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Distance between the Visible Copper and Cytochrome *a* in Bovine Heart Cytochrome Oxidase†

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ABSTRACT: Electron paramagnetic resonance (EPR) at 15 K was used to probe the magnetic interaction between the visible copper Cu_A^{2+} and ferric cytochrome *a* in the carbon monoxide compound of beef heart cytochrome oxidase. At pH 8.6, the midpoint potentials (E_m's) for one-electron oxidation of Cu_A^+ and cytochrome a^{2+} were found to be 195 and 235 mV, respectively. Because the E_m of Cu_A is well below that of cytochrome *a* under these conditions, the microwave power saturation of Cu_A could be measured as a function of percentage cytochrome *a* oxidized. Although progressive power saturation data directly provide only the product of the spin-lattice and transverse relaxation rates $\Delta[1/(T_1T_2)]$, Castner's theory for the saturation of inhomogeneously broadened lines [Castner, T. G., Jr. (1959) *Phys. Rev. 115* (*6*), 1506-1515], along with our own theoretical formulation of the dipolar T_2, enabled us to determine the change in T_1 of Cu_A due to dipolar relaxation by cytochrome *a*. The orientation of the principal *g* values of Cu_A with respect to those of cytochrome *a* was evaluated in partially oriented membranous multilayers. When allowance was made for uncertainties in the relative Cu_A-cytochrome *a* configuration and in the dipolar axis-magnetic field orientation, a range for the spin-spin distance *r* was calculated on the basis of the dipolar T_1 of the g_x component of Cu_A. This distance range was further restricted by consideration of T_1 for the nonunique orientations of Cu_A giving rise to the g_y signal. Only those values of *r* are possible for which the calculated T_1 ratio (g_x/g_y) is equal to the experimentally determined ratio. To allow for T_1 effects due to scalar exchange interaction, a small correction to the dipolar-only distance was calculated. No line broadening consistent with either dipolar or exchange coupling was detected. Taken together, all of the available data lead us to conclude that the Cu_A-cytochrome *a* distance falls within the range 8 Å $\leq r \leq$ 13 Å.

Cytochrome oxidase (ferrocytochrome *c*:O_2 oxidoreductase; EC 1.9.3.1), the terminal enzyme of the mitochondrial electron-transfer chain, is responsible for the concerted transfer of four electrons to molecular oxygen in an energy-yielding redox reaction, resulting ultimately in ADP phosphorylation. Four metal ions are present, each capable of one-electron oxidation/reduction: the two heme iron atoms of cytochromes *a* and a_3 and two copper atoms, Cu_A and Cu_B.

Stopped-flow optical absorbance experiments revealed the sequence of events in the reaction with cytochrome *c* to be reduction of cytochrome *a* (Gibson & Greenwood, 1965) with a second-order rate constant of $k = 10^7$ M^{-1} s^{-1} (Andréasson et al., 1972) followed by much slower transfer to cytochrome a_3 and, presumably, Cu_B (Brunori et al., 1979). Gibson & Greenwood (1965) monitored the 820-nm band and observed a rapid decrease in absorbance, slightly less rapid than the change at 605 nm but much more rapid than the slower phase. They concluded that copper (i.e., Cu_A) is involved in the initial stages of electron transfer from cytochrome *c*. Using a rapid-freeze technique, Beinert et al. (1976) followed changes in the electron paramagnetic resonance (EPR) signals of Cu_A and cytochromes *a* and a_3 upon reaction with reduced cytochrome *c*. They found that both Cu_A and cytochrome *a* are reduced in the rapid phase of electron transfer to cytochrome

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oxidase. Andréasson (1975) showed that only one electron equivalent is accepted by the enzyme in this rapid phase, consistent with a simple equilibrium between cytochrome c^{2+} and an electron accepter with $E_m = 285 \pm 15$ mV, i.e., cytochrome a^{3+} (Tiesjema et al., 1973). However, Andréasson (1975) also found that ΔA at 605 nm for the rapid reaction is only 50% of the fully reduced minus oxidized difference.

A reasonable interpretation of the available data is that Cu_A and cytochrome *a* are both approximately 50% reduced during the rapid phase, in which one electron is transferred from cytochrome *c*. This conclusion was arrived at previously by Wikström et al. (1981). It is not clear whether cytochrome *c* interacts directly with Cu_A or if Cu_A acts only as an intermediary between cytochrome *a* and the remaining electron acceptors, as suggested by Beinert et al. (1976).

In asking questions about functional relationships between Cu_A and each of the two hemes, it is useful to know the distance between the three pairs of spins: Cu_A–*a*, Cu_A–a_3, and *a*–a_3. In this paper a novel approach to calculation of the spin–spin distance between Cu_A and cytochrome *a* is described. Progressive microwave power saturation is shown to be capable of yielding accurate distance information when the orientation dependence of magnetic relaxation is taken into consideration. This is the first time that static EPR relaxation has been used to measure distances between unperturbed metal ions within the same molecule. While this work was in progress, magnetic relaxation of *a* by a_3–NO was observed (Ohnishi et al., 1982), implying that the two hemes are in close proximity. The electronic *g* value dependence of a_3–N_3 on the redox state of *a* (Goodman, 1983, 1984) is anticipated by this result. In future work[1] it will be shown that magnetic relaxation of low-spin a_3–N_3 by cytochrome *a* can likewise be measured by static EPR power saturation.

The temperature dependence of the EPR saturation of Cu_A in resting (i.e., air oxidized) cytochrome oxidase has been investigated by other workers. Because they found a T^7 temperature dependence below 20 K, which is unusual for a Cu(II) protein and which mirrors that of cytochrome *a*, Chan et al. (1982) concluded that cytochrome *a* probably relaxes Cu_A. Furthermore, they suggested that the limit on dipolar interaction effects is ~10 Å and that this therefore is the maximum Cu_A–cytochrome *a* distance. After submission of our paper a paper by Scholes et al. (1984) appeared that described a pulsed, saturation recovery EPR study of the magnetic relaxation properties of Cu_A and cytochrome *a* in the resting state. The T_1 values for Cu_A and cytochrome *a* which they obtained by direct measurement over the range 1.5–20 K are in general agreement with the T_1 values calculated in the present work from T_1T_2 products for the carbon monoxide compound at 15 K. The Scholes group reasoned that either Cu_A and cytochrome *a* are in similar protein backbone environments or else Cu_A is being relaxed by "nearby" cytochrome *a*. The Scholes paper brought to our attention the work of Blair et al. (1983), who also measured progressive saturation of Cu_A in the carbon monoxide compound and who found that the T_1T_2 product for Cu_A is decreased upon partial reduction. The interpretation given was that the relaxation properties of Cu_A are a function of the oxidation state of cytochrome *a*, which therefore must be in close proximity. However, as pointed out by Scholes et al., Blair and co-workers did not follow the redox titration behavior of the saturation effect, which we have done in the present work. We have utilized the previously unreported pH dependence of the Cu_A midpoint potential in the CO complex to obtain a state (above pH 8) in which cytochrome *a* is reduced in >90% of the molecules having Cu_A oxidized.

[1] G. Goodman and J. S. Leigh, Jr., unpublished data.

Materials and Methods

Enzyme Purification. Cytochrome oxidase was isolated from frozen beef heart mitochondria by the method of Yu et al. (1975) or by the Triton extraction method of Sun et al. (1968) followed by solubilization in cholate and by ammonium sulfate precipitation of impurities. Details of the purification procedure have been described elsewhere (Goodman, 1984).

Sample Preparation. Redox titrations were performed under a 1:1 atmosphere of argon (Airco grade 5)/CO (Airco grade 4). The calomel and platinum electrodes were calibrated before each use with 10 mM 1:1 potassium ferricyanide/ferrocyanide in 0.1 M potassium phosphate, pH 7.0. The midpoint potential of this standard was taken to be 425 mV at 25 °C plus 2.4 mV/°C below 25 °C (O'Reilly, 1973). Titrations were performed at room temperature; the potentials of the redox components were not corrected for the 2–3 °C day-to-day variation.

The titration medium contained cytochrome oxidase plus 110 mM glucose, 1.7 units of glucose oxidase (Sigma, type IV), 2.5 μg of catalase (Sigma), 7 nM superoxide dismutase from yeast (gift of Dr. Henry J. Forman), and the following concentrations of redox mediators: 7 μM phenazine methosulfate, 8 μM 1,2-naphthoquinone-4-sulfonic acid, 30 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine, and 7 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride. Following incubation with CO and argon, enzymes, mediators, and NADH were introduced. The enzyme was titrated with microliter quantities of 0.1 M potassium ferricyanide in 0.1 M potassium phosphate, pH 7.0. Samples were transferred anaerobically to 3-mm OD quartz EPR tubes, frozen in 5:1 isopentane/methylcyclohexane at the freezing point, and removed rapidly to liquid nitrogen. Carbon monoxide bound samples were kept from light during storage in liquid nitrogen to prevent photolysis.

Oriented Multilayers. Membranous cytochrome oxidase prepared according to Sun et al. (1968) was the generous gift of Dr. T. G. Frey. Partially oriented multilayers were formed by drying the enzyme on strips of mylar previously coated with nitrocellulose.

Electron Paramagnetic Resonance. EPR spectra were taken on a Varian E-109 spectrometer equipped with a Varian E-231 TE 102 rectangular cavity; modulation frequency and amplitude were 100 kHz and 16 G unless stated otherwise. An Air Products LTD-3-110 flowing helium cryostat cooled the quartz Dewar; the temperature was monitored continuously with a calibrated carbon resistor placed directly below the sample. Temperature regulation was via a Johnson Foundation thermostated heater.

Progressive Microwave Saturation. The microwave field strength H_1 is related to the incident power by a constant of multiplication which is a function of the cavity. This constant was determined by measuring the saturation behavior of a cytochrome *c* standard of known T_1 and T_2. In these experiments, a microwave power of 1.0 mW corresponds to an H_1 of 1.0×10^{-2} G.

The microwave saturation parameter $H_{1/2}'$ was taken from a log–log plot of signal amplitude vs. H_1. $H_{1/2}'$ is defined as that value of H_1 at which the straight line (with slope = 2) through the unsaturated points at low power intersects the tangent to the maximum (saturated) amplitude achieved at high power. This parameter was defined originally by Castner (1959), who termed it "$H_{1/2,\text{uncorrected}}$".

In order to quantitate the spin relaxation effect of cytochrome *a* on Cu_A, it is necessary to express the saturation parameter in terms of the longitudinal and transverse relaxation times, T_1 and T_2:

$$H_{1/2}^2 = \frac{1}{\gamma^2 T_1 T_2} \quad (1)$$

where γ is the gyromagnetic ratio. Note that this equation is written for the corrected saturation parameter $H_{1/2}$; the correction factor k ($1 \leq k \leq 4$), such that $H_{1/2}^2 = kH_{1/2}'^2$, is estimated from the shape of the saturation curve. The magnitude of k is a function of the degree to which the line is inhomogeneously broadened (Castner, 1959; Blum & Ohnishi, 1980). In these experiments, line broadening approaches the completely inhomogeneous case, with $k \approx 1.0$.

Equation 1 requires that the saturation experiment satisfies the adiabatic condition:

$$\omega_m H_m \ll \gamma H_{1/2}^2 \quad (2)$$

where ω_m is the modulation frequency and H_m is the modulation amplitude [see also Pake (1962)]. Since decreasing the modulation frequency and amplitude results in a diminished signal-to-noise ratio, the approach to adiabaticity is limited by the enzyme concentration.

Dipolar Spin Relaxation. If two unlike, isotropic spins I and S are coupled to one another via a dipolar interaction, the dipolar component of the relaxation times T_1 and T_2 of spin I due to spin S can be derived (Bloembergen et al., 1948; Abragam, 1961):

$$\left(\frac{1}{T_2}\right)_D = \gamma_I^2\gamma_S^2\hbar^2 S(S+1)\left(\frac{1}{3}A + \frac{1}{12}B + \frac{3}{2}C + 3D + \frac{3}{4}E\right) \quad (3)$$

and

$$\left(\frac{1}{T_1}\right)_D = \gamma_I^2\gamma_S^2\hbar^2 S(S+1)\left(\frac{1}{6}B + 3C + \frac{3}{2}E\right) \quad (4)$$

where

$$A = \tau_1 r^{-6}(1 - 3\cos^2\theta)^2$$

$$B = \frac{\tau_2}{1 + (\omega_I - \omega_S)^2\tau_2^2} r^{-6}(1 - 3\cos^2\theta)^2$$

$$C = \frac{\tau_1}{1 + \omega_I^2\tau_1^2} r^{-6}\sin^2\theta\cos^2\theta$$

$$D = \frac{\tau_2}{1 + \omega_S^2\tau_2^2} r^{-6}\sin^2\theta\cos^2\theta$$

$$E = \frac{\tau_2}{1 + (\omega_I + \omega_S)^2\tau_2^2} r^{-6}\sin^4\theta$$

τ_1 and τ_2 are the longitudinal and transverse relaxation times of spin S, the ω_i's are the resonance frequencies, r is the spin–spin distance, and θ is the angle between the magnetic field direction and the vector r. It should be noted that Hyde & Rao (1978) derived a similar expression for $(1/T_1)_D$ on the basis of the original calculations of Bloembergen et al. (1948) and of Abragam (1961); however, they assumed $\tau_1 = \tau_2$. Other workers have attempted to apply the Hyde and Rao equation to cases in which $\tau_2 < \tau_1$ (Ohnishi et al., 1982, and references therein).

Equation 3 is only valid when τ_1 of spin S is short compared with the induced change in T_2 of spin I. Likewise, for eq 4 to have validity, τ_2 of spin S must be short compared with the induced change in T_1 of spin I.

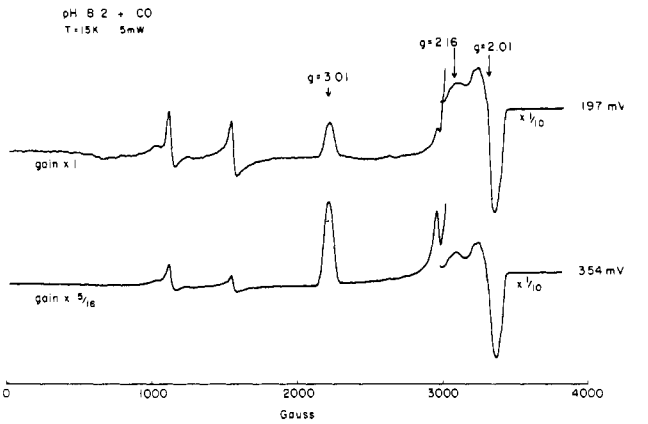

FIGURE 1: EPR spectra of 0.62 mM cytochrome oxidase at pH 8.2 in the presence of 0.5 atm of carbon monoxide, at two redox potentials. See Materials and Methods for details. Additional EPR conditions: T = 15 K and microwave power 5 mW. The g_z and g_y components of low-spin ferric cytochrome *a* are apparent at g = 3.01 and g = 2.24. The complete Cu_A(II) signal is seen above 3000 G. The signals at g = 6 and g = 4 are due to high-spin ferric heme and non-heme iron, respectively; they contribute insignificant intensity.

In the following, expressions for $(1/T_1)_D$ and $(1/T_2)_D$ will be developed in terms of experimentally and theoretically determined quantities. It can be demonstrated that eq 4 is obeyed in the present set of experiments and therefore can be solved to yield the Cu_A–cytochrome *a* spin–spin distance r. It will be shown also that eq 3 is not useful under these same conditions, and in fact, $(1/T_2)_D$ is independent of r in this case.

Results and Discussion

In Figure 1, EPR spectra of the carbon monoxide compound of cytochrome oxidase at pH 8.2 are shown at two oxidation potentials. Since cytochrome a_3 and Cu_B are both reduced upon binding of CO to cytochrome a_3 (Lindsay et al., 1975), the only EPR signals in the spectrum (Leigh et al., 1974) arise from cytochrome a^{3+}, with prinicpal g values of 3.01, 2.24 and 1.45, and Cu_A^{2+}, with principal g values of 2.17, 2.03, and 1.99 (Greenaway et al., 1977). The Cu_A signal amplitude from the trough ($g \approx 2.0$) to the high field base line overlaps both the g_x and g_y components; this is termed "g_{xy}".

In Figure 2, amplitudes of the g = 3 signal (cytochrome *a*) and g_{xy} of Cu_A are plotted vs. oxidation potential for the carbon monoxide bound enzyme at pH 8.6. At this pH, the two signals have midpoint potentials of 235 and 195 mV, respectively. Complete titration analysis shows no indication of cooperativity between these two centers. The separation of midpoint potentials is sufficient to ensure that, at low potentials (i.e., <200 mV), only a small percentage of molecules having Cu_A oxidized will also have cytochrome *a* oxidized. Thus, the saturation of the Cu_A EPR signal at low potentials will be a measure of the intrinsic spin lifetime, nearly devoid of ferric cytochrome *a* effects. Similarly, at redox potentials above 350 mV, saturation of Cu_A reflects the fact that nearly 100% of the molecules have cytochrome *a* oxidized. These considerations hold for pH 8.2, where the midpoint potentials for oxidation of Cu_A and cytochrome *a* are respectively 20 and 10 mV higher.

Figure 3 shows saturation curves for g_x of Cu_A at 15 K for the samples of Figure 1 under nonadiabatic (A) and adiabatic (B) conditions. The reason for measuring the g_x component separately from the g_y component will become apparent when orientation dependence of dipolar spin relaxation is discussed. The arrows indicate the values of the uncorrected saturation

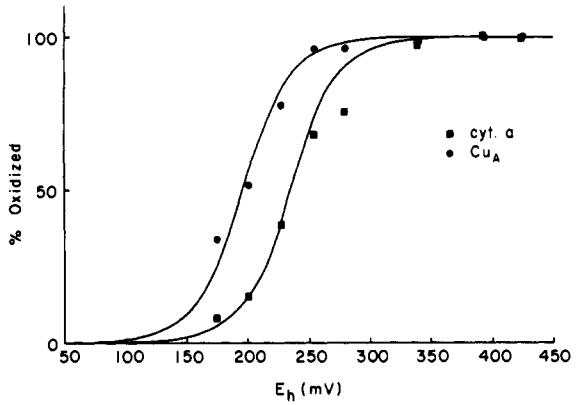

FIGURE 2: Redox potential dependence of cytochrome *a* and Cu_A at pH 8.6 in the presence of 0.5 atm of carbon monoxide. Cytochrome oxidase at 50 μM concentration was titrated anaerobically with ferricyanide (see Materials and Methods). The amplitudes of the cytochrome *a* (g = 3) and Cu_A (trough at g = 2.0 to high-field base line) signals were measured at T = 15 K with 5-mW microwave power. The solid lines are theoretical Nernst curves for an n = 1 oxidation.

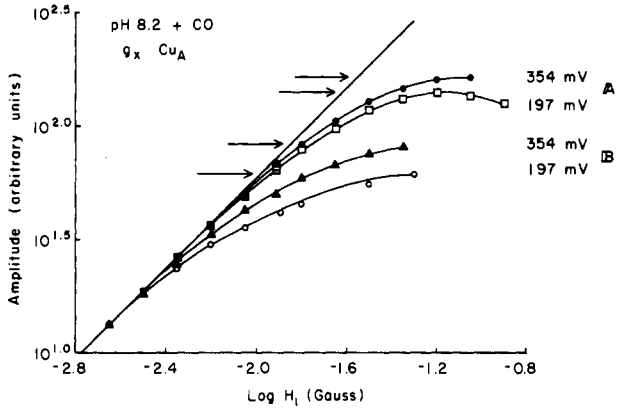

FIGURE 3: Microwave field strength dependence of the g_x component of Cu_A at 15 K; samples some as those as Figure 1. EPR conditions: (A) microwave frequency 100 kHz and modulation amplitude 16 G; (B) microwave frequency 1 kHz and modulation amplitude 4.0 G.

parameter, $H_{1/2}'$. The corrected saturation parameter $H_{1/2}$ was obtained by fitting the adiabatic curves to a set of computer-generated saturation curves differing only in the ratio of Lorentzian to Gaussian (i.e., overall) line widths. In addition to providing the best experimental value for $H_{1/2}$, the curve-fitting procedure enabled us to estimate T_2 for these signals (see below).

In Table I the squared, corrected adiabatic saturation parameters for g_x of Cu_A at 15 K (from Figure 3B) are listed, along with those for the g_y component; g_y is measured from the high-field peak to the low-field base line, and g_x is estimated from the shoulder (at g = 1.97) of the trough formed by the overlapping g_x and g_y signals. The product of the relaxation rates $1/(T_1T_2)$ is obtained from $H_{1/2}^2$ by using eq 1. We were unable to measure the saturation of the much broader g_z signal under adiabatic conditions. The nonadiabatic result indicates that the g_z component is relaxed an order of magnitude faster than g_y or g_x.

The effect of progressive cytochrome *a* oxidation on Cu_A saturation can be followed in the pH 8.6 titration series of Figure 2. This was at lower concentration than the pH 8.2 set of Figure 3 (50 μM vs. 0.62 mM cytochrome oxidase); consequently, it was not possible to obtain adiabatic saturation parameters. When saturation of the g_{xy} component was followed nonadiabatically (modulation frequency and amplitude of 100 kHz and 16 G) at 15 K, the resulting values of $H_{1/2}'^2$ were found to have a linear dependence on ferric cytochrome

Table I: Saturation Parameters (Squared) and Relaxation Rates for Cu_A at High and Low Potential

	mV	$H_{1/2}^2$ (G^2)	$1/(T_1T_2)$ (s^{-2})
g_y	354	$(2.3 \pm 0.2) \times 10^{-4}$	$(7.3 \pm 0.7) \times 10^{10}$
	197	$(1.1 \pm 0.2) \times 10^{-4}$	$(3.5 \pm 0.6) \times 10^{10}$
g_x	354	$(2.0 \pm 0.1) \times 10^{-4}$	$(6.1 \pm 0.3) \times 10^{10}$
	197	$(1.0 \pm 0.1) \times 10^{-4}$	$(3.1 \pm 0.3) \times 10^{10}$

[a] EPR conditions: temperature 15 K; modulation frequency 10^3 Hz; modulation amplitude 4.0 G. T = 15 K. Enzyme: 0.62 mM at pH 8.2, titrated in the presence of 0.5 atm of carbon monoxide.

a (data not shown). The line of least squares through these points had a slope of 0.050×10^{-4} $G^2/\%$ a^{3+} and a correlation coefficient of 0.968. The y intercept is 1.3×10^{-4} G^2; this can be identified with the intrinsic saturation parameter of Cu_A in the absence of any relaxation by a^{3+}, i.e., when *a* is in its ferrous, nonparamagnetic, S = 0 state. The corresponding product of relaxation rates (from eq 1) is 4.1×10^{10} s^{-2}. This nonadiabatic result is not distinguishable from the pH 8.2 adiabatic relaxation rate products given in Table I for the separate g_x and g_y components of Cu_A at 197 mV.

All of our work on the saturation of Cu_A in the absence of ligands, in the presence of azide, or in the CO compound at lower pH provides ample evidence that there is no primary correlation between percent Cu_A oxidation and Cu_A saturation. Furthermore, we do not think that spin diffusion would be an effective source of relaxation in the magnetically isolated enzyme. Spin coupling between distant Cu atoms in neighboring protein molecules within the noncrystalline array is expected to be negligible due to random orientation of the anisotropic **g** tensors. The only close magnetic neighbor of Cu_A is cytochrome *a*; their interaction is best described as being isolated and pairwise. The decreased saturation that we see at higher oxidation potential is entirely attributable to spin relaxation by cytochrome *a*.

At 197 mV, the quantity $1/(T_1T_2)$ must be a function only of the intrinsic Cu_A relaxation times; since cytochrome *a* is reduced, no dipolar relaxation occurs. At 354 mV, $1/(T_1T_2)$ includes both intrinsic and dipolar contributions, i.e., the combined or total relaxation rates. Letting the total, intrinsic, and dipolar relaxation rates be indicated by the subscripts T, I, and D, respectively, one can write

$$\left(\frac{1}{T_1}\right)_T = \left(\frac{1}{T_1}\right)_I + \left(\frac{1}{T_1}\right)_D \tag{5a}$$

and

$$\left(\frac{1}{T_2}\right)_T = \left(\frac{1}{T_2}\right)_I + \left(\frac{1}{T_2}\right)_D \tag{5b}$$

Equations 5a and 5b are just statements of the rule for addition of relaxation rates. Multiplying eq 5a by 5b

$$\left(\frac{1}{T_1T_2}\right)_T = \left(\frac{1}{T_1T_2}\right)_I + \left(\frac{1}{T_1T_2}\right)_D + \left(\frac{1}{T_2}\right)_I\left(\frac{1}{T_1}\right)_D + \left(\frac{1}{T_1}\right)_I\left(\frac{1}{T_2}\right)_D \tag{6}$$

Equation 6 can be rearranged to

$$\left(\frac{1}{T_1}\right)_D = \frac{\Delta[1/(T_1T_2)] - (1/T_1)_I(1/T_2)_D}{(1/T_2)_D + (1/T_2)_I} \tag{7}$$

where

$$\Delta\frac{1}{T_1T_2} \equiv \left(\frac{1}{T_1T_2}\right)_T - \left(\frac{1}{T_1T_2}\right)_I$$

is the difference between the high and low potential values obtained from the saturation experiments.

The transverse relaxation time T_2 can be written as

$$T_2 = \frac{2}{\gamma \Delta H_L} \tag{8}$$

where ΔH_L is the Lorentzian or homogeneous line width of an individual spin. The intrinsic and total T_2's can be obtained by fitting the adiabatic saturation curves (Figure 3B) to a set of stimulated curves generated with a range of ratios of the Lorentzian to Gaussian line widths. The g_x saturation curves for both the 197- and 354-mV samples at 15 K are adequately simulated when $(\Delta H_L/\Delta H_G) = 0.004 \pm 0.001$. Since the temperature-independent line width of the g_x component is 45 G [Greenaway et al., 1977], then, at 15 K, $\Delta H_L = 0.180 \pm 0.045$ G. By use of eq 8 for the relaxation rates, $(1/T_2)_T \simeq (1/T_2)_I = (1.6 \pm 0.4) \times 10^6$ s^{-1}. Spin-echo measurements of the 197- and 354-mV samples gave values for $1/T_2$ of 0.9×10^6 and 2×10^6 s^{-1}, respectively.[2] This implies that the dipolar contribution to the total transverse relaxation rate is small compared to the intrinsic rate. Knowing $(1/T_2)_I$ allows one to estimate the corresponding spin–lattice relaxation rate. The experimental value of the product of the intrinsic relaxation rate (Table I; g_x at 197 mV) is divided by $(1/T_2)_I$, yielding the result $(1/T_1)_I = (2.0 \pm 0.5) \times 10^4$ s^{-1}.

At this point, we have experimentally determined or calculated numerical values for every expression on the right hand side of eq 7 except $(1/T_2)_D$. Equation 3 for $(1/T_2)_D$ may be extended into the range where $\tau_1 \gtrsim (T_2)_D$ by analogy with two-site chemical exchange processes (Hoffman & Forsen, 1966). In this case, the Cu_A electron spin is experiencing two environments due to the slow relaxation (τ_1) between the two spin states ($S = \pm 1/2$) of cytochrome *a*. Ignoring the small Boltzmann factor, we assume the states to be equally populated and identical except for the difference in resonance frequency experienced by spin I due to the A term of the dipolar interaction. Equation 18 of Leigh (1971) can be written as

$$\left(\frac{1}{T_2}\right)_D = \frac{1}{2\tau_1} + \left(\frac{1}{4\tau_1^2} - \Delta\omega_D^2\right)^{1/2} \tag{9}$$

where $\Delta\omega_D^2 = (1/3)\gamma_1^2\gamma_2^2\hbar^2 S(S+1)(1 - 3\cos^2\theta)^2 r^{-6}$. In the long τ_1 limit $[1/(2\tau_1) < \Delta\omega_D]$, the expression under the square root becomes negative. Only the real part $1/(2\tau_1)$ represents relaxation while the imaginary part describes the dipolar splitting. Note that the real part of the dipolar T_2 is then independent of the spin–spin distance r of the field orientation and is equal to twice the spin–lattice relaxation time τ_1 of the other species.

To obtain τ_1 and τ_2 for cytochrome *a* at 15 K, the progressive microwave power saturation of the $g = 3$ signal in the highest potential CO-bound sample was measured under adiabatic conditions (modulation frequency and amplitude of 10^3 Hz and 4.0 G). The saturation parameter $H_{1/2}^2 \simeq H_{1/2}'^2 = (6.9 \pm 0.2) \times 10^{-4}$ G^2 of the inhomogeneously broadened line was related to the product $\tau_1\tau_2$ by using eq 3, with $T_1T_2 \equiv \tau_1\tau_2 = 2.1 \times 10^{12}$ s^2. The transverse relaxation time τ_2 is a function of the Lorentzian line width ΔH_L (eq 8). An estimate of ΔH_L at 15 K was obtained by extrapolation from a log–log plot of line width vs. temperature at higher temperatures (Blum & Ohnishi, 1980) resulting in $\Delta H_L = 2.1$ G (Figure 4). Hence, $\tau_2 = (3.6 \pm 0.7) \times 10^{-8}$ s, and therefore, $\tau_1 = (5.8 \pm 1.5) \times 10^{-5}$ s at 15 K. We do not include points

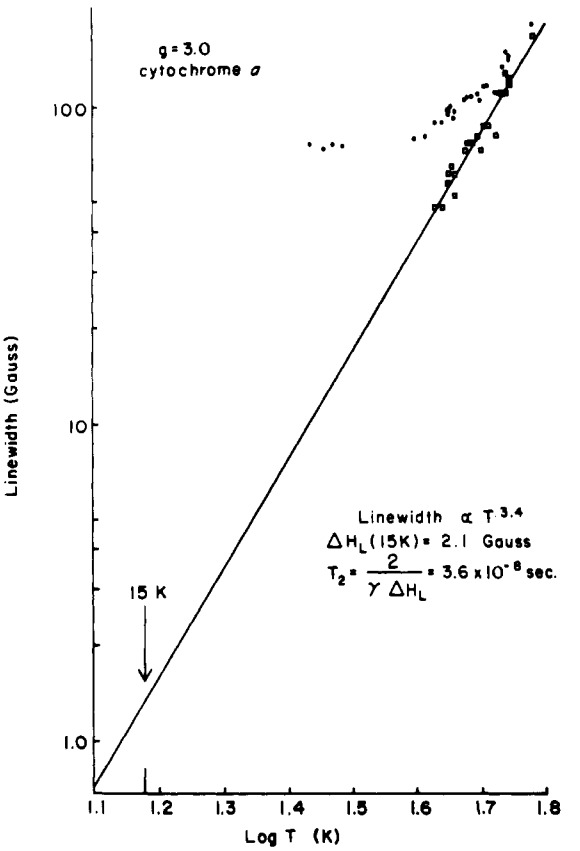

FIGURE 4: log line width vs. log temperature for the $g = 3$ signal of cytochrome *a* in the CO compound at 354 mV. Measured line width ΔH_T (●); Lorentzian contribution to the line width ΔH_L (□), calculated by subtracting the temperature-independent Gaussian line width ΔH_G observed at low temperature, $\Delta H_L = (\Delta H_T^2 - \Delta H_G^2)^{1/2}$. The validity of this formula was checked by using a computer program capable of convoluting Gaussian and Lorentzian line shapes. The straight line is the line of least squares through points above 40 K. EPR conditions: 10^3 Hz modulation frequency, 16 G modulation amplitude, and 20 mW microwave power. Enzyme: see legend to Figure 1.

below 40 K in the extrapolation because the overall linewidth becomes nearly independent of temperature; ΔH_L is so small by comparison that the Gaussian line width dominates. Imprecision in the correction for the temperature-independent Gaussian line width therefore becomes large at $T < \sim 40$ K.

This procedure for predicting τ_1's and τ_2's has been checked against spin-echo EPR data at 6–9 K for cytchrome *c*; the two methods agree within a factor of 4.[3] For low-spin heme signals, we found that the line width vs. temperature extrapolation was a more reliable method for finding T_2 than fitting the saturation curves to theoretical Castner curves of given $\Delta H_L/\Delta H_G$ ratios. For the nearly isotropic Cu_A signal, significant line-width overlap at higher temperatures complicates interpretation of the temperature dependence of a particular orientation of the **g** tensor. The Castner theory is derived for isotropic lines; therefore, it is not surprising that the saturation curve method gives accurate results for Cu_A. In all cases, our criteria for accuracy were the spin-echo results, usually at $T < 10$ K. A detailed comparison between static and time-resolved EPR measurements will be given in future work.[3]

Knowing $(1/T_2)_D = 1/(2\tau_1)$, it is now possible to solve eq 7. The dipolar T_1 is small compared to the intrinsic rate, as predicted. For relaxation of the g_x component of Cu_A by cytochrome *a* at 15 K, $(1/T_1)_D = (1.9 \pm 0.5) \times 10^4$ s^{-1}, with

[2] R. LoBrutto, G. Goodman, and J. S. Leigh, Jr., unpublished data.

[3] G. Goodman and R. LoBrutto, unpublished data.

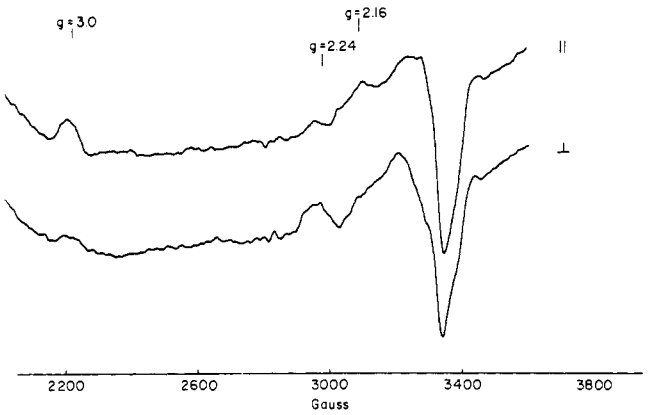

FIGURE 5: EPR spectra of partially oriented membranous multilayers of cytochrome oxidase oriented parallel and perpendicular to the magnetic field. T = 11 K; microwave power 5 mW.

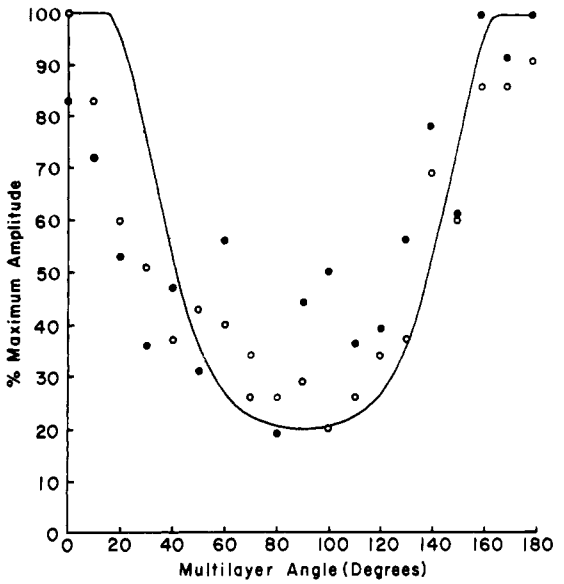

FIGURE 6: Amplitudes of the g_z components of cytochrome a (○) and Cu_A (●) as a function of the rotation angle of the membrane multilayer with respect to the magnetic field. At 0°, the membrane is parallel to the field; at 90°, it is perpendicular. The solid line is from simulation of the orientation dependence of the cytochrome a g_z signal. EPR conditions: temperature 11 K; microwave power 5 mW.

uncertainty in the various parameters being taken into account. By an entirely analogous procedure the g_y component was found to have $(1/T_1)_D = (1.6 \pm 0.9) \times 10^4$ s^{-1} at 15 K. It is clear that since τ_2 is sufficiently short in comparison to the dipolar T_1, eq 4 is applicable.

Spin Orientation. Calculation of the spin–spin distance from the spin relaxation effect also requires attention to the relative orientation of the two spin systems. When partially oriented multilayers of membranous cytochrome oxidase were rotated in the magnetic field of the EPR spectrometer, the amplitude of the g_z (g = 2.16–2.17) component of Cu_A followed that of the g_z (g = 3.0) component of cytochrome a (Figures 5 and 6). The amplitude of the g_y component of Cu_A varied with the angle by 30% with maxima at 48 ± 5° from the membrane normal. The orientation of cytochrome a with respect to the membrane was determined previously by Blum et al. (1978) and Erecińska et al. (1979) and is sketched in Figure 7. The EPR spectra of oriented multilayers presented in the paper by Blum et al. clearly show that the g_z component of Cu_A has the same orientation as the g = 3.0 signal, in agreement with our results. Simulation of the g = 3.0 orientation dependence using their analysis yields the solid line in Figure 6. Both the

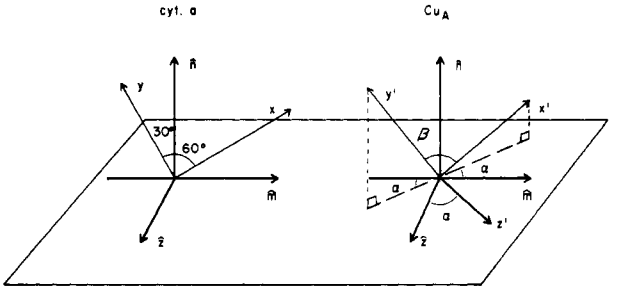

FIGURE 7: Orientation of the Cu_A and cytochrome a **g** tensors with respect to one another and the mitochondrial membrane. The vector $\hat{\mathbf{m}}$ lies in the plane of the membrane; $\hat{\mathbf{n}}$ is normal to the membrane.

g = 2.17 and g = 3.0 signals are seen to take values consistent with a mosaic spread of 25° when corrected for 20% disorientation (Figure 6).

The qualitative dependence of the g_y amplitude on field angle can be reproduced by simulation of the Cu_A signal but is not very sensitive to the choice of the angle β between g_y and the membrane normal. Only the angle that a given component makes to the plane of the multilayer (i.e., membrane) or its normal can be measured; even though the g_z directions of both Cu_A and cytochrome a are in the membrane plane, the angle α between them remains undetermined. The angular relationships are shown in Figure 7. For any given value of α, the magnitude of the cytochrome a **g** tensor along the g_z direction of the Cu_A **g** tensor can be computed; i.e., **g**(cytochrome a) → $\vec{g}_z(Cu_A)$. To determine the effective g value of cytochrome a along the g_x or g_y principal axes of Cu_A, the uncertainty in β must also be taken into account.

In this quasi-isotropic formulation, the dipolar angle θ for $\vec{g}_i$ is just the angle between the magnetic field $\vec{H}$ and the spin–spin vector $\vec{r}$ when $\vec{g}_i$ is along $\vec{H}$, where $i = x$, y, or z. The dipolar angle "seen" by any direction of the Cu_A spin is determined by the fixed geometry of the system: for $\vec{g}_z$, $\cos^2\theta = \cos^2\theta_r$; for $\vec{g}_y$, $\cos^2\theta = \sin^2\theta_r \sin^2\phi_r$; for $\vec{g}_x$, $\cos^2\theta = \sin^2\theta_r \cos^2\phi_r$, where the angles have their usual definitions [θ_r is the angle between $\vec{g}_z$ and $\vec{r}$, and ϕ_r is the angle between $\vec{g}_x$ and the projection of $\vec{r}$ onto the $\vec{g}_x$–$\vec{g}_y$ plane (Leigh, 1970)].

In simulations, angles β and α were varied in 15° and 3° increments, respectively. At each (α,β) point, the matrix of cosines of (α,β) for rotating the cytochrome a **g** tensor into the Cu_A **g** tensor coordinate system was calculated. (Because of the symmetry of the system, only two angles are needed to define the rotation operation.) This rotation matrix was used to calculate the effective g value of cytochrome a when the magnetic field is along the g_x axis of Cu_A. For each pair of rotation angles (α,β), 100 possible values of the dipolar angle θ were computed by allowing $\cos^2\theta_r$ and $\cos^2\phi_r$ each to take 10 equally spaced values between 0 and 1. By use of the relaxation rate $(1/T_1)_D$ for g_x of Cu_A, eq 2 was solved for r^6 at each dipolar angle θ over the range $0 \leq \cos^2\theta \leq 1$. The entire process was repeated at a different set of rotation angles (α,β), resulting in a table of r^6 as a function of α and β.

Since α and β are unknown, there is a large uncertainty in $\omega_I - \omega_S$; as $\omega_I - \omega_S$ becomes smaller the maximum calculated distance increases. The minimum distance remains constant since only the B term in eq 2 depends on $\omega_I - \omega_S$. At values of the dipolar angle θ such that $(1 - 3\cos^2\theta)^2$ is very small, the B term becomes insignificant compared to the C and E terms. Since the C and E terms depend on $\omega_I + \omega_S$ and ω_I rather than on $\omega_I - \omega_S$, they are otherwise much smaller than the B term. Therefore, the minimum distance is calculated when the dipolar angle takes values such that $\sin^2\theta \gg \cos^2\theta$, independent of $\omega_I - \omega_S$.

It is possible to place tighter limits on the distance by taking into consideration $(1/T_1)_D$ for g_y of Cu_A. Whereas the g_x signal corresponds to a unique orientation in space along the g tensor axis of smallest magnitude, the g_y signal consists of contributions from all $\vec{g}$ vectors with magnitude g_y, including the one that happens to lie along the tensor principal axis "g_y". These fall into two circles of points which satisfy the equation

$$g_y^2 = g_z^2 \cos^2 \theta_g + g_y^2 \sin^2 \theta_g \sin^2 \phi_g + g_x^2 \sin^2 \theta_g \cos^2 \phi_g \quad (10)$$

where θ_g and ϕ_g are the usual polar coordinates describing the orientation of the magnetic field in the g_x,g_y,g_z principal axis coordinate system of Cu_A. For each (θ_g, ϕ_g) pair which is a solution to eq 10, there is a cooresponding (θ_g', ϕ_g') describing the field orientation in the g_x,g_y,g_z coordinate system of cytochrome *a*. However, each (θ_g', ϕ_g') will give rise to a different effective *g* value for the heme iron. Thus, a range of cytochrome *a* *g* values (and hence, ω_S values) are responsible for relaxation of the Cu_A g_y signal.

To obtain the angles (θ_g', ϕ_g'), eq 10 was first solved for cos θ_g at 50 equally spaced values of ϕ_g between 0° and 180°. At each (θ_g, ϕ_g) pair, the magnetic field vector $\vec{H}(\theta_g, \phi_g)$ was rotated from the unprimed into the primed coordinate system (inverse of the rotation operation used to transform the cytochrome *a* tensor into the Cu_A principal axis system). The effective cytochrome *a* *g* value was calculated at each (θ_g', ϕ_g') point. The unique value of r^6 for the g_x direction calculated at each dipolar angle θ was used to compute a range of dipolar $(T_1)_y$ values as a function of (θ_g', ϕ_g').

Relaxation of the g_y signal is due to a composite of the separate relaxation times of the various orientations. A stimulated relaxation curve (log V_R vs. log H_1, where V_R is the EPR signal amplitude) can be constructed for each $(T_1)y$. The saturation parameter $H_{1/2}$ is proportional to $(1/T_1)^{1/2}$ for each curve. When $\sum V_R$ vs. log H_1 is plotted, $(T_1)_y^e$ (where the superscript stands for "effective") for all orientations that give rise to the g_y signal can be deduced from the apparent $H_{1/2}$. We found that 0.36 times the average of $(T_1)_y$ is a good approximation to $(T_1)_y^e$ for 50 values of $(T_1)_y$ varying over a range of 10^5; the two numbers were within 20% of one another in every case examined. The quantity $0.36\overline{(T_1)_y}$ was compared with the single $(T_1)_x$ [i.e., $(T_1)_D$] value from the g_x calculation. The experimentally determined ratio of relaxation times is equal to 0.84 (range 0.29–1.79) for x/y. By selection of only those values of r^6 such that this ratio falls within its experimentally determined limits, the distance range becomes 6–11 Å.

In general, if the distance r between any two spins is 8–14 Å, it is expected that the scalar superexchange interaction energy may be comparable with that due to dipolar interaction (Coffman & Buettner, 1979a,b). The homogeneous line width of the individual spins would therefore be split by an amount $\Delta H^{ex} = 2J^{ex}$, where J^{ex} is defined by the Hamiltonian operator $\mathcal{H}^{ex} = J^{ex}S_1 \cdot S_2$ (Abragam, 1961). If such a splitting is present, the change in the overall Gaussian inhomogeneous line width resulting from the superposition of the individual spins can be predicted by simulation.

Measurement of the half-width (from the g_y center line to the g_x trough) of the Cu_A signal reveals that the 354-mV line is not detectably broader than the 197-mV line at either 15 or 150 K. In our estimation, an upper limit on the undetected broadening would be 2% at either temperature. Simulation of inhomogeneously broadened lines (with $\Delta H_L/\Delta H_G$ from 0.002 to 0.02) indicates that ΔH^{ex} must be $\leq$ 10% of ΔH_G for the overall broadening to be $\leq$2%. In the case of g_x and g_y of Cu_A, with line widths of 45 and 65 G, respectively, this implies that $J^{ex} \leq 3 \times 10^{-4}$ cm^{-1}.

Coffman & Buettner (1979a) have developed an empirical outer limit function for log J^{ex} vs. r. For $J^{ex} = 3 \times 10^{-4}$ cm^{-1}, $r \lesssim 13.5$ Å. The available data compiled by these workers for the distance dependence of antiferromagnetic exchange coupling suggest that 8 Å is a reasonable lower limit for the spin–spin distance when $J^{ex} = 3 \times 10^{-4}$ cm^{-1}.

The absence of observable line broadening places the same constraints upon the dipolar splitting $\Delta\omega_D/\gamma_I$ as on the exchange splitting. But since $\Delta\omega_D$ is a function of $1 - 3\cos^2\theta$, it can disappear entirely, whereas the scalar quantity ΔH^{ex} is independent of the dipolar angle θ. The same maximum distance is in effect for $\Delta\omega_D/\gamma_I \leq 5\%$ of ΔH_G, as well. The results of our distance calculations allow us to specify further the relative orientation of the two spins: $21° < \alpha < 60°$ for any value of β. If $\beta \approx 45°$, as suggested by the oriented multilayer studies, then α is between 37° and 44°.

In calculating relaxation times from the saturation parameters, the effect of any line splitting has been neglected. This is justifiable since the apparent relaxation parameter is unaffected by even relatively large line splittings. We have simulated relaxation curves for highly inhomogeneously broadened spin packets both with and without splitting of the individual Lorentzian lines. When the splitting is 12.5% of the overall line width ΔH_G, there is no measurable effect on the saturation parameter, even when the homogeneous line width ΔH_L of the individual line is more than an order of magnitude smaller than the splitting. (We made no attempt to simulate relaxation curves with larger splittings.)

A question arises as to the correctness of applying a dipolar-only relaxation equation to the distance calculation. The spin–lattice relaxation enhancement due to the scalar exchange can be written as (Abragam, 1961)

$$\left(\frac{1}{T_1}\right)_{ex} = \frac{2\,(J^{ex})^2 S(S+1)}{3} \frac{\tau_2}{1 + (\omega_I - \omega_S)^2 \tau_2^2} \quad (11)$$

Our distance calculation revealed that only the A term contributes significantly to $(1/T_1)_D$ (eq 4). $(1/T_1)_{ex}$ is virtually identical with $(1/T_1)_D$ when $1 - 3\cos^2\theta = 1$; for smaller values of the angular term, $(1/T_1)_{ex}/(1/T_1)_D$ increases proportionately. In our calculations of r^6 based on $(1/T_1)_D$, we found that the experimental restrictions on $(T_1)_x/(T_1)_y$ and $\Delta\omega_D/\gamma_I$ imply that the magnetic field angles θ_r and ϕ_r only take values such that $\sin^2\theta_r \cos^2\phi_r$ (i.e., $\cos^2\theta$ for $\vec{r} \cdot \vec{g}_x$) is between 0.15 and 0.52. Therefore, $(1/T_1)_{ex}$ is 1–1.8 times the dipolar rate. What we have called $(1/T_1)_D$ is just the sum of the true dipolar spin–lattice relaxation rate with $(1/T_1)_{ex}$. Since the ratio of $(1/T_1)_D$ to the actual dipolar rate is between 2 and 2.8, the minimum and maximum values for the distance r are underestimated by a factor of $2^{1/6}$ (=1.12) and $2.8^{1/6}$ (=1.19), respectively. Our implicit assumption that the relaxation may be treated as a dipolar-only effect would appear to be justified, and the corrected estimate of the spin–spin distance is 8 Å $\leq r \leq$ 13 Å.

It is possible to predict the maximum tunnelling rate W_{ab} for electron transfer between cytochrome *a* and Cu_A from the upper limit on the exchange coupling constant J^{ex}. The tunnelling matrix element T_{ab} is related to J^{ex} by

$$J^{ex} = \frac{|T_{ab}|^2}{E_a - E_b - \Delta} \quad (12)$$

where E_a and E_b are the midpoint potentials of cytochrome *a* and Cu_A in the resting enzyme and Δ is the energy of the relevant optical absorption band (Okamura et al., 1979;

Hopfield, 1974). Hopfield (1974) suggests that $\Delta \simeq 1$ eV for electronic charge transfer in cytochromes. For $J^{ex} \leq 3 \times 10^{-4}$ cm^{-1}, $|T_{ab}|^2 \leq 3.8 \times 10^{-8}$ eV^2. For this value of $|T_{ab}|^2$, solution of Hopfield's eq 8 results in $W_{ab} \leq 2.0 \times 10^7$ s^{-1}. This is a factor of 10^6 higher than reported maximal turnover numbers for the dissociation-limited reaction (Ferguson-Miller et al., 1978). If $\Delta = 2.05$ eV, corresponding to the α band transition at 605 nm, then $|T_{ab}|^2 \leq 7.8 \times 10^{-8}$ eV^2, and $W_{ab} \leq 2.6 \times 10^3$ s^{-1}; this rate is also sufficiently fast to accommodate the observed turnover numbers. For the Soret band transition at 420 nm, $\Delta = 2.96$ eV and $W_{ab} \leq 8.6 \times 10^{-3}$ s^{-1}, which is far too slow.

Hopfield (1974) approximated the distance dependence of the tunnelling matrix element when the barrier height was 2 eV: $|T_{ab}| = 2.7 \exp(-0.72r)$. For $|T_{ab}|^2 \leq 7.8 \times 10^{-8}$ eV^2, this equation predicts $r \geq 12.6$ Å. Thus, the upper limit on our distance calculated from dipolar spin–relaxation rates is consistent with the Hopfield theory of electron tunnelling between donor and acceptor hemes in proteins. It must be cautioned that Cu_A is not a heme, and therefore, somewhat different effective barrier conditions may apply.

Recently, the cytochrome *a*–cytochrome a_3 distance was estimated as 12–16 Å by Ohnishi et al. (1982) and 15 Å by Mascarenhas et al. (1983) by analysis of dipolar spin–relaxation and EPR spectral line-shape changes, respectively, in nitric oxide complexes. Given the results presented here, the maximum distance that could be spanned by the three metal centers would be ~30 Å for a linear array: Cu_A–*a*–a_3.

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Registry No. Cu, 7440-50-8; cytochrome *a*, 9035-34-1; cytochrome oxidase, 9001-16-5.

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