of superoxide anion dismutation) to $^1O_2$ levels in systems containing superoxide anion should be carefully assessed.

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Susceptibility Studies of Laccase and Oxyhemocyanin Using an Ultrasensitive Magnetometer. Antiferromagnetic Behavior of the Type 3 Copper in *Rhus* Laccase

Sir:

The laccases are enzymes (*p*-diphenol: O_2 oxidoreductase, EC 1.10.3.2) that contain four copper atoms in three distinct sites.[1] In addition to the two EPR-detectable centers (types 1 and 2), there are two EPR-nondetectable (or type 3) copper atoms. The type 3 coppers, which are responsible for an absorption band at 330 nm, together function as a two-electron acceptor in the enzyme. Most investigators have considered a type 3 site to contain either an antiferromagnetically coupled Cu(II) pair[2] or two Cu(I) units associated with another two-electron acceptor (e.g., a disulfide[3]). Another possibility that suggests itself from work[4] on D-galactose oxidase is a low-spin Cu(III)–Cu(I) unit.

The temperature dependence of the magnetic susceptibility is the best method for obtaining direct evidence on the state of the type 3 copper atoms. For an antiferromagnetically coupled Cu(II) dimer the variation of the susceptibility with temperature will not follow the Curie law ($\chi \propto 1/T$) for paramagnetism, but will exhibit a maximum at a position dependent on the strength of the coupling.[5] Both low-spin Cu(III)–Cu(I) and binuclear Cu(I) units will be diamagnetic and exhibit no temperature dependence of the susceptibility. Previous measurements of χ vs. T have been made for *Rhus vernicifera* laccase, but antiferromagnetic components were not resolved.[6] As very high sensitivity would be required for such resolution, we have measured the temperature dependence of the volume susceptibility of *Rhus* laccase over a wide range with a magnetometer utilizing quantum flux detection methods.

Rhus vernicifera laccase was purified to an A_{280}/A_{614} ratio of 15.4 by a standard method.[7] One sample was dialyzed extensively against distilled, deionized water and then concentrated by ultrafiltration to 1.25 mM with a ratio of 15.6. A second sample was dialyzed for 24 h against 0.001 M EDTA, 25 mM Tris buffer (pH 8.1). This sample was then dialyzed against buffer alone for another 24 h and concentrated to 3.4 mM. At a concentration of 3.4 mM, A_{280}/A_{614} increased to 19, but A_{280}/A_{330} remained constant. Apo laccase was prepared by extensive dialysis against 0.01 M NaCN in Tris buffer. Copper content was checked by atomic absorption and found to be negligible.

The magnetic susceptibilities were measured using an oscillating sample[8] superconducting magnetometer.[9] The instrument was calibrated with a 17 mM $NiCl_2$ solution using $\chi_M^{Ni} = 4434 \times 10^{-6}$ cgs/mol at 20 °C. The high sensitivity of this instrument allows changes in volume susceptibility of 0.5% of the diamagnetism of water to be resolved. Sample volumes of 0.12 ml were measured over a 30–210 K range with a reproducibility of 1.5% of water diamagnetism between runs. Several measurements of protein susceptibilities were made at each temperature and the error was taken as the maximum deviation from the mean. Compensation for sample rod contraction was made by repositioning the sample after every temperature change. Experimental difficulties with the high temperature system prevented obtaining data above 140 K for the 3.4 mM laccase sample.

The measured volume susceptibilities of *Rhus* laccase samples as a function of $1/T$ are presented in Figure 1. Paramagnetism is observed as a decrease from solution diamagnetism. The intersection of the extrapolated low temperature lines gives $\chi_{cc}^{\infty} = -0.669 \times 10^{-6}$ cgs, which is close to the volume susceptibility of water ($\simeq -0.68 \times 10^{-6}$ cgs at 223 K). The slope of the best-fit line for the apo

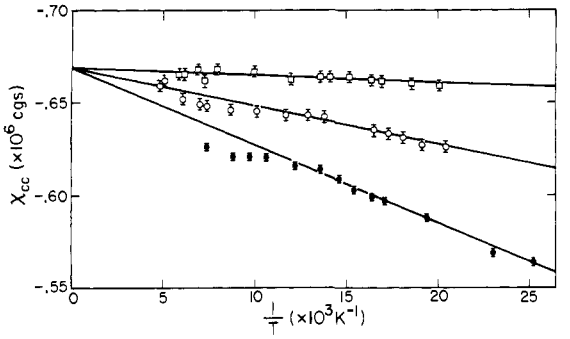

Figure 1. Temperature dependence of the volume susceptibility of *Rhus* laccase solutions: □, 1.24 mM apo; ○, 1.25 mM native; and ●, 3.4 mM native.

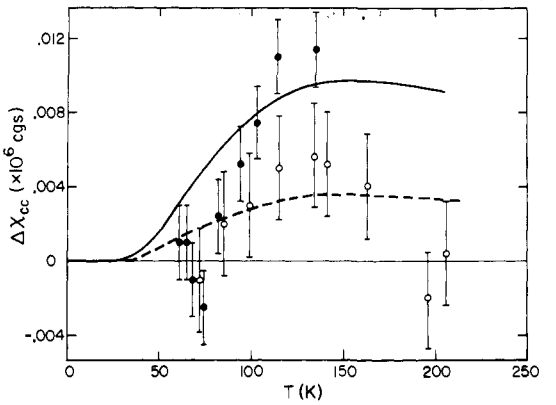

Figure 2. Deviation of the volume susceptibilities of native *Rhus* laccase solutions from the linear $1/T$ dependence observed at low temperatures: ○, 1.25 mM; ●, 3.4 mM. Theoretical lines (see text) are: - - -, 1.25 mM; —, 3.4 mM.

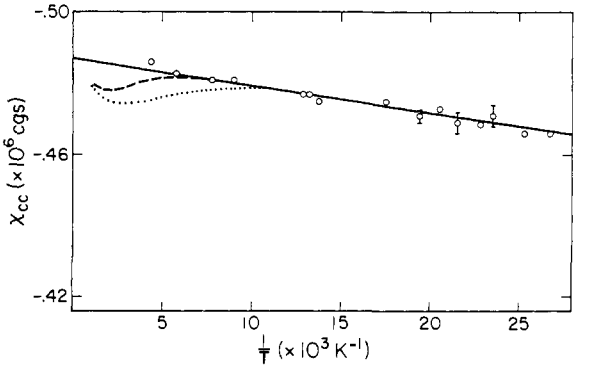

Figure 3. Temperature dependence of the volume susceptibility of a gel of Keyhole limpet oxyhemocyanin (0.12 ml, 9 mM). Calculated deviations from linear χ vs. $1/T$ behavior for J values of 400 and 625 cm^{-1} are shown in dotted (···) and dashed (- - -) lines, respectively.

sample is 0.4×10^{-6} cgs K, which was taken as a measure of the amount of dissolved oxygen present. The simple $1/T$ dependence in this case excludes any anomalous volume susceptibility changes owing to sample density effects. The lines for the native samples represent best fits to the $T < 70$ K points. As a result of the larger error associated with the less concentrated sample, the line was constrained to intersect at the same χ_{cc}^{∞} value as that for apo laccase. The observed slopes for the 1.25 and 3.4 mM samples are 2.1×10^{-6} and 4.3×10^{-6} cgs K, respectively. Theoretical slopes, calculated assuming Curie law behavior for each of the two paramagnetic coppers (μ/Cu = 1.93 μ_B) and corrected for the observed behavior of the apo sample, are 1.6×10^{-6} and 3.6×10^{-6} cgs K. The difference between the observed and calculated slopes in each case is attributed to the presence of a very small amount of paramagnetic impurities (e.g., 0.01% high-spin Fe^{3+}).

At higher temperatures the χ_{cc} values for *Rhus* laccase deviate from the extrapolated low temperature line. The nature of the deviation establishes the presence of an antiferromagnetically coupled Cu(II) dimer in the enzyme. For such a dimer, the variation of the susceptibility with temperature is given by $\chi_M = (g^2N\beta^2/3kT)\,(1 + \frac{1}{3}e^{J/kT})^{-1}$, neglecting the temperature-independent paramagnetism.[5] The exchange energy, J, is defined by the interaction Hamiltonian $\mathcal{H} = JS_1 \cdot S_2$ ($S = \frac{1}{2}$ for each Cu(II)). Taking $g = 2.17$,[10] a best fit of the observed deviation ($\Delta\chi_{cc}$) for each sample to the above theoretical expression gives a J of 170 ± 30 cm^{-1} (Figure 2). An additional check of this value is afforded by the observed maximum deviation in the 1.25 mM sample. For two $S = \frac{1}{2}$ Cu(II) atoms, theory predicts $J = 1.6kT_{max}$, which gives a J of approximately 150–160 cm^{-1}.

We have also measured the temperature dependence of the volume susceptibility of oxyhemocyanin,[11] as certain physical properties of this protein are similar to those characteristic of type 3 copper.[1] All the data points shown in Figure 3 are well represented by the best-fit solid line. Thus, only a weak paramagnetic contribution ($\chi = C/T$) with $C = 7.5 \pm 0.6 \times 10^{-7}$ cgs K is observed outside the large diamagnetic background of the protein gel. The value of the Curie constant, C, is readily rationalized as the contribution from dissolved oxygen (assumed to be 0.3 mM, and 0.8 mM paramagnetic Cu^{2+}). The latter estimate is based on ageing curves and represents about 4% of the total protein-bound copper in a week-old sample.[12]

The observation of linear χ vs. $1/T$ behavior for oxyhemocyanin at high temperature contrasts strikingly to the results obtained for *Rhus* laccase. Deviations predicted by theory are shown in Figure 3 for J values of 400 and 625 cm^{-1}. It is apparent that any structural model for oxyhemocyanin based on the presence of antiferromagnetically coupled copper(II) dimers must take $J \geq 625$ cm^{-1}. The lower limit of 625 cm^{-1} for J is much larger than that proposed previously[13] for oxyhemocyanin, owing to the higher sensitivity of the magnetometer used in the present study.

Resonance Raman spectral studies have shown the oxygen in oxyhemocyanin to be bound in a peroxy form.[14] Thus, two electrons must be transferred from the binding site to the oxygen. Assuming that it is the copper that is oxidized, models based on strongly superexchange-coupled, bridged binuclear units are most attractive. It should be clear that exact identification of the bridging group (or groups) in either oxyhemocyanin or *Rhus* laccase cannot be made from the results of the present experiments. For *Rhus* laccase, however, it is important to emphasize that the type 3 site is an antiferromagnetically coupled Cu(II) dimer, and that any model based either on two Cu(I) atoms or a Cu(I)-Cu(III) unit cannot be correct.

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Carbon-13 Nuclear Magnetic Resonance of Polymers Spinning at the Magic Angle

Sir:

Cross polarization (CP) of carbons by protons is a practical way of obtaining high sensitivity natural abundance ^{13}C NMR spectra of solids.[1] In the Hartmann–Hahn version of the CP experiment,[1,2] polarization transfers from the protons to nearby carbons via static dipolar interactions in a time characterized by a spin-lock CP relaxation time in the rotating frame, T_{CH}.[3] In many cases T_{CH} is on the order of 100 μs.[1,4] After the CP transfer, the carbon free-induction decay can be observed while the protons are being dipolar decoupled. Although dipolar broadening has been removed by strong decoupling during data acquisition, chemical shift anisotropy may still severely complicate the ^{13}C spectrum. For sufficiently simple systems involving only a few chemically different types of carbons, resolved or partially resolved chemical shift anisotropy can be a useful source of information about the geometry and electronic structure of the solid.[1] But in many systems of interest to the chemist, overlapping anisotropies are not interpretable, and the ^{13}C spectrum of the solid becomes a poorly resolved and disappointing wide-line spectrum.

In this situation the information contained in the chemical shift anisotropies may be sacrificed for the improvement in resolution which can often be achieved by high-speed mechanical sample rotation at the magic angle.[5] By fashioning the solid into a rotor[4] (or placing the solid inside a hollow rotor) and aligning the axis of rotation of the rotor at 54.7 $\pm$ 1° relative to H_0, chemical shift anisotropic dispersions are averaged to their isotropic values by sample rotation of 2–3 kHz, spinning frequencies which are somewhat greater than the width of the dispersion.[4,5]

The question arises as to the practical limits to the achievable resolution and hence the complexity of chemical systems which can, in fact, be approached by CP ^{13}C NMR with spinning at the magic angle. We have investigated, at room temperature, over a dozen synthetic and natural macromolecular systems by magic-angle CP ^{13}C NMR and present here results on three such systems, illustrating what we feel are the present capabilities and limitations of the technique.

As shown in Figure 1, the magic-angle CP spectrum of solid polysulfone, an engineering plastic, is nearly as detailed as the standard FT spectrum of the polymer in solution. (The CP spectra were obtained by the single-contact Hartmann–Hahn procedure, using ^1H and ^{13}C rf fields of 8 and 32 G, respectively. The data for each CP spectrum were acquired from 500 sequence repetitions requiring a

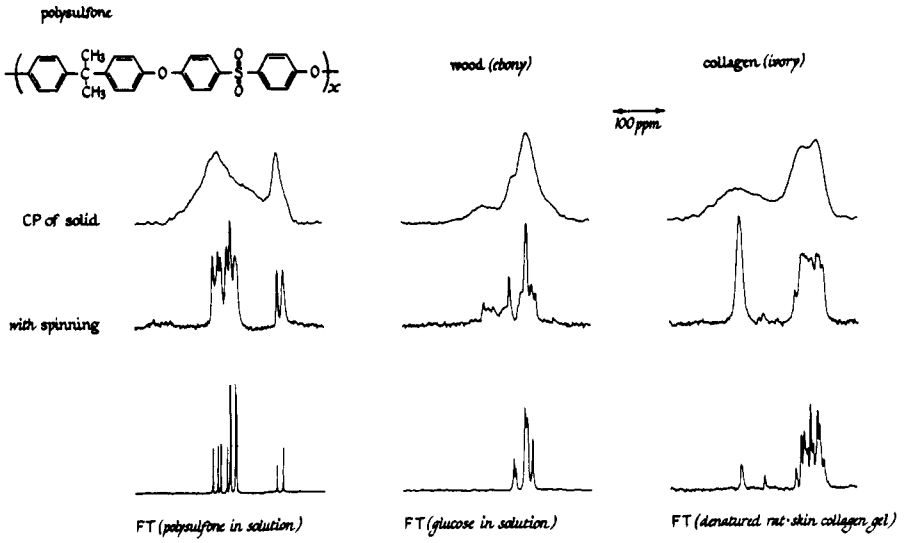

Figure 1. Dipolar-decoupled natural abundance ^{13}C NMR spectra of some solids obtained using single Hartmann–Hahn cross-polarization contacts of 1 ms duration. The cross-polarization spectra, obtained both with and without magic-angle spinning, are compared to some standard Fourier transform ^{13}C NMR spectra of various materials in solution. (The FT spectrum of the protein gel, bottom right, is not fully relaxed, and the intensity of the lowest field line has been discriminated against.) Each spectrum is 8 kHz wide (at 22.6 MHz). The magnetic field increases from left to right.