TEMPERATURE DEPENDENCE OF THE ELECTRON SPIN-LATTICE RELAXATION RATE FROM PULSED EPR OF Cu_A AND HEME *a* IN CYTOCHROME *c* OXIDASE

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ABSTRACT This work shows the feasibility of using pulsed, saturation recovery EPR to study directly the magnetic relaxation properties of metal centers in cytochrome *c* oxidase in the 1.5–20 K range. Heme *a* and Cu_A both showed remarkably similar T^n temperature dependences in their spin-lattice relaxation rates. Either both are in environments with very similar protein backbone configurations (Stapleton, H. J., J. P. Allen, C. P. Flynn, D. G. Stinson, and S. R. Kurtz, 1980, *Phys. Rev. Lett.*, 45:1456–1459; Allen, J. P., J. T. Colvin, D. G. Stinson, C. P. Flynn, and H. J. Stapleton, 1982, *Biophys. J.*, 38:299–310), or the Cu_A is relaxed by nearby heme *a*. Spin-lattice relaxation of the nitrosylferrocytochrome a_3 center in mixed valence oxidase showed enhancement of relaxation by a nearby paramagnetic center, most likely heme *a*.

INTRODUCTION

Cytochrome *c* oxidase accepts electrons from cytochrome *c* and transfers them to oxygen. It concomitantly couples the electron flow to ATP production and to proton gradients (1). Several of the four oxidase metal centers, two copper and two heme A, must be physically close enough to transfer electrons. The Cu_A and heme *a* of the oxidized resting enzyme are EPR detectable. Our goal has been to understand the details of metal centers by EPR-ENDOR[1] (2, 3) and to obtain evidence for magnetic interactions between them (4). Electron spin-lattice relaxation (T_1) measurements reveal the immediate environs of such centers and might suggest magnetic interactions between the metals.

For low spin ($S = 1/2$) heme and non-heme iron proteins, the spin-lattice relaxation rate temperature dependence has been determined (5, 6) as

$$1/T_1 = AT^n + BT. \quad (1)$$

The term in T^n predominates above ~2 K. It shows a Raman process where a high energy phonon scatters inelastically off a spin and simultaneously flips that spin. The spin-phonon coupling constant, A, becomes larger if there are low-lying excited electronic states. The T^n dependence is thought due to protein vibrations, and the exponent, $n = 3 + 2d$, thought to reflect the fractal dimension, d, of the protein backbone (5, 6). Many protein backbones describe a self-avoiding random walk, where $d = 5/3$. (The rms end-to-end length of a polymer, R_{rms}, is related to the number, N, of monomer units by $R_{rms} \propto N^{[1/d]}$.) Values of d from T_1 behavior have agreed well with values computed from x-ray coordinates (6). The linear term, BT, which predominates below ~2 K, indicates a direct absorption of a low energy phonon and simultaneous flip of the electron spin.

A slowly relaxing spin (e.g., that on nitrosylferrocytochrome a_3) may have its relaxation rate ($1/T_{1s}$) enhanced by magnetic interaction with a nearby faster relaxing spin (e.g., on heme *a*). If the fast rate is less than electron

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[1]Abbreviations used in this paper: T_1, spin-lattice relaxation time; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; rms, root mean square; a_3-NO, nitrosylferrocytochrome a_3; resting oxidase, $a^{3+} \cdot Cu_A^{2+} \cdot a_3^{3+} \cdot Cu_B^{2+}$; fully reduced a_3-NO, $a^{2+} \cdot Cu_A^{1+} \cdot a_3^{2+}NO \cdot Cu_B^{1+}$; mixed valence a_3-NO, $a^{3+} \cdot Cu_A^{2+} \cdot a_3^{2+}NO \cdot Cu_B^{1+}$; T_2, spin-spin relaxation time; ν_e, EPR frequency; T, temperature.

Larmor frequencies, $1/T_{1s}$ will be proportional to $1/T_{1f}$, the faster relaxation rate. For nitrosylferrocytochrome a_3 in mixed valence oxidase, relaxation enhancement was noted from a change in the T_1T_2 product inferred from progressive microwave saturation (7), which is a less direct technique than the one used here for measuring relaxation times. A dipolar interaction between the NO-ligated a_3 center and paramagnetic heme a was proposed to account for the change. For dipolar relaxation there will in general be several contributions (7, 8) having different angular and frequency dependences, but all depending on the inverse sixth power of inter-center distances. A simplified, single-term expression was developed to account for relaxation of the $g = 1.98$ feature of NO-ligated a_3 center. The simplification arose because the relative directions of the $g = 1.98$ tensor component and the g tensor of the heme a were known from EPR work on membrane-oriented oxidase (7). Thus

$$1/T_{1s} = 1/2\ b^2/[(\Delta\omega)^2 T_{1f}]. \quad (2)$$

$\Delta\omega$ is the difference in EPR angular frequencies of the interacting centers. b^2 is a spin-spin interaction constant, which depends on the inverse sixth power of distance between centers; b^2 also depends on $(3\cos^2\theta - 1)^2$, where θ is the angle between the applied magnetic field and the vector between metal centers. (See footnote 2.)

METHODS AND MATERIALS

Bovine cytochrome *c* oxidase was prepared according to previous methods (9, 10), and the sample of fully oxidized, resting state oxidase was similar to that used for previous ENDOR work (2). Nitric oxide derivatives were those of reference 4.

The pulsed, saturation recovery method was used to obtain T_1's. We modified our ER 420 spectrometer (Bruker Instruments, Inc., Billerica, MA) for superheterodyne detection. Saturating, high-power pulses and low-power monitoring pulses, attenuated by 30 dB, were provided by shunted microwave switches (6). Proper saturating powers were determined in initial studies. A crystal-clocked pulse programmer provided on and off pulse times in the 100 ns–10 s range. For $T_1 > 1$ ms, the EPR signal was collected by a 570 signal averager (Tracor Northern, Middleton, WI); for 20 μs $< T_1 <$ 1 ms, a Biomation 805 transient recorder (Gould Inc., Instruments Div., Santa Clara, CA) was used; for 1 μs $< T_1 <$ 20 μs a boxcar integrator (model 164; EG & G Princeton Applied Research, Princeton, NJ) was used.

Below 4.2 K the temperature was controlled by maintaining the pressure over the liquid helium in which the EPR cavity was immersed. Above 4.2 K the temperature was controlled by the combination of a carbon resistor adjacent to the EPR cavity, a heater on the waveguide next to the cavity, a boil-off coil in liquid helium below the cavity, and a temperature controller (LR-130; Linear Research Inc., San Diego, CA). The temperature at the sample site was measured by a silicon diode (Lake Shore Cryotronics, Inc., Westerville, OH).

EPR was done at 9.1–9.4 GHz and T_1's determined from the recovery of the EPR signal after a long pulse ($>T_1$) of saturating microwave power (11). It was recommended in reference 11 to estimate the T_1 from longest time transient behavior to avoid possible complications from rapid initial spectral diffusion, non-T_1, processes. Semilog plots, (superimposed on Figs. 1 and 3) were used to estimate T_1's. Sometimes an initial rapid recovery was observed (Fig.1 *b–d*), and the value of T_1 was determined from long time transient behavior. Our experience was that the decay time of the initial rapid recovery was at most 40% shorter than the long time T_1.

The data obtained for Fig. 2 were obtained at respective intermediate g values of 2.02 (Cu_A) and 2.30 (heme *a*); EPR signals and sensitivity are largest at intermediate g values of a frozen solution pattern. If the T_1 process is orientation dependent, there might be a variation of T_1 with the g value where measured. Since a number of orientations contribute to the EPR signal at intermediate g values, it is plausible that a spectrum of different T_1's might contribute to the EPR signal at intermediate g values. Therefore, T_1's were measured at several temperatures (2.2, 4.2, 6.5, 9.2,

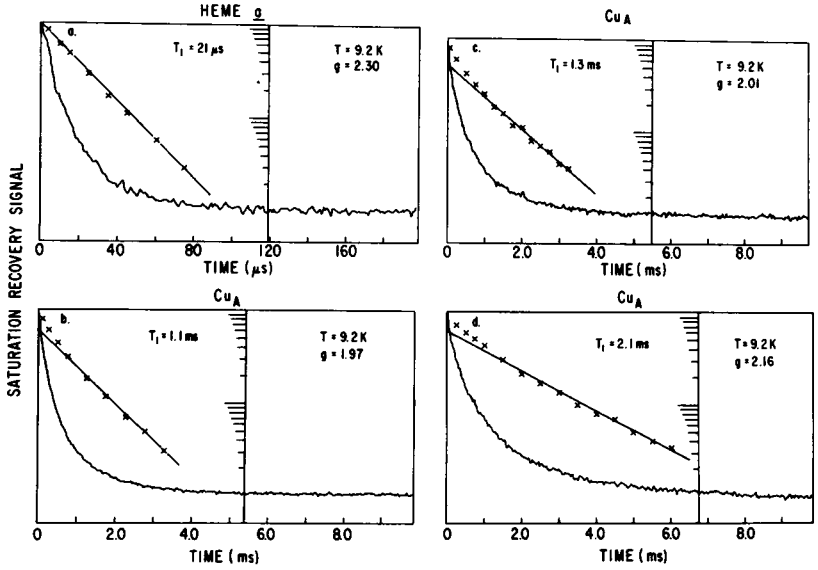

FIGURE 1 Saturation recovery for (*a*) heme *a* at $g = 2.30$, (*b*) Cu_A at $g = 1.97$, (*c*) $g = 2.01$ and (*d*) $g = 2.16$. In *a* the saturating power was ~30 mW and monitoring power 30 μW; on and off pulses were 1.6 and 3.4 ms, respectively. In *b*, *c*, and *d* the saturating power was ~3 mW and monitoring power 3 μW; on and off pulses were 17 and 35 ms, respectively. $T = 9.2$ K. Superimposed semilog plots show the exponential nature of saturation recovery.

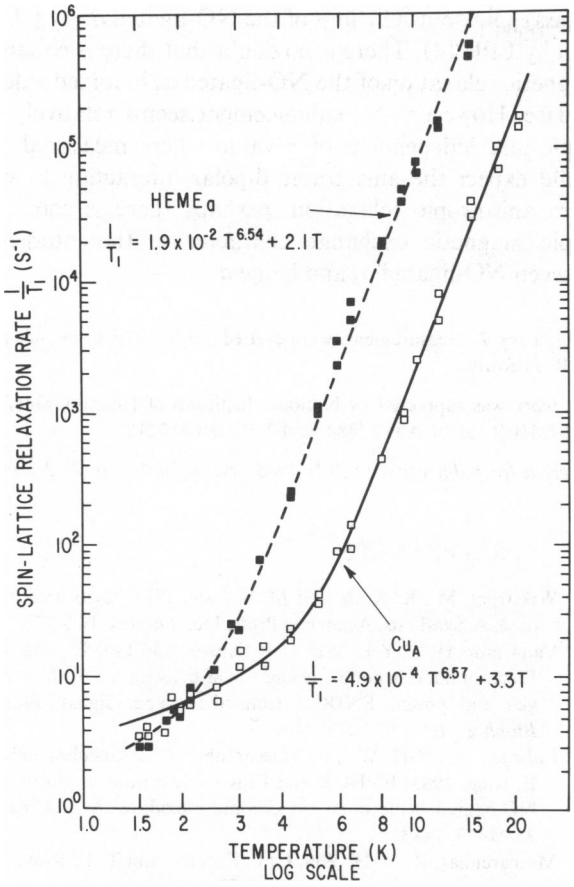

FIGURE 2 A log-log plot of electron spin-lattice relaxation vs. temperature for heme *a* and Cu_A in resting oxidase. These measurements were collected at $g = 2.30$ for heme *a* and $g = 2.02$ for Cu_A.

12.0 K) at extremal *g* values (2.16 and 1.97 for Cu_A and 2.90 for heme *a*) as well as at the intermediate *g* values. The T_1's of heme *a* at $g = 2.90$ and 2.30 were within 20% of each other. The T_1's of Cu_A at $g = 2.16$, 2.02 and 1.97 were within a factor of 2 of each other, with those at 2.16 the largest. The overall temperature dependence of T_1 was independent of *g* value. Although the traces of Fig. 1 *b–d* from Cu_A do show evidence for fast initial saturation recovery, there was no more evidence for a spectrum of T_1's at the intermediate *g* value than at extremal *g* values. The T_1's of NO-ligated oxidase in fully reduced and mixed-valence forms were also measured at extremal *g* values of 2.085 and 1.975 as well as the intermediate *g* value of 2.005 and at temperatures of 9.2, 12, and 15 K.

RESULTS AND DISCUSSION

Relaxation rates vs. temperature for Cu_A and heme *a* are shown in Fig. 2, and the least-squares fit parameters to Eq. 1 are given in Table I. The parameters for heme *a* are similar to those of cytochrome *c*, just as its *g* values are similar. The T^n temperature dependence for the Raman term of Cu_A is remarkably similar to that of heme *a*. Progressive EPR saturation (12) had indicated that heme *a* and Cu_A saturate with similar temperature dependence. Our work shows that it is explicitly the T_1 temperature dependence that is the same. One explanation is that the relaxation rates of Cu_A and heme *a* are affected by a

TABLE I
CONSTANTS DETERMINED FROM SPIN-LATTICE RELAXATION RATE $1/T_1 = AT^n + BT$

Metal	*A*	*n*	*B*
Heme *a** (this work)	$1.9\ (\pm 0.6) \times 10^{-2}$	6.54 ± 0.16	2.1 ± 0.2
Cu_A§ (this work)	$4.9\ (\pm 1.5) \times 10^{-4}$	6.57 ± 0.13	3.3 ± 0.3
Cytochrome *c* (reference 6)	2.21×10^{-2}	6.34 ± 0.06	4.85

Quoted error equals one SD determined by least-squares (14).
*Performed at $g = 2.30$.
§Performed at $g = 2.02$.

similar or identical protein environment, whose backbone has the same $d = 1.78$ fractal dimension. The difference in the *A* term (Eq. 1) between the two metals would simply reflect lower lying excited states for ferric heme. A faster relaxing heme *a* could also act as magnetic relaxer of

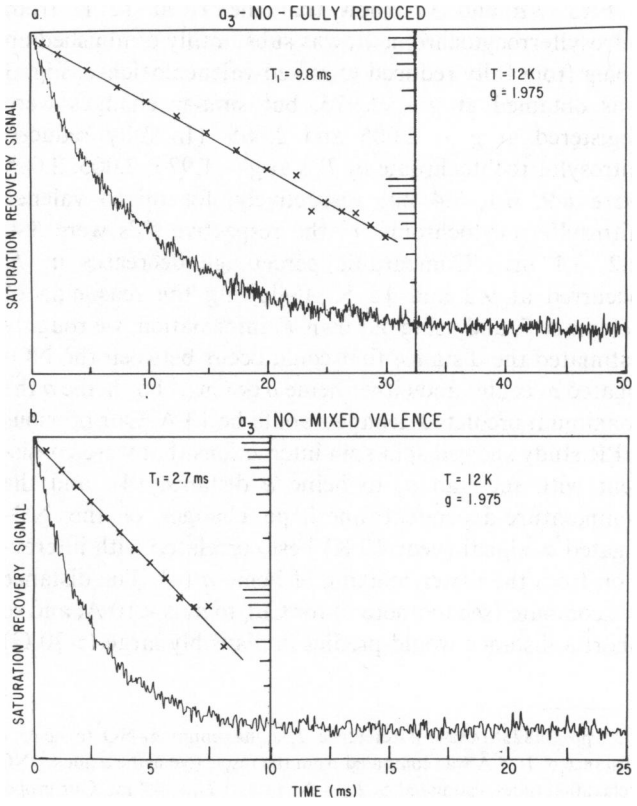

FIGURE 3 A comparison of saturation recovery for the a_3-NO in (*a*) fully reduced and (*b*) mixed valence oxidase. The measurements were carried out at $g = 1.975$, saturating powers were ~1 mW and monitoring powers 1 μW. On and off pulses were 50 ms. $T = 12$ K. The superimposed semilog plots show the exponential nature of saturation recovery. The difference in relaxation rate ($1/T_1$) between mixed valence (3.7×10^2 s^{-1}) and fully reduced ($1.0 \times 10^{2\ -1}$) is accounted for by relaxation enhancement of $1/T_{1s} = 2.7 \times 10^2\ s^{-1}$. Thus $T_{1s} = 3.7$ ms.

nearby Cu_A. Related to this interpretation is the work of reference 13, where progressive saturation of Cu_A was measured in mixed-valence CO-liganded oxidase, which had its heme *a* and Cu_A additionally reduced, each by ~75%. The T_1T_2 product decreased twofold as a result. The interpretation was that the reduction of heme *a* near Cu_A increased the Cu_A relaxation time (13). There is, however, an assumption that the similar redox potentials of heme *a* and Cu_A are uncorrelated so that the majority of oxidized Cu_A should have a reduced diamagnetic heme *a* as neighbor. We believe that this assumption should be critically investigated by careful redox titrations, and T_1 studies then carried out on the product of the redox titrations. A final explanation, which we believe implausible, for the similar temperature dependence of Cu_A and heme *a* relaxation is that both are relaxed by the binuclear a_3 center. The a_3 center, although EPR silent in oxidized resting oxidase, is still an $S = 2$ center, which could act as a relaxer. Progressive saturation studies in references 12 and 13 have shown negligible effect of the a_3 magnetic state (either CN^--liganded, CO-liganded, or resting) on heme *a* or Cu_A relaxation.

Figs. 3 *a* and 3 *b* show that the T_1 at 12 K from nitrosylferrocytochrome a_3 was substantilly diminished on going from fully reduced to mixed-valence oxidase. Fig. 3 was obtained at $g = 1.975$, but similar changes were registered at $g = 2.005$ and 2.085. (In fully reduced nitrosylferrochtochrome a_3 T_1's at $g = 1.975$, 2.005, 2.085 were 8.9, 6.1, 7.4 ms, respectively; for mixed valence nitrosylferrocytochrome a_3, the respective T_1's were 3.4, 2.2, 3.1 ms.) Comparable percentage decreases in T_1 occurred at 9.2 and 15 K. Following the reasoning of reference 7, and using our own T_1 information, we roughly estimated the distance that could occur between the NO-ligated a_3 center and either heme *a* or Cu_A.[2] For heme *a* the maximum predicted distance would be 13 Å. Our previous EPR study showed spin-spin interactions that were consistent with such an a_3 to heme *a* distance (4), and the temperature-dependent lineshape changes of the NO-ligated a_3 signal (near 40 K) best correlated with interaction from the faster relaxing of heme *a* (4). The distance we compute (see footnote 2) for Cu_A to a_3 is <10 Å, and so short a distance would predict implausibly large (>20 G) (gauss) spin-spin splittings of the NO-ligated a_3 signal, not seen by EPR (4). There is no doubt that there is enhanced magnetic relaxation of the NO-ligated a_3 in mixed valence oxidase. However, this enhancement seems relatively isotropic and independent of *g* value where measured. We would expect the anisotropic dipolar interaction to yield more anisotropic relaxation; perhaps there is some isotropic magnetic exchange character to the interaction between NO-ligated a_3 and heme *a*.

[2]On pp. 14823–14824 of reference 7, a maximum a_3-NO to heme *a* distance of 16.4 Å was computed from the respective heme *a* and a_3-NO relaxation times, estimated as $T_{1f} = 2.5$ μs and $T_{1s} = 4.8$ ms. Our proper $T_{1f} = 6.5$ μs and $T_{1s} = 3.7$ ms measured at 12 K reduce the maximum distance down to 13 Å. Relaxation measurements were carried out at $g = 1.975$ on the a_3-NO signal. The $g = 2.10$ signal for heme *a* is known to occur along the same direction as the $g = 1.975$ signal (7), and thus when $\nu_c = 9.3$ GHz, $\Delta\omega = 3.5$ GHz. The angular factor $(3\cos^2\theta - 1)^2$ in b^2 is taken at its maximum value of 4 (7). In estimating a distance <10 Å between a_3-NO Cu_A, we have used our measured $T_{1f} = 165$ μs for Cu_A. The *g* values for Cu_A extend over a 2.18–1.99 range, and because their orientation with respect to a_3-NO is unknown, we have arbitrarily kept $\Delta\omega = 3.5$ GHz for Cu_A.

Preliminary T_1 measurements were carried out in 1979-80 in our lab by Dr. R. LoBrutto.

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