Multiquantum EPR of the mixed valence copper site in nitrous oxide reductase

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ABSTRACT This work demonstrates the use of multiquantum EPR to study the magnetic properties of copper complexes and copper proteins. Pure absorption spectra are obtained because of the absence of field modulation. The signal intensity of 3-quantum spectra is proportional to the spin lattice relaxation time T_1, while its linewidth in a frequency difference sweep is T_1^{-1}. A change in lineshape for the EPR detectable mixed valence [Cu(1.5) . . . Cu(1.5)] site in nitrous oxide reductase is attributed to suppression of the forbidden transitions. The data confirm the unusually fast relaxation time for this site, which requires temperatures of less than 100 K to resolve hyperfine structure. The T_1's for the mixed valence [Cu(1.5) . . . Cu(1.5)] site in nitrous oxide reductase are very similar to T_1's for the Cu_a site in cytochrome *c* oxidase. The similar relaxation properties, together with previous multifrequency EPR results, support the hypothesis that the EPR detectable sites in cytochrome *c* oxidase and nitrous oxide reductase are mixed valence [Cu(1.5) . . . Cu(1.5)] configurations.

INTRODUCTION

An EPR method to detect intermodulation sidebands produced from irradiation of a spin system by two closely spaced microwave frequencies (ω_1, ω_2) has recently been applied to the study of free radicals (1) and nitroxides (2). Double frequency irradiation results in a periodic stimulation of the spin system and the elements of the spin density matrix oscillate due to successive absorption and emission of photons at two different frequencies (3). The signal intensity of a 3-quantum spectrum for a simple two-level system is proportional to the spin lattice relaxation time T_1, while the width of an inhomogeneous line in a frequency difference sweep (i.e., sweep of $\omega_1 - \omega_2$) is independent of the distribution of Larmor frequencies and is equal to T_1^{-1} (2) when $T_2 \ll T_1$. Thus, T_1 can be measured at different *g*-values throughout the spectrum. For a multilevel system, T_1 is replaced by the saturation parameter of the observed transition. This parameter includes all relaxation pathways that couple the transition to the lattice and can be calculated from the transition probability matrix (4). Thus, the notation T_1 in this paper could alternatively be called $T_{1\,\mathrm{eff}}$ (4). The oscillation of the transverse magnetization at $(n + 1)\omega_{1(2)} - n\omega_{2(1)}$, where n is the number of photons involved, means that this technique requires no field modulation and the display of the signal is an absorption rather than the first derivative. The absorption display is particularly interesting for EPR of metal complexes because the signals cover wide spans of the field, whereas the first derivative EPR signals return to the baseline between the turning points. The severe loss of signal due to the limited field modulation when $B_m/\Delta B \ll 1$, where B_m is the field modulation amplitude and ΔB is the width of the resonance line, is avoided and absolute susceptibilities are obtained.

The electron spin lattice relaxation time for the EPR detectable Cu_A site in cytochrome *c* oxidase (COX) is unusual for copper sites in that the relaxation time is much faster (5). An optimum temperature to resolve hyperfine structure is ~10–30 K. Recently, it was shown by multifrequency EPR that in nitrous oxide reductase (N_2OR) there is a [Cu(1.5) . . . Cu(1.5)] mixed valence Cu site that can be used as a model for the EPR detectable Cu_A site in COX (6–8). The mixed valence center in N_2OR is characterized by a seven-line pattern in the $g_\parallel$ region, which identifies [Cu(1.5) . . . Cu(1.5)], and by the absence of a half-field $\Delta m = 2$ transition. Analysis of first harmonic spectra for the EPR sites in N_2OR and COX at four frequencies, Q-band (35 GHz), X-band (9 GHz), C-band (5 GHz), and S-band (3 GHz), shows that the EPR parameters are similar, but not identical (6–8). The inference is that signals from both COX and N_2OR belong to the same type.

If the EPR detectable signal from N_2OR is from the same type of configuration as is found in COX and the dominant relaxation mechanism is intrinsic relaxation, then the spin lattice relaxation rates should be comparable. Furthermore, the signal from N_2OR is not superimposed on a heme *a* signal, as is the case for COX, and the analysis of the $g_\parallel$ region is less complicated. T_1's for COX have been carefully measured using saturation recovery EPR (9).

The temperature dependence for T_1 was observed in reference 9 to be similar for the copper detectable site and the heme *a* site in COX. These authors concluded that either the copper detectable site and the heme *a* site are in environments with very similar protein backbone configurations or the copper detectable site in COX is relaxed by heme *a* (9). The T_1's of Cu_A at $g_\parallel = 2.16$ were longer by a factor of about two than in the $g_\perp$ region, i.e., $g_y = 2.02$, and $g_x = 1.97$ as measured at 9.2 K. In contrast

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to the data of reference 9, Goodman and Leigh, using nonadiabatic progressive power saturation data find that the $g_\parallel$ component is relaxed an order of magnitude faster than $g_\perp$ (10). In the present study, multiquantum EPR data from N_2OR are compared to data of reference 9 for COX with the intent of understanding relaxation properties of both sites. The saturation parameter is determined using the frequency-difference swept lineshape. Cross-relaxation rates such as copper nuclear relaxation were assumed to have minimal contribution to the saturation parameter. However, our study does not rule out the possibility of copper nuclear relaxation contributing to the measured parameter. Given the complications of forbidden transitions and suppression of forbidden transitions with the multiquantum method (work in progress), it is not surprising that continuous wave measurements of T_1 are more complicated than saturation recovery and multiquantum techniques.

MATERIALS AND METHODS

Nitrous oxide reductase, N_2OR, from *Pseudomonas stutzeri* ATCC 14405 (11) was obtained from Professor W. G. Zumft, Karlsruhe, Germany. Pure N_2OR contained 7.6 ± 0.7 Cu atoms/M_r as determined by atomic absorption spectroscopy; there is no iron, manganese, cobalt, nickel, or molybdenum. In some samples, zinc was found at about 0.2 atoms/M_r. Thus, the contribution of trace paramagnetic metals to the relaxation of the mixed valence binuclear site is assumed to be negligible. Data from the EPR active site are attributed to two Cu atoms from a mixed valence [Cu(1.5) . . . Cu(1.5)], $S = 1/2$ complex. In addition, an underlying broad featureless signal is observed. This featureless signal is absent in an inactive form of the enzyme isolated from a transposon $-T_n5$-induced mutant defective in chromophore biosynthesis. Continuous wave data from the mutant are similar to data for the native form of N_2OR (6) and will be included in a full paper, which is in preparation. It is concluded that the broad underlying signal plays no role in the multiquantum EPR data presented here. The X-band multiquantum apparatus is essentially the same as previously described (1). A 4 mm five-loop four-gap resonator (Medical Advances, Inc., Milwaukee, WI) was used in the present experiment (12).

Temperatures were maintained with a Helitron flow system comprised of a transfer line and a digital indicator/controller from Air Products (Allentown, PA), and a quartz dewar. A chromel-gold thermocouple (Scientific Instruments, Inc., West Palm Beach, FL) was inserted into a 4 mm EPR tube to the top of the sample to monitor the temperature. The first harmonic of the multiquantum spectra is obtained with SUMSPC92, a computer program available upon request from the National Biomedical ESR Center in Milwaukee (13).

RESULTS AND DISCUSSION

The CW EPR spectrum of nitrous oxide reductase is shown in Fig. 1 *a*. Fig. 1 *c* shows the corresponding low power 3-quantum signal. Low power is used for $T_{1\,\mathrm{eff}}$ measurements since the frequency difference swept lineshape can be power broadened (3). The pure absorption signal is recorded since no field modulation is employed. For comparison with first derivative EPR spectra, the first harmonic using pseudomodulation (13) is shown in Fig. 1 *b*. A substantial change in lineshape occurs in the center of the spectrum at low microwave field as indicated by the arrow in Fig. 1. It is assumed that the difference in lineshape between the multiquantum and conventional spectrum arises from the enhanced suppression of the forbidden transitions in the 3-quantum spectrum. Additional studies are underway to measure quadrupole couplings from metal complexes (Pfenninger et al., work in progress).

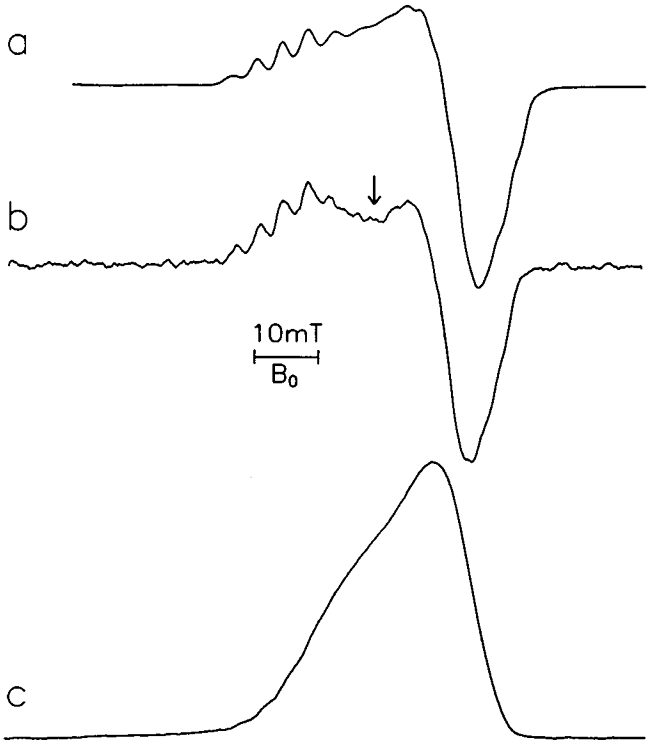

FIGURE 1 X-band EPR spectrum for N_2OR substituted with ^{63}Cu at 20 K: (*a*) CW spectrum at 1 mW with 100 kHz field modulation and 5 G for the amplitude; (*b*) first harmonic of spectrum *c* using 1% pseudomodulation; (*c*) 3-quantum EPR spectrum with 10 μW of incident power. Arrow indicates the most obvious difference in lineshape for the 3-quantum spectrum.

To measure $T_{1\,\mathrm{eff}}$, using the 3-quantum display, a series of field-swept 3-quantum spectra are obtained as the frequency difference between the two microwave frequencies, ω_1 and ω_2, is varied between 1 and 40 kHz (Fig. 2). $T_{1\,\mathrm{eff}}$ is determined at $(\omega_1 - \omega_2)T_{1\,\mathrm{eff}} = 1$, where the 3-quantum intensity drops to 1/2 (3). Values of $1.3 \times 10^5\ \mathrm{s}^{-1}$ and $2.7 \times 10^5\ \mathrm{s}^{-1}$ at 20 K were obtained for the effective spin lattice relaxation rate of the EPR detectable site in N_2OR from two samples determined in separate experiments. While the measurements are within a factor of two, the precision for a five degree temperature change is better for the same sample than the accuracy for two different experimental conditions. This is attributed to measuring the temperature at the high end of the sample where it is difficult to obtain an identical flow of helium and identical temperature gradients. Multiquantum data taken over a range from 15 to 25 K for the EPR detectable site in N_2OR are compared with saturation data for COX from Scholes et al. (9) in Fig. 3. The data

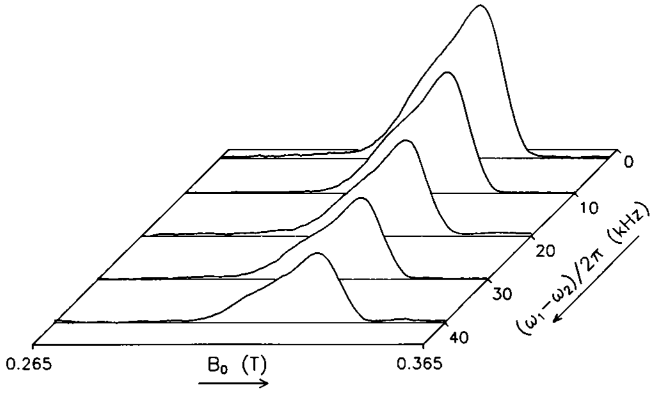

FIGURE 2 Field-swept 3-quantum EPR spectra of N_2OR at $T = 20$ K as a function of the separation of the microwave frequencies.

between 15 and 25 K attest to the remarkable similarity of the spin relaxation times, T_1, for the EPR sites in N_2OR and COX.

The $T_{1\,\mathrm{eff}}$'s for the EPR site in N_2OR were within a factor of two of each other at $g_\parallel$ and $g_\perp$ (Fig. 4). N_2OR does not contain the EPR detectable heme *a* moiety. The relaxation is attributed primarily to the mixed valence bi-nuclear [Cu(1.5) . . . Cu(1.5)] site, i.e., intrinsic relaxation. Given the similarity between the relaxation parameters for the copper EPR detectable site in N_2OR and COX, both of which are in environments with similar protein backbone configurations, it seems likely that the unusually fast relaxation time for the signal in COX is primarily intrinsic relaxation as a result of the configuration of the EPR site. Most notably, the primary structures of COX subunit II from several sources and the primary structure of N_2OR have two invariant histidines, two cysteines, and one methionine, which are highly conserved (14, 15). Assuming a bridging ligand, it is suggested that the unusually fast relaxation for the binuclear site in N_2OR and COX may be attributed to vibrational modes from the bridged binuclear copper site (16).

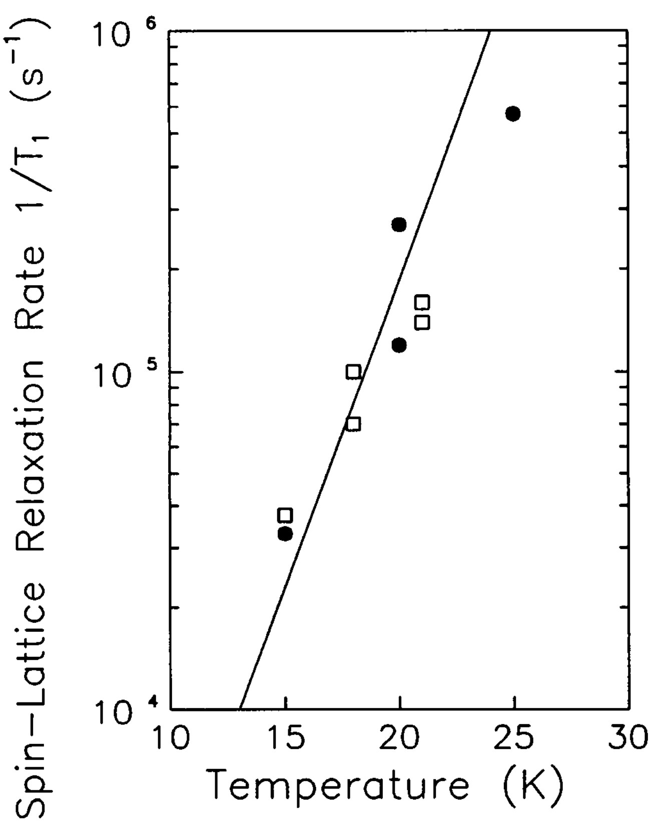

FIGURE 3 Plot of spin-lattice relaxation rates (●) for N_2OR versus temperature. Note data (☐) for cytochrome *c* oxidase are taken from Scholes et al., reference 8.

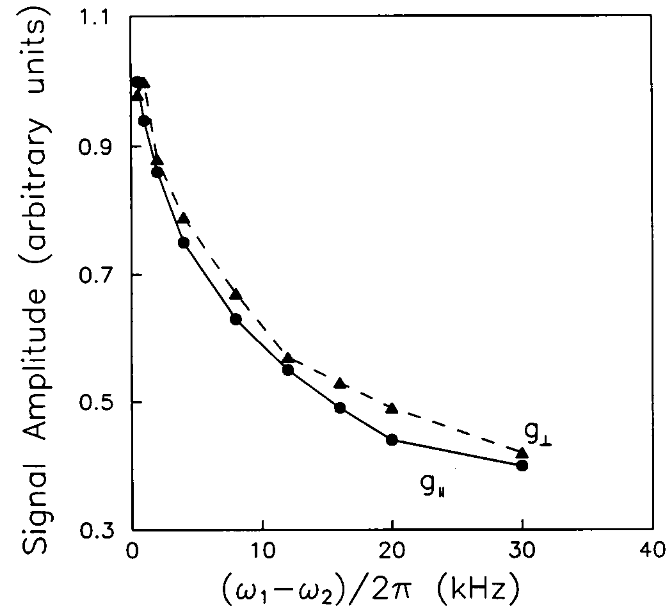

FIGURE 4 Plot of signal intensity from 3-quantum spectra of ^{65}Cu-N_2OR at $T = 20$°K versus the separation of the microwave frequencies.

In summary, multiquantum EPR is a viable alternative to conventional EPR for analysis of paramagnetic metal centers. $T_{1\,\mathrm{eff}}$'s were obtained simultaneously for all regions of the spectrum. The $T_{1\,\mathrm{eff}}$ data for N_2OR from the multiquantum method are very similar to previous saturation-recovery data for COX.

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