hours at 10^{-4} Torr and 25 °C and then reoxygenated with $^{17}O_2$. $^{17}O_2$ (70% ^{17}O) was obtained from ICON Services, Inc. (Summit, NJ).

Erythrocytes were removed from citrated blood (donated by the Champaign County Blood Bank, Champaign, IL) by centrifugation, and were washed five times with 0.9% NaCl containing 0.5 mM EDTA and 0.01 M phosphate buffer at pH 8. The packed red cells were lysed by mixing with an equal volume of deionized water at room temperature, for 15 min, and the stromata removed by centrifugation at 30000*g* for 60 min. The resultant hemolysate was purified on a Sephadex (Pharmacia, Piscataway, NJ) G-25 coarse column that had been equilibrated with 0.01 M phosphate buffer, pH 8, containing 0.05 mM EDTA.

Sperm whale myoglobin was obtained from Fluka (Ronkonkoma, NY) and was dissolved in 0.05 M Tris–HCl buffer, pH 8, containing 0.5 mM EDTA. After reduction with sodium dithionite, the protein was immediately run down a Sephadex G-25 column, equilibrated with 0.05 M Tris–HCl buffer, pH 8, containing 0.5 mM EDTA. The purified oxyhemoglobin and oxymyoglobin solutions were then concentrated in Amicon (Danvers, MA) ultrafiltration cells, using PM-10 membranes. Each concentrated (~7.5 mM) protein solution was then diluted using an approximately 4-fold excess of ^{17}O-depleted water (Cambridge Isotope Labs, Woburn, MA), followed by reconcentration. This procedure was repeated twice for each protein. $^{17}O_2$-labeled oxyhemeproteins were prepared by evacuating the purified oxyhemeproteins to their deoxy forms and then reoxygenating with $^{17}O_2$ gas (~60% enriched, ICON Services, Inc., Summit, NJ). Maximum final concentration of [$^{17}O_2$]oxyhemeprotein was achieved by ultrafiltration, or by polyethyleneglycol protein exclusion.[28,29] Unless otherwise indicated, all protein preparations were carried out between 4 and 10 °C. Protein concentrations were estimated using a millimolar (heme) extinction coefficient of 11.0 at 540 nm, according to Van Assendelft and Zijlstra.[30]

NMR Aspects. ^{17}O NMR spectra were obtained at 3.52, 8.45, and 11.7 T (corresponding to ^{17}O NMR resonance frequencies of 20.5, 48.8, and 67.8 MHz, respectively) on "home-built" spectrometers, which consist of 102-mm (3.52 T), 89-mm (8.45 T), and 52-mm (11.7 T) bore superconducting solenoids (Nalorac Cryogenics, Concord, CA (3.5 T) and Oxford Instruments, Osney Mead, U.K. (8.45 and 11.7 T)) and a Nicolet 1180 computer and 2090 transient recorder (Madison, WI), together with a variety of other digital and radiofrequency electronics. We also used a home-built solenoidal radiofrequency coil probe (sample volume ~0.5 cm^3), together with a spin-echo sequence,[31] for data acquisition. The "solid" 90° pulse width was ~2 μs. Chemical shifts are reported referenced to $H_2{}^{17}O$ in an external sample of tap water at 0 ppm, with high-frequency, low-field, paramagnetic, or deshielded values taken as positive (IUPAC δ scale). The chemical shift anisotropy tensor component convention used is that δ_{33} is the most deshielded element.

Results and Discussion

Picket Fence Porphyrin. We show in Figure 1 the solid-state ^{17}O NMR spectra of a powdered sample of [$^{17}O_2$] picket fence porphyrin at 298 K, at two different magnetic field strengths: 8.45 T (Figure 1A) and 11.7 T (Figure 1C). It can be seen that the total line breadth of the powder spectrum (in ppm) is independent of the magnetic field strength, indicating that the predominant line-broadening interaction is chemical shift anisotropy (CSA). A second-order quadrupolar interaction, which in general is the main contributor to the powder line shapes of quadrupolar nuclei, would cause the total line breadth of the powder spectrum (in Hz) to be *inversely* proportional to magnetic field strength. Thus, the total line breadth of the powder spectrum at low magnetic field strength would be greater (in Hz) than that at a higher magnetic field strength if the second-order quadrupolar interaction were a major contributor to the observed line width. However, we observe no such pronounced extra broadening of the powder spectrum at the lower field strengths we have used, and thus conclude that the ^{17}O powder line shape of the [$^{17}O_2$] picket fence porphyrin is overwhelmingly dominated by the chemical shift anisotropy, certainly at the higher mangetic field strengths (8.45 and 11.7 T) we have used (Figure 1A,C) for most spectra.

We should comment on a number of aspects of the data acquisition process for such heme systems. First, we have obtained a total of over 400 such spectra of oxy picket fence porphyrin, varying the field, temperature, recycle time, pulse width, pulse spacing, carrier frequency offset, and results on a number of different samples. The spectra shown represent those judged to be minimally distorted by instrumental conditions, and typically require many hundreds of thousands of transients. At low field (3.5 T), spectral signal-to-noise ratios are much lower, while at high field, the increased overall spectral width (~200 kHz) and longer pulse widths (due to our use of lower rf power levels in our narrow bore magnet, to prevent arcing) cause some attenuation

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Table I. Principal Components of Shift Tensors (ppm)[a] for Terminal and Bridging Oxygens in Oxy Picket Fence Porphyrin at 298 and 77 K

	δ_i[b]	δ_{11}[b]	δ_{22}[b]	δ_{33}[b]	$(\delta_{33}-\delta_{11})$[b]	δ_i[c]	δ_{11}[c]	δ_{22}[c]	δ_{33}[c]	$(\delta_{33}-\delta_{11})$[c]
terminal	2017	1600	1600	2850	1250	1967	850	850	4200	3350
bridging	1600	0	2150	2650	2650	1190	100	1170	2300	2200

[a] Chemical shifts are in parts per million from $H_2{}^{17}O$. Chemical shift anisotropies ($\delta_{33}-\delta_{11}$) are in parts per million. Estimated errors are ±100–200 ppm. The computer program used to deduce the shielding tensor elements is described in ref 41. [b] 298 K. [c] 77 K.

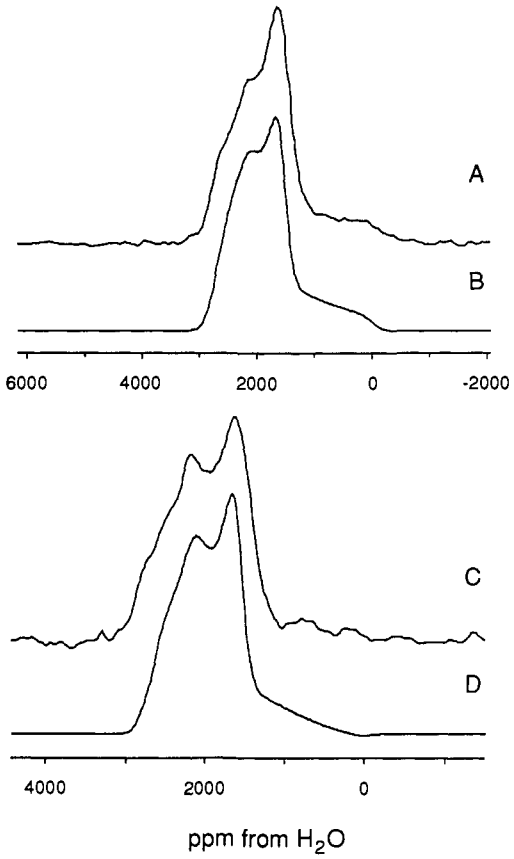

Figure 1. Solid-state ^{17}O NMR spectra and computer simulations of a powdered sample of $[^{17}O_2]$ picket fence porphyrin at 298 K, at two different magnetic field strengths: (A) spin-echo spectrum at 8.45 T; (B) computer simulation (ref 41) of (A) using δ_{ii} = 1600, 2850 ppm, QCC = 2 MHz, η = 0.1, line broadening 15 kHz, intensity 0.50 and δ_{ii} = 0, 2150, 2650 ppm, intensity 0.50, Euler angles 60, 60, 0° (see ref 41 for details); (C) spin-echo spectrum at 11.7 T; (D) computer simulation (ref 41) of (C). All parameters are as in (B).

in the wings of the spectrum. Nevertheless, as noted below, our results clearly indicate that the chemical shift anisotropy broadening mechanism dominates the observed spectra.

We have carried out a very large number of computer simulations in an effort to analyze our picket fence porphyrin results, using a wide range of initial parameters, and we find that the best agreement between simulation and experiment is obtained when using simulation parameters which are overwhelmingly dominated by the chemical shift anisotropy interaction. Large quadrupole coupling constant (QCC) values result in much worse simulations, and do not reflect the experimentally observed field dependence results. On the other hand, a dominant CSA interaction explains each of the following observations: (1) The positions of the major singularities in the spectra are field independent. At 11.7 T (Figure 1C) there are clearly peaks at ~2200 ± 50 ppm and at 1650 ± 50 ppm. There are also singularities at ~2200 ± 100 and 1650 ± 100 ppm at 8.45 T (Figure 1A). (2) At 3.52 T (data not shown), although the signal-to-noise ratio is poor, the major peak clearly occurs at ~1900 ± 100 ppm, essentially the midpoint of the two peaks seen at higher field ((1650 + 2150)/2 = 1900 ppm). (3) At both 8.45 and 11.7 T, the spectra show a more-or-less well-defined high-frequency cutoff at ~3000 ppm, while the low-frequency cutoff is around 0 ppm (this is more apparent when the carrier frequency is located further upfield). On the basis of these three pieces of information, it seems clear that, within achievable spectral signal-to-noise ratios, there is little or no change in the overall spectral width (in ppm) or shape, as a function of magnetic field strength. If second-order quadrupolar interactions were large, they would have major effects on the line shapes, line widths, and perhaps most important, on the peak positions, and none of these effects are seen experimentally, Figure 1A,C.

We also find that recording ^{17}O NMR spectra of oxy picket fence porphyrin using much longer interpulse separation times than those used in Figure 1 has the effect of letting much of the spectrum decay away in a spin–spin relaxation process (T_2), and the major spectral singularities (later assigned to δ_{11} and δ_{22} of the nonbridging site, and δ_{22} of the bridging oxygen) are considerably enhanced, as is a feature at ~2800 ppm (assigned to δ_{33} of the nonbridging oxygen). At 8.45 T, the major peaks are at 1650, 2200, and 2700 ppm (all ±50–70 ppm) downfield from H_2O, while at 11.7 T the corresponding peak positions are at 1650, 2250, and 2800 ppm (±50–70 ppm). We believe these observations also strongly suggest the dominance of the chemical shift anisotropy line-broadening mechanism.

We therefore show in Figure 1 simulations of the ^{17}O NMR powder spectra of $[^{17}O_2]$ picket fence porphyrin, at both 8.45 and 11.7 T. We used the CSA tensor elements given in Table I, together with a small (2 MHz) QCC contribution, in an effort to estimate the approximate magnitude of the QCC. Increasing QCC much above 2 MHz led to poor simulations which did not match the experimental widths or shapes. Smaller QCC values had a relatively minor effect on the simulations, as did the relative orientations of the chemical shift and electric field gradient tensors. We thus conclude that the principal components of the chemical shift tensors for the two oxygens are as listed in Table I. These results show that one component has an axially symmetric shift tensor with an isotropic chemical shift (δ_i) of ~2000 ppm, while the other component has a slightly asymmetric shift tensor with a δ_i of ~1600 ppm (at 298 K). These isotropic chemical shifts are deshielded from the recent solution ^{17}O NMR results of Gerothanassis et al.[25] of ~2500 and ~1750 ppm. We tentatively assign the axially symmetric ~2000 ppm component to the terminal oxygen, and the asymmetric component having $\delta_i \approx 1600$ ppm to the bridging oxygen, in general agreement with the assignments published previously by Gerothanassis et al.[24,25]

We obtain quite different ^{17}O NMR spectra from the dioxygen complex at lower temperatures, although the positions of the major spectral features change in a continuous manner. Figure 2 shows ^{17}O NMR spectra of the picket fence porphyrin complex at 8.45 T, and at a variety of temperatures between 298 and ~4.2 K. It can be seen that the powder line shape of the spectrum changes with temperature, and becomes much wider at lower temperatures. However, below ~130 K, the line shape remains approximately constant. Thus, the spectrum at 77 K is basically the same as that at 4.2 K, but it is significantly different from that at 298 K. The change in spectral appearance between 298 and ~133 K, and the lack of change below ~133 K, suggests that the temperature dependence of the spectra is predominantly due to motional averaging. Specifically, we believe it to be due to fast internal motion of the dioxygen ligand at room temperature, which freezes out below ~133 K, resulting in rigid-lattice ^{17}O NMR spectra—although of course small angle librations may remain at even the lowest temperatures investigated, and there may be frozen-in static disorder at very low temperatures. The idea of rapid FeO_2 motion freezing out is not new, and has been used by Spartalian et al. to interpret their Mossbauer data[32] and by

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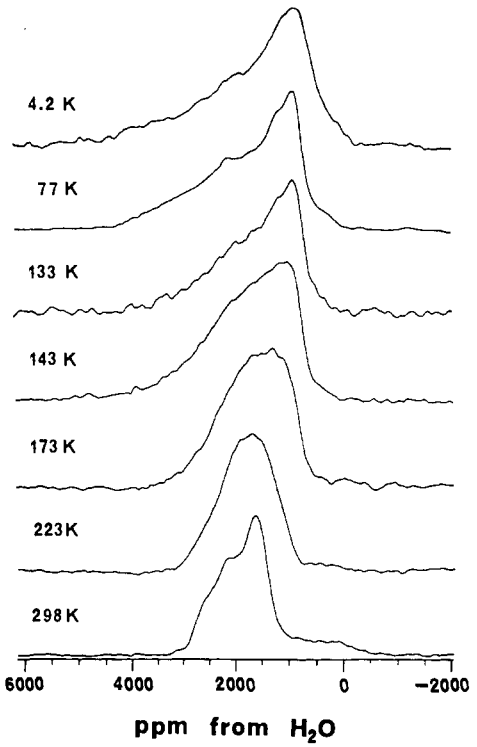

Figure 2. ^{17}O NMR spectra of [$^{17}O_2$] picket fence porphyrin at a variety of temperatures, at 8.45 T.

Gerothanassis et al. to interpret their NMR results.[25]

We show in Figure 3 the 8.45-T ^{17}O NMR spectrum of the [$^{17}O_2$] picket fence porphyrin at 77 K. The results of numerous additional experiments confirmed that the spectrum covered the range ~100–4200 ppm downfield from H_2O, and the major singularities occur at ~850 and 1170 ppm. Once again, we carried out a large number of computer simulations of the spectrum shown in Figure 3A, and were only able to obtain reasonable agreement by using a very large CSA interaction. Figure 3B shows the result of a simulation, in which most of the major features are reproduced using solely the CSA elements given in Table I. An improved result was obtained by incorporating a second-order quadrupole interaction, as well as a correction for pulse-power fall off, and is shown in Figure 3C. In this simulation, we also varied the relative orientations of the chemical shift and quadrupolar tensor, as well as the relative contributions from the two sites. Even when all simulation parameters were varied, we were unable to improve the fit to the experimental spectrum by use of chemical shift tensor elements significantly different from those given in Table I. Our best fit simulation (Figure 3C) used a 2:1 intensity ratio for the terminal/bridging oxygen sites, due we believe to different T_1 values at low temperatures. Although differential nonselective echo excitation responses (and motional averaging and T_2 effects) can all affect signal intensities, none of these effects appear significant at room temperature, where T_1 is known to be short, due to ligand rotation.

The results of Table I indicate that one of the shielding tensors is axially symmetric, while the other is not (although δ_{22} and δ_{33} for the bridging oxygen could be brought into agreement by an experimental error of ±250 ppm, simulations support our view as stated). The overall breadth of the axially symmetric shift tensor is significantly reduced at 298 K compared to that at 77 K. We believe this indicates that the axially symmetric shielding tensor may be partially averaged at 298 K by rapid anisotropic motion of the dioxygen ligand. Indeed, X-ray results on the same dioxygen complex[3] show that the terminal oxygen in the Fe–O–O fragment is statistically disordered, with the Fe–O–O plane bisecting the N_{pyr}–Fe–N_{pyr} bond angle, indicating some rotational freedom of the terminal oxygen. A barrier to rotation of less than 7 kcal mol^{-1} has been reported[19] from an extended Hückel calculation. This is relatively low, and presumably allows relatively

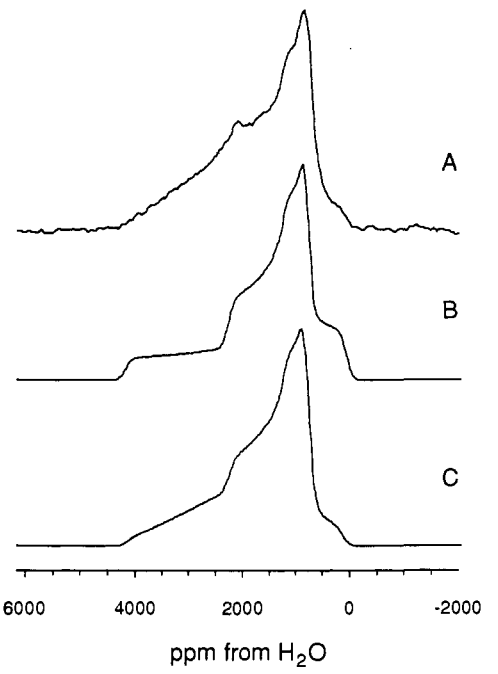

Figure 3. 8.45-T ^{17}O NMR spectrum of [$^{17}O_2$] picket fence porphyrin at 77 K, and its computer simulation: (A) experimental spectrum; (B) computer simulation (ref 41) using δ_{ii} = 850, 850, 4200 ppm, QCC = 0, line broadening 10 kHz and δ_{ii} = 100, 1170, 2300 ppm, QCC = 0, intensities 0.5; (C) computer simulation (ref 41) using δ_{ii} = 850, 850, 4200 ppm, QCC = 2 MHz, η = 0.1, line broadening 11 kHz, Euler angles 60, 60, 0°, intensity 0.5 and δ_{ii} = 100, 1080, 2300 ppm, QCC = 4 MHz, η = 0.1, line broadening 10 kHz, Euler angles 10, 10, 0°, intensity 0.25.

free rotation at higher temperatures. Thus, we assign the axially symmetric shielding tensor to the terminal oxygen, and attribute the much smaller shielding anisotropy at room temperature to partial averaging, presumably due to a 4-fold rotational jump, about the Fe–O bond axis. The assignment of the more deshielded resonance to the terminal oxygen is in agreement with the solution NMR results of Gerothanassis.[24,25]

For rapid axial rotation, the averaged shift tensor will be axially symmetric and will have averaged principal components, $\bar{\delta}_\perp$ and $\bar{\delta}_\parallel$, given by

$$\bar{\delta}_\perp = \tfrac{1}{2}(1 - \cos^2\alpha \sin^2\beta)\delta_{11} + \tfrac{1}{2}(1 - \sin^2\alpha \sin^2\beta)\delta_{22} + \tfrac{1}{2}(\sin^2\beta)\delta_{33} \quad (1)$$

$$\bar{\delta}_\parallel = (\cos^2\alpha \sin^2\beta)\delta_{11} + (\sin^2\alpha \sin^2\beta)\delta_{22} + (\cos^2\beta)\delta_{33} \quad (2)$$

where β is the angle formed by δ_{33} and the rotation axis, and α is the angle formed by δ_{11} and $\delta_\perp$. When the rigid-lattice shielding tensor is axially symmetric, as is the case for the terminal oxygen, eqs 1 and 2 are further simplified, and the averaged shift anisotropy, $\overline{\Delta\delta} = \bar{\delta}_\parallel - \bar{\delta}_\perp$, is related to the rigid-lattice value, $\Delta\delta$, by

$$\overline{\Delta\delta} = \tfrac{1}{2}(3\cos^2\beta - 1)\Delta\delta \quad (3)$$

With the observed $\overline{\Delta\delta}$ of 1250 ppm (298 K) and $\Delta\delta$ of 3350 ppm (77 K) for the terminal oxygen, we find $\beta = 40°$, the angle between the O–O bond and the rotation axis (Fe–O bond axis). This is in excellent agreement with the angle of 45° determined[3] by X-ray diffraction methods, and we believe when taken together with the independent solution NMR assignments, it supports our assignments and simulation results.

Rapid rotation of O_2 about the Fe–O bond axis should also axially average the shielding tensor of the bridging oxygen, and our simulations indicate that this is approximately true within the stated uncertainties, although the best simulations have slightly different values for δ_{22} and δ_{33}. This apparent asymmetry might be due to the quadrupole interaction. However, because the

chemical shift interaction so dominates the spectra, varying the electric field gradient tensor parameters, and their orientation relative to the CS tensor, adds too many parameters to the fitting procedure relative to the slight differences produced in the simulations, leading to a flat, broad minimum in parameter space.

Our temperature dependence results for the bridging oxygen are also more difficult to understand, since the isotropic chemical shift becomes ~400 ppm more shielded on cooling from 298 to 77 K, and the high- and low-temperature chemical shift tensors are not simply related, as was found with the terminal oxygen. We believe that the observed chemical shift (and chemical shift anisotropy) of the bridging oxygen is due to the presence of several rapidly interconverting (at 298 K) substates, one of which "freezes in" at low temperature. This hypothesis is based in part on the Mössbauer work of Spartalian et al.,[32] and is exactly the same argument used recently by Gerothanassis et al.[25] to explain the anomalous temperature-dependent line widths they observed using solution NMR techniques. Thus, solution-state ^{17}O NMR, solid-state ^{17}O NMR, and Mössbauer spectroscopy all show unusual temperature-dependent behavior.

Spartalian et al.[32] observed a peculiar temperature dependence of the quadrupole splitting in ^{57}Fe Mössbauer measurements of the same dioxygen complex that we have investigated. They proposed that the temperature dependence of the quadrupole splitting reflects a thermal equilibrium between two energetically different conformational states, and deduced significantly different electric field gradient tensors of the iron nucleus for the high- and low-energy conformers. X-ray results on the same complex[3] suggest that these two states could result from the presence of the imidazole plane, which makes an angle of either 20° or 70° with the iron oxygen plane. It seems possible that the bridging oxygen, which is directly bonded to the iron, may also have different electronic environments (and shielding tensors) in the two states. Rapid rotation of the dioxygen ligand at room temperature would simply cause an averaging of the shift tensors of the two states. We thus believe that the shift tensor of the bridging oxygen at 298 K is the average value of the two conformers, while the shielding tensor at 77 K corresponds primarily to the low-energy conformer.

Our results show that the anisotropies of the chemical shift tensors are very large for both oxygens ($\Delta\delta$ = 2200 ppm for the bridging oxygen and 3350 ppm for the terminal oxygen, at 77 K), and the isotropic chemical shifts of both oxygens are also both unusually deshielded. The optical spectra of oxyhemoglobin indicate[14,15] that a number of low-lying excited states exist in the $Fe-O_2$ unit (at least four excited states with excitation energies of between 0.85 and 1.6 eV), and theoretical calculations also predict[9] similar low-lying excited states for a model compound. When we consider the typical average excitation energies of 5–10 eV for the majority of other compounds,[33] the excitation energy ΔE of ~1 eV for the $Fe-O_2$ unit is exceptionally low, and could be a contributor to both the unusual low-field shifts and the large chemical shift anisotropies.

Myoglobin and Hemoglobin. We have obtained over 100 solid-state ^{17}O NMR spectra of frozen solutions and polyethylene glycol induced microcrystalline precipitates of oxymyoglobin and oxyhemoglobin, as a function of both temperature and instrumental conditions, basically as described previously for oxy picket fence porphyrin, and representative spectra for MbO_2 and HbO_2 are shown in Figure 4. There is clearly a close general similarity between the proteins and the picket fence porphyrin, which reflects the close similarity in bonding between all three systems. Unfortunately, currently achievable spectral signal-to-noise ratios do not warrant any detailed spectral simulations—to investigate, e.g., the effects of bond angle on shift, or to differentiate between the α- and β-subunits in HbO_2. Such studies may in the future be feasible, but will require higher field strengths, combined with field-sweep/spin-echo techniques to obtain more detailed line shape information. Nevertheless, the results of Figure 4 are important

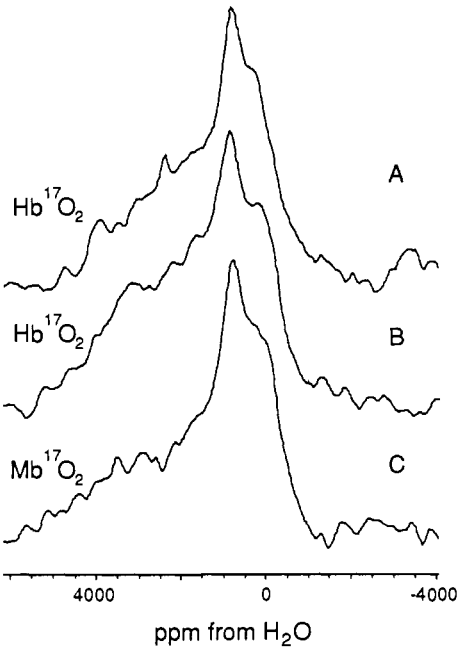

Figure 4. 8.45-T ^{17}O NMR spectra at 77 K of [$^{17}O_2$]hemoglobin and -myoglobin: (A) hemoglobin, frozen solution; (B) hemoglobin, polyethylene glycol microcrystals; (C) myoglobin, polyethylene glycol microcrystals.

in that they do represent the first actual observation of the ^{17}O NMR resonances of oxymyoglobin and oxyhemoglobin. The overall spectral breadths observed (~4000 ppm) as well as the position of the major singularity (~850 ± 70 ppm, possibly δ_{11} of the terminal oxygen) are close to the values found for oxy picket fence porphyrin, at low temperatures, implying no major difference in Fe–O–O bonding, and suggest a rigid FeO_2 subunit, at 77 K. Unfortunately, at 298 K, the $^{17}O_2$ signals are obscured by residual $H_2{}^{17}O$ (data not shown).

According to Goddard and Olafson,[8] the $Fe-O_2$ moiety in HbO_2 can be represented by an ozone-like structure, with Fe(II) (S = 1) O_2 (S = 1) bonding. Although the isotropic chemical shift alone may not be a true measure of the electronic environment of a nucleus, which may be better represented by the chemical shift tensor, our values of ~2000 and ~1200–1600 ppm for the terminal and bridging oxygens are somewhat comparable with the values of 1598 and 1032 ppm for the ozone molecule,[34,35] suggesting some similarities in the electronic environments between the Fe–O–O fragment and the ozone molecule, basically as recounted by Gerothanassis and Momenteau.[24,25] On the other hand, our results also indicate that the nuclear quadrupole coupling constants of the two oxygens in the picket fence porphyrin $Fe-O_2$ unit are *much* smaller than the reported values[36] of 19.95 and 8.52 MHz, for the terminal and bridging oxygens in ozone, suggesting, not surprisingly, major differences between them.

We now briefly consider possible origins for the solution/solid-state chemical shift differences we have observed.

Recent progress in the theory of chemical shifts for ^{13}CO and $C^{17}O$ bound to heme proteins by Dykstra et al. has pointed to the importance of weak electrical interactions in governing the observed chemical shifts, an interaction mediated via the shielding polarizability, A.[37,38] While computation of the chemical shifts and shielding polarizabilities of fragments as large as FeO_2 (or hemes!) is not currently practical, it nevertheless seems plausible

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that the highly polarizable Fe=O^+—O^- moiety will have very large $A°$ values, which could contribute to large solid-state/solution-state chemical shift differences. The ^{17}O chemical shifts, and chemical shift anisotropies, are the largest ever observed for a diamagnetic solid, and it thus seems reasonable to suppose that the changes in such shifts with electric fields will likewise be extremely large.

Experimentally, our results indicate at room temperature ~150 and ~500 ppm chemical shift differences between the solid-state and solution-state NMR results, for the bridging and terminal oxygens, respectively, while the low-temperature results are both ~500 ppm more shielded than the solution values. While we can of course invoke Fe–O–O bond angle/length changes as Gerothanassis et al. have done in order to explain their significant modifications of the electric field gradient at both oxygen sites,[25] we must also consider solvent or hydrogen-bonding[39] effects.

We have shown previously[38] that weak electric field interactions may cause sizeable chemical shifts, e.g., for $C^{17}O$ ligands in heme proteins, an interaction mediated via the shielding polarizability. For free $C^{17}O$, we found $A° \approx 2270$ ppm/au (1 au $\approx 5.14225 \times 10^9$ V cm^{-1}), and CO has an ^{17}O chemical shift anisotropy of ~700 ppm. As a crude estimate of $A°$ for the O_2 ligand oxygens, we might simply scale the $A°$ term by the increase in the apparent CSA, say to $\sim 2270 \times 3350/700 \approx 10000$ ppm/au. For a CONH group in the pivalamido picket ~4-Å distant, we can estimate a field of ~0.01 au so the maximal E field shift is ~100 ppm. We estimate our experimental uncertainty on the isotropic chemical shift to be ~±200 ppm—due primarily to uncertainties in determining the cutoff in the wings of the spectra.

For the bridging oxygen at room temperature, our solid-state NMR results on the picket fence porphyrin ($\delta_i = 1600$ ppm) are in quite good agreement with the solution NMR results ($\delta_i \approx 1750$ ppm)—certainly within the range of experimental error. Only when we compare the low-temperature solid-state results with the room-temperature solution results are there significant differences for the bridging oxygen, which once again could imply a different structure at low T, consistent with the X-ray and Mössbauer results.[32] Thus, the main solid–solution difference is observed for the terminal oxygen, although in the solid state the high and low T solid-state results ($\delta_i = 2017$ ppm, 298 K; $\delta_i = 1967$ ppm, 77 K) are quite consistent.

Conclusion

In summary, we believe that the results we have described above are important for a number of reasons. First, we have obtained the first solid-state ^{17}O NMR spectra of an $^{17}O_2$ ligand bound to a model heme system. Our results indicate that both bridging and nonbridging oxygen resonances are highly shifted, that the chemical shift anisotropies are the largest ever measured, that the actual shifts are moderately close to those of O_3, but that the quadrupole coupling constants are a factor of ~2–4 less than those found for O_3. Second, we have obtained the first ^{17}O NMR spectra of oxyhemoglobin and oxymyoglobin, and our results reveal strong similarities to the model compound previously studied. Third, we have shown that ^{17}O NMR is capable of monitoring ligand rotation in the solid state in a model heme system, and thereby permits an estimation of the Fe–O–O bond angle. Fourth, our results, obtained down to 4.2 K, reveal no evidence for antiferromagnetic *met*-superoxide bonding between Fe and O_2, although of course the limited accuracy of our line shape analyses cannot absolutely rule out a very small contribution from such states.

Our results are in many ways complementary to those of Gerothanassis et al.,[24,25] who have carried out a number of ^{17}O NMR investigations of oxyheme model systems using solution NMR methods. The advantages of solution methods are clear: enhanced peak signal-to-noise ratios, and accurate chemical shifts. On the other hand, essentially all information on the chemical shift and electric field gradient tensors is lost, only a very limited temperature range can be investigated, and to date we are unaware of any ^{17}O NMR spectra being obtained from $^{17}O_2$ ligated proteins, e.g., oxyhemoglobin or oxymyoglobin, due presumably to excessive line widths caused either by exchange, or by quadrupolar relaxation.

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