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Magnetic susceptibility studies of laccase and oxyhemocyanin

(copper proteins/variable temperature measurements/antiferromagnetism)

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ABSTRACT **The magnetic susceptibility of *Rhus vernicifera* laccase has been remeasured over the temperature range 5–260 K. In contrast to our previous results [Solomon, E. I., Dooley, D. M., Wang, R. -H., Gray, H. B., Cerdonio, M., Mogno, F. & Romani, G. L. (1975) *J. Am. Chem. Soc.* 98, 1029–1031] linear χ versus T^{-1} behavior was observed. The susceptibility of *Limulus polyphemus* oxyhemocyanin has also been measured in the range 5–260 K. Only weak paramagnetism, attributable to dissolved oxygen and a small amount of paramagnetic impurities, was observed. Analysis of the data establishes a lower limit of 550 cm^{-1} for J, consistent with our earlier work. The temperature dependence of the susceptibility of laccase is quantitatively accounted for by the presence of two paramagnetic copper ions (types 1 and 2) per enzyme molecule. Curie law behavior at low temperatures rules out significant interaction between the two copper types, indicating that these redox centers are well separated (several angstroms) and are not connected by bridging ligands. Formulation of the type 3 site as binuclear Cu(II) requires $J \geq 500$ cm^{-1}.**

A number of metalloenzymes that interact with dioxygen are known or postulated to contain a binuclear copper site: hemocyanin (1), tyrosinase (1, 2), and the oxidases laccase, ascorbate oxidase, and ceruloplasmin (3). Additionally cytochrome *c* oxidase has been proposed to contain a coupled heme iron–copper pair as the site of dioxygen binding and reduction (4). In no case has any electron paramagnetic resonance (EPR) signal attributable to a binuclear site been observed in the native protein (5). Although other structural possibilities are consistent with this observation, it is generally thought that the metal ions involved are strongly antiferromagnetically coupled, resulting in a diamagnetic or even spin ground state (5, 6). The temperature dependence of the magnetic susceptibility of such systems is distinctive and may be used to distinguish antiferromagnetic coupling from a truly diamagnetic structure—e.g., a Cu(I) dimer. For two antiferromagnetically coupled $S = 1/2$ Cu(II) ions, the susceptibility will be a maximum at a temperature that is simply related to the energy difference, J, between the diamagnetic ground state and the paramagnetic excited state (7). Antiferromagnetic coupling between two copper ions has been observed in a wide variety of dimeric Cu(II) complexes (8) and in the four-copper derivative of bovine superoxide dismutase (9). We have focused our efforts on oxyhemocyanin and laccase. It is generally accepted that the dioxygen binding site in oxyhemocyanin is a binuclear copper(II) peroxo complex, the evidence coming mainly from resonance Raman (10) and other spectroscopic (11, 12) experiments. The four copper atoms in laccase are distributed in types 1 (paramagnetic), 2 (paramagnetic), and 3 (binuclear; EPR nondetectable) sites. The type 3 coppers apparently function as a two-electron acceptor and are thought to be the site of interaction with dioxygen (3).

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Previous magnetic susceptibility measurements showed oxyhemocyanin to be diamagnetic over the temperature range 35–250 K (13, 14). Over this same range, the susceptibility of *Rhus* laccase showed, in addition to the Curie law paramagnetism of copper types 1 and 2, non-Curie paramagnetism at temperatures above 80 K that was consistent with an antiferromagnetically coupled Cu(II) pair with $J = 170 \pm 30$ cm^{-1} (13).

Owing to the fact that the fit of theory to the observed deviation from Curie law in laccase was not entirely satisfactory (due in part to the low precision of the measurements) and realizing the importance of these observations to understanding the copper-site electronic structure of hemocyanin and laccase, we have remeasured the susceptibilities over a wider temperature range by using susceptometers recently developed at SHE Corporation, San Diego, CA. Although oxyhemocyanin was again observed to be essentially diamagnetic over the entire temperature range, laccase, in contrast to our previous results, displayed only the Curie law behavior of copper types 1 and 2 over the temperature range examined.

MATERIALS AND METHODS

Rhus vernicifera laccase was purified by the method of Reinhammar (15) from the acetone powder (Saito & Co., Ltd., Japan) to a A_{280}/A_{614} value <15. A sample was then dialyzed extensively against distilled, deionized water and concentrated in an Amicon ultrafiltration apparatus to a final concentration of 2.2 mM, determined from the extinction coefficient (ϵ_{614} = 5700 M^{-1} cm^{-1}). *Limulus polyphemus* hemolymph was strained through cheesecloth, centrifuged, and dialyzed against 50 mM Tris·glycine/10 mM EDTA, pH 8.9, and then against 50 mM Tris/10 mM EDTA, pH = 8.0. This procedure ensured that the oxyhemocyanin was disaggregated into a mixture of subunits of molecular weight ~ 70,000 (16). Copper concentration was determined by atomic absorption and by optical absorption (ϵ_{340} = 10,000 M^{-1} cm^{-1}) (17) and found to be 4.70 ± 0.05 mM (mean ± SEM) by both methods.

Magnetic susceptibility measurements as a function of temperature were performed by using two different prototype instruments constructed at SHE Corporation; these instruments are fully described elsewhere.¶ Both versions detected the total magnetic moment in a sample by using a superconducting quantum interference device (SQUID). The earlier instrument used two single-loop SQUID pickup coils arranged concentri-

Abbreviations: EPR, electron paramagnetic resonance; SQUID, superconducting quantum interference device; BM, Bohr magneton.

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¶ SHE Corporation (1977), Technical Description, Variable Temperature Superconducting Susceptometer Systems.

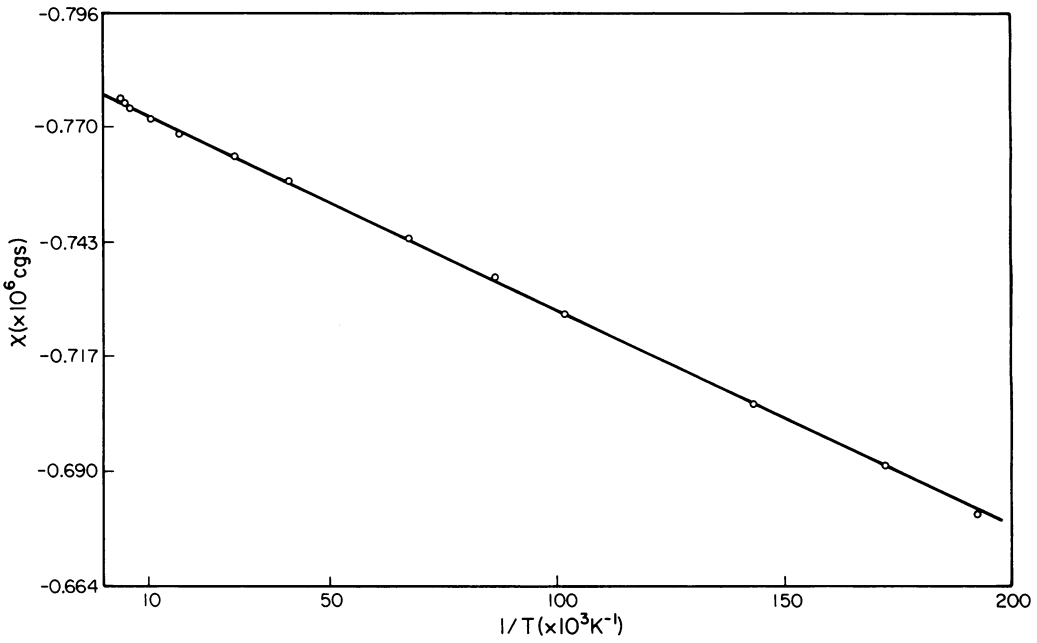

FIG. 1. Temperature dependence of the volume susceptibility of *Limulus* oxyhemocyanin. O, Six standard deviations of the SQUID output at each temperature.

cally relative to the sample space and thus was sensitive to the exact sample geometry. (Corrections could be made during conversion of the SQUID output to a volume susceptibility.) The newer instrument used two counterwound Helmholtz coil pairs compensated to respond uniformly (within 5%) over a volume 5 mm in diameter and 8 mm long, thereby allowing the mass susceptibility to be measured directly. Degassed distilled deionized water, $HgCo(SCN)_4$, and platinum metal were used to calibrate the susceptometers. The temperature was monitored by using a resistance thermometer calibrated *in situ* against platinum and carbon/glass standards from Lake-Shore Cryogenics. At least five measurements were recorded and averaged at each temperature. Temperature regulation was better than 0.4% maximum deviation from the set point over the entire range. The sample holders were measured separately over the same temperature range as the samples and their contribution to the observed signals was subtracted. No effort was made to subtract the diamagnetism of the water or buffer from the total sample signal.

Laccase was degassed in the susceptometer airlock by several cycles of complete evacuation followed by flushing with helium. This procedure was avoided for oxyhemocyanin in order to prevent significant deoxygenation. Instead, oxyhemocyanin was exposed briefly to a partial vacuum and then quickly flushed with helium. No changes in the samples were observed to occur during this process. Approximately 0.6 ml of solution

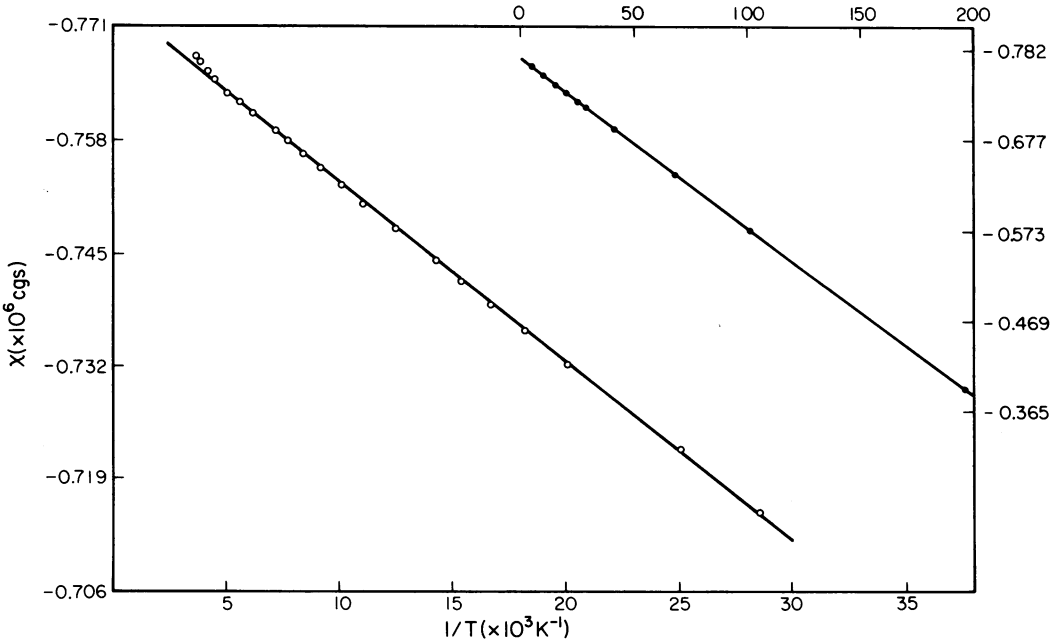

FIG. 2. Temperature dependence of the volume susceptibility of *Rhus* laccase. O and ●, Three and 25 standard deviations of the SQUID output at each temperature.

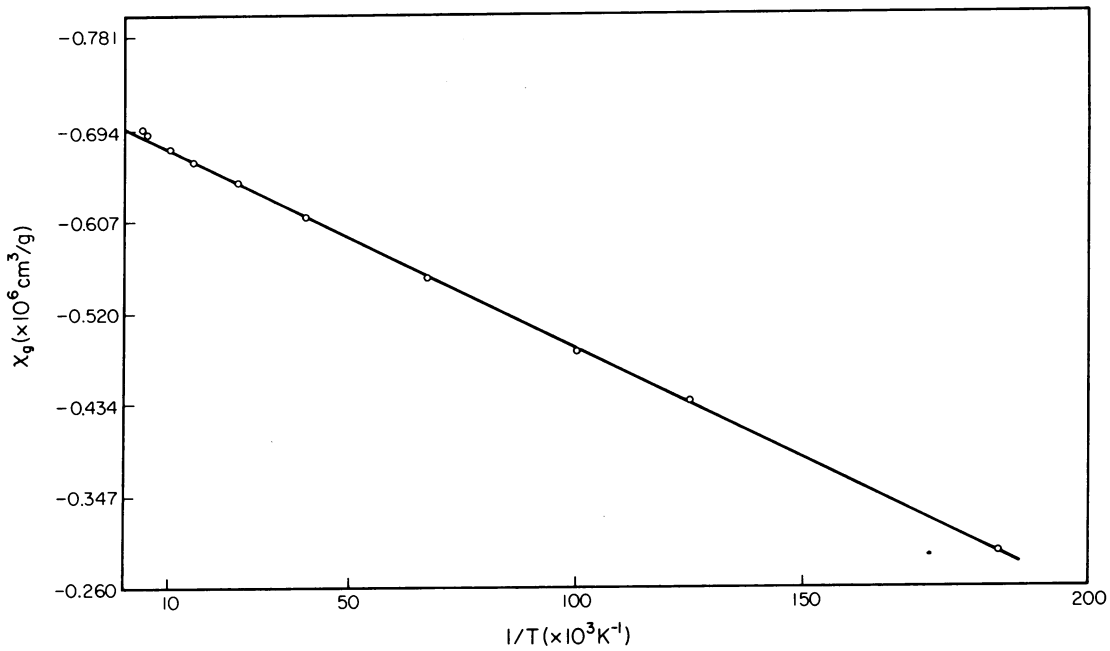

FIG. 3. Temperature dependence of the gram susceptibility (based on total sample weight) of *Rhus* laccase measured on the new prototype susceptometer. O, Approximately 15 standard deviations of the SQUID output at each temperature.

was measured with the first prototype; only ~0.05 ml was required with the newer instrument.

RESULTS AND DISCUSSION

The volume susceptibility of *Limulus* oxyhemocyanin as a function of T^{-1} is shown in Fig. 1. A weak Curie law ($\chi = CT^{-1}$) paramagnetism, observed as a decrease in the solution diamagnetism, is evident with a least squares fit slope of -0.50×10^{-6} cgs·K. This is less than 25% of the slope calculated—assuming 4.7×10^{-3} M Cu^{2+} and $\mu = 1.93$ Bohr magnetons (BM), ($= -2.2 \times 10^{-6}$ cgs·K)—and is attributed to the presence of dissolved dioxygen and paramagnetic impurities.‖ Our present data agree well with previous measurements of *Megathura crenulata* (13) and *Limulus* oxyhemocyanin (unpublished data) and, taken together, establish that the binuclear copper pair in oxyhemocyanin is diamagnetic over the entire temperature range 5–260 K, indicating that the antiferromagnetic coupling must be very strong. With a Heisenberg–Dirac–Van Vleck hamiltonian, $\mathcal{H} = JS_1 \cdot S_2$, to define the exchange energy, the present data require $J \geq 550$ cm^{-1}. This lower limit is somewhat below our estimate for *M. crenulata* hemocyanin ($J \geq 625$ cm^{-1}), owing to the much lower copper concentration in these experiments. Antiferromagnetic coupling is usually mediated by a superexchange pathway involving the atomic or molecular orbitals of bridging groups. Because such large values of J have not been observed in any singly bridged binuclear Cu(II) complexes (19–21), a multiply bridged dioxygen–copper center is a more attractive structural possibility for oxyhemocyanin.

Fig. 2 depicts the volume magnetic susceptibility of *Rhus* laccase *versus* T^{-1}. In contrast to our earlier results (13), only a simple Curie law paramagnetism is observed from 5 to 260 K. The slope of the least squares fit line is -1.96×10^{-6} cgs·K, which is identical within 5% of the theoretical slope calculated for two paramagnetic Cu(II) ions with $\mu = 1.93$ BM (per Cu) per laccase molecule. The lack of any contribution to the observed temperature dependence from dissolved O_2 or paramagnetic impurities (as seen in the oxyhemocyanin data) is probably due to the more extensive degassing and the dialysis against pure water used for the laccase sample.

The mass susceptibility of the same sample of *Rhus* laccase as a function of T^{-1} is plotted in Fig. 3. Again, only Curie law paramagnetism is observed. The slope of the least squares line is -0.00787 (cm^3/g_{Cu})·K,** which agrees with the theoretical slope for two paramagnetic coppers [-0.00733 (cm^3/g_{Cu})·K]. We are confident that the present results are correct for two reasons: (*i*) the measurements (which are identical within experimental error) were made on the same sample several months apart on entirely different susceptometers; and (*ii*) it is extremely difficult to conceive of effects that would give fortuitous cancellation of a complicated temperature dependence [i.e., $\chi_M = (g^2N\beta^2/3KT)(1 + 1/3e^{J/KT})$], due to an antiferromagnetically coupled Cu(II) dimer, in order to obtain precisely the Curie law behavior predicted for the copper types 1 and 2.

Because Curie law behavior is observed to ~5 K, any interaction between the copper types 1 and 2 must be quite small, consistent with the idea that these ions are separated by several angstroms (3, 22) in the native laccase molecule. Furthermore, it is unlikely that the copper types 1 and 2 redox centers are connected by ligand bridging groups, because such structures

‖ Assuming the O_2 concentration in solution to be 0.30×10^{-3} M (18) and $\mu = 2.83$ BM, the slope is calculated to be -0.30×10^{-6} cgs·K. Iron content of the oxyhemocyanin solution was determined by atomic absorption to be 27 μM; if $\mu = 5.9$ BM, then its contribution to the slope is -0.12×10^{-6} cgs·K. Thus, nearly the entire temperature dependence of oxyhemocyanin is accounted for without any contribution from the intrinsic copper.

** This gram susceptibility may be converted to an approximate volume susceptibility by use of the relationship $\chi_g = \chi/\rho$ in which ρ is the density g_{Cu}/ml. A value of -2.2×10^{-6} cgs·K obtains, in good agreement with the measurement on the older susceptometer. The slightly increased paramagnetism could arise from less efficient deoxygenation or from impurities, because the sample was not redialyzed against pure water after several months of storage at −10°. The A_{280}/A_{614} ratio was ~15.0 after this period, compared to ~13.5 initially.

would be expected to give rise to measurable superexchange interactions.

Our finding that the type 3 site in oxidized *Rhus* laccase is magnetically similar to the binuclear copper redox center in oxyhemocyanin may have structural significance. If the type 3 site contains Cu(II), then strong antiferromagnetic coupling ($J \geq 500\ cm^{-1}$) must be present. Further evidence that the type 3 site has some structural similarity to the binuclear copper center in oxyhemocyanin has been obtained recently from spectroscopic examination of laccase–dioxygen intermediates during turnover conditions. At 3° and pH 6.0, a laccase–dioxygen complex is observed with an optical spectrum (λ_{max} = 340 nm and a weaker peak at 475 nm) that is similar to that of oxyhemocyanin; what is more, a circular dichroism maximum is observed for this species at 362 nm, a region in which signals are present in the spectrum of oxyhemocyanin (unpublished data). Further spectroscopic and magnetic studies of this laccase-dioxygen complex are required to define the oxidation levels of the coppers and the dioxygen, as well as the relationship, if any, of the type 3 site in this species to the binuclear copper(II)–peroxo unit in oxyhemocyanin.

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