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–dioxoxygen complex of "picket fence porphyrin" ($S$,10,15,20-tetrakis(c,a,a,a'-O-pivalamidophenyl)porphyrinatoiron) (II) (1-MeIm)O$_2$, as well as oxyhemoglobin and oxymyoglobin 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 Gerohanassis 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 subspecies. 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 ($\leq 55$ MHz) in all systems, much smaller than the 8.5- and 20-MHz values found for ozone, suggesting extensive 3-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 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$_2$ and HbO$_2$, and on model compounds, support Pauling's bent end-on structure over Griffith's triangular, sideways structures, 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.

Pauling first proposed a totally spin paired Fe($III$)O$_2$ configuration, based on the observation of diamagnetism in oxyhemoglobin. Weiss then suggested the superoxide formulation (Fe($III$)O$_2^-$) in which the low-spin ferric iron has its unpaired electron antiferromagnetically coupled to the coordinated superoxide oxygen. These two models should be capable of predicting (or fitting) exchange broadening or to long correlation times, were overcome by us previously by use of spin-echo methods, 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, Gerohanassis et al. have used spin-echo methods to observe the $^{17}$O NMR of several oxy picket fence porphyrins, in solution, but to date, no spectra

$^{17}$O NMR resonances of HbO$_2$ or MbO$_2$, have failed. Experimental and theoretical studies have been used to support both models. In some theoretical calculations, Goddard and Olafson postulated an ozone-like Fe–O–O fragment with Fe($II$) [S = 1] O$_2$ [S = 1] bonding, while Case et al. 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.

Similar early failures to observe $^{17}$O NMR from the peroxidic "model" Vaskas and Pt dioxoxygen complexes, attributed either to exchange broadening or to long correlation times, were overcome by us previously by use of spin-echo methods, 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, Gerohanassis et al. have used spin-echo methods to observe the $^{17}$O NMR of several oxy picket fence porphyrins, in solution, but to date, no spectra

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 subspecies. 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 ($\leq 55$ MHz) in all systems, much smaller than the 8.5- and 20-MHz values found for ozone, suggesting extensive 3-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.

of oxyhemoglobin or oxyhemoglobin have been reported. We believe that the observation of high-resolution $^{17}$O NMR spectra of oxyhemoglobin 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 carbonmonoxyhemoglobin and carbonmonoxymyoglobin 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 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, oxyhemoglobin, 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 chemical shift, observed in solution NMR as a scalar quantity, cause very extensive homogeneous broadening, and conventional solution NMR methods are rendered ineffective. Moreover, in solution, it certainly seems plausible that $Q$ 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 $-3 \text{ K}$) 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 fact seen.

In any case, we believe that our observation of the $^{17}$O NMR spectra of oxy picket fence porphyrin, oxyhemoglobin, 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 $\epsilon^2\gamma^2Q/h$ values, a value much smaller than that found for either oxygen or azide. 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, oxyhemoglobin, and oxyhemoglobin, concentrating on the static and dynamic aspects of the model system.

Experimental Section

Chemical Aspects. The dioxigen complex of "picket fence porphyrin", (5,10,15,20-tetraakis(a,a,a,a-O-pivalamidoethyl)porphyrinato)iron(II), was prepared using a method described previously, from free a,a,a,a-T$_{pp}$b, purchased from Mid-Century Chemicals (Posen, IL). For the preparation of a powdered sample of Fe(a,a,a,a-T$_{pp}$b)(II), $^{16}$O, the Fe(II) complex of the picket fence porphyrin was deoxygenated in the solid state by evacuating for several hours at 10$^4$ Torr and 25 $\text{°C}$ and then reoxidized with $^{18}$O$_2$. $^{12}$O (70% $^{18}$O) was obtained from ICON Chemicals, Inc. (Summit, NJ).

The heme protein in solution was removed from citrated blood (donated by the Saint John's Blood Bank, Piscataway, NJ) 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 25 min, and the stroma removed by centrifugation at 3000g for 60 min. The resultant hemolysate was purified on a Sephadex G-25 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 dithionate, 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 oxyhemoglobin 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 deoxygenated water (using degassed water from Sigma, Chemicals, Piscataway, NJ).

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 National (11.0 T computer and 1180 transient receiver) together with a variety of other digital and radiofrequency electronics. We also used a home-built solenoidal radiofrequency coil probe (sample volume $\approx 0.5 \text{ cm}^3$), together with a spin-echo sequence, for data acquisition. The "solid" $^{17}$O pulse width was $\approx 2 \mu \text{s}$. Chemical shifts are reported external to H$_2$O, relative to the TMS resonance. Spectra were acquired at temperatures ranging from 8-298 $\text{°C}$, with high-frequency, low-field, paramagnetic, or deshielded values taken as positive (IUPAC 8 scale). The chemical shift anisotropy tensor component convention used is that $\Delta_3$ 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 [I$^{17}$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 shape 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 [I$^{17}$O$_2$] picket fence porphyrin is overwhelmingly dominated by the chemical shift anisotropy, certainly at the higher magnetic 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 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 ($\approx 200 \text{ KHz}$) and longer pulse widths (due to our use of lower rf power levels in our narrow bore magnet, to prevent arcing) cause some attenuation.


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

<table>
<thead>
<tr>
<th>Component</th>
<th>δ^b</th>
<th>δ_1^b</th>
<th>δ_2^b</th>
<th>δ^g</th>
<th>δ_1^g</th>
<th>δ_2^g</th>
<th>δ^g_S</th>
<th>δ_1^g_S</th>
<th>δ_2^g_S</th>
</tr>
</thead>
<tbody>
<tr>
<td>terminal</td>
<td>1600</td>
<td>1600</td>
<td>1600</td>
<td>1250</td>
<td>1967</td>
<td>850</td>
<td>850</td>
<td>4200</td>
<td>3350</td>
</tr>
<tr>
<td>bridging</td>
<td>0</td>
<td>2150</td>
<td>2650</td>
<td>2650</td>
<td>1190</td>
<td>100</td>
<td>1170</td>
<td>2300</td>
<td>2200</td>
</tr>
</tbody>
</table>

*Chemical shifts are in parts per million from H_2^17O. Chemical shift anisotropies (δ^g - δ^b) 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. a 298 K. b 77 K.

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 ^1^H NMR spectra of 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 (T2), and the major spectral singularities (later assigned to δ_1^b and δ_2^b of the nonbridging site, and δ_2^g of the bridging oxygen) are considerably enhanced, as is a feature at ~2800 ppm (assigned to δ_2^g 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 ^17O NMR powder spectra of [^17O_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_0) of ~2000 ppm, while the other component has a slightly asymmetric shift tensor with a δ_0 of ~1600 ppm (at 298 K). These isotropic chemical shifts are deshielded from the recent solution ^17O NMR results of Gerothanassis et al. of ~430–70 ppm. We tentatively assign the axially symmetric ~2000 ppm component to the terminal oxygen, and the asymmetric component having δ_0 = 1600 ppm to the bridging oxygen, in general agreement with the assignments published previously by Gerothanassis et al.24,25

We obtain quite different ^17O 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 ^17O 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 ^17O NMR spectra—albeit 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 data22 and by

experimental error of A250 ppm, simulations support our view

out a large number of computer simulations of the spectrum shown

of temperatures, at 8.45 T.

elements significantly different from those given in Table

all simulation parameters were varied, we were unable to improve

using solely the

interaction, as well as a correction for pulse-power fall off, and

result was obtained by incorporating a second-order quadrupole

values at low temperatures. Although differential nonselective

fragment is statistically disordered, with the Fe-0-0 plane bi-

freedom of the terminal oxygen. A barrier to rotation of less than

kcal mol$^{-1}$ has been reported$^{19}$ from an extended Hückel cal-

The results of Table I indicate that one of the shielding tensors

is axially symmetric, while the other is not (although $\delta_{12}$ and $\delta_{33}$ 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$^{2}$ show that the terminal oxygen in the Fe-O-O fragment is statistically disordered, with the Fe-O-O plane bisecting the N$_{py}$-Fe-N$_{py}$ 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

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 Gerothanassios.$^{24,25}$

For rapid axial rotation, the averaged shift tensor will be axially symmetric and will have averaged principal components $\bar{\delta}_{1}$ and $\bar{\delta}_{2}$, given by

\[
\bar{\delta}_{1} = \frac{1}{2}(1 - \cos^{2}\beta)\delta_{11} + \frac{1}{2}(1 - \sin^{2}\beta)\delta_{22} + \frac{1}{2}(\sin^{2}\beta)\delta_{33}
\]

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

where $\beta$ is the angle formed by $\delta_{11}$ and the rotation axis, and $\alpha$ is the angle formed by $\delta_{11}$ and $\delta_{22}$. 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, $\Delta\bar{\delta} = \bar{\delta}_{1} - \bar{\delta}_{2}$, is related to the rigid-lattice value, $\Delta\delta$, by

\[
\Delta\bar{\delta} = \frac{1}{2}(3\cos^{2}\beta - 1)\Delta\delta
\]

With the observed $\Delta\delta$ of 1250 ppm (298 K) and $\Delta\delta$ of 3350 ppm (77 K) for the terminal oxygen, we find $\beta = 40^\circ$, 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$_{3}$ 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 $\delta_{22}$ and $\delta_{33}$. 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 Gerothannasis et al.33 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 dioxyn 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 complex7 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 dioxyn 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 ($\Delta \sigma = 2200$ 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 oxymyoglobin indicate14,15 that a number of low-lying excited states exist in the Fe-$O_2$ unit (at least four excited states with excitation energies of 0.85 and 1.6 eV), and theoretical calculations also predict6 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,13 the excitation energy $\Delta E$ 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 pikelet ferric porphyrin, and representative spectra for Mb$O_2$ and Hb$O_2$ are shown in Figure 4. There is clearly a close general similarity between the proteins and the pikelet ferric 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 $\alpha$- and $\beta$-subunits in Hb$O_2$. 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 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 $\delta_1$, of the terminal oxygen) are close to the values found for oxy pikelet ferric porphyrin, at low temperatures, implying no major difference in Fe-O-O bonding, and suggest a rigid Fe-$O_2$ subunit, at 77 K. Unfortunately, at 298 K, the $^{17}$O signals are obscured by residual H$_2^{17}$O (data not shown).

According to Goddard and Olafson,4 the Fe-$O_2$ moiety in Hb$O_2$ 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,14,36 suggesting some similarities in the electronic environments between the Fe-O-O fragment and the ozone molecule, basically as reported by Gerothannasis and Momenteau.37,38 On the other hand, our results also indicate that the nuclear quadrupole coupling constants of the two oxygens in the pikelet ferric porphyrin Fe-$O_2$ unit are much smaller than the reported values36 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 $^{15}$C 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 Fe$O_2$ (or hemes!) is not currently practical, it nevertheless seems plausible.

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(35) Lee, H.; Oldfield, E. Unpublished results confirming the $^{17}$O chemical shifts of Solomon et al. (ref 34).
that the highly polarizable Fe=O+—O− moiety will have very large $A^o$ 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 measured, that the chemical shift anisotropies are the largest ever measured, that the actual shifts are moderately close to those of O₂, but that the quadrupole coupling constants are a factor of ~2-4 less than those found for O₂. 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 O₂, 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 oxyhemeprotein 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 ligated proteins, e.g., oxyhemoglobin or oxymyoglobin, due presumably to excessive line widths caused either by exchange, or by quadrupolar relaxation.

Acknowledgment. We thank Drs. P. D. Ellis and R. E. Wasylishen for generously providing copies of their line shape simulation programs (ref 40 and 41).

