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The distance between cytochromes *a* and a_3 in the azide compound of bovine-heart cytochrome oxidase

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The electron-spin relaxation rates of the two species of cytochrome a_3^{3+}-azide found in the azide compound of bovine-heart cytochrome oxidase were measured by progressive microwave saturation at $T = 10$ K. It has been shown previously that Cyt a_3^{+3}-azide gives rise to two distinct EPR resonances, depending upon the oxidation state of Cyt *a*. When Cyt *a* is ferrous, Cyt a_3^{3+}-azide has $g = 2.88$, 2.19 and 1.64; upon oxidation of Cyt *a*, the a_3^{3+}-azide *g*-values become $g = 2.77$, 2.18, and 1.74 (Goodman, G. (1984) J. Biol. Chem. 259, 15094–15099). The relaxation effect of Cyt *a* on Cyt a_3 could be measured as the difference in microwave field saturation parameter $H_{1/2}$ between the $g = 2.77$ and $g = 2.88$ species. For each signal the spin–lattice relaxation time T_1 was determined from $H_{1/2}$ using the transverse relaxation time T_2. The value of T_2 at 10 K was extrapolated from a plot of line-width vs. temperature at higher temperature. The dipolar contribution to T_1 was related to the Cyt *a*-Cyt a_3 spin–spin distance utilizing available information on the relative orientation of Cyt a_3-azide and Cyt *a* (Erecińska, M., Wilson, D.F. and Blasie, J.K. (1979) Biochim. Biophys. Acta 545, 352–364). By taking into account the relaxation parameters for both g_x and g_z components of the Cyt a_3-azide *g*-tensor, the angle between the g_z components of the Cyt *a* and Cyt a_3 *g*-tensors was determined to be between 0 and 18°, and the Cyt *a*–Cyt a_3 spin–spin distance was found to be 19 ± 8 Å.

Introduction

Cytochrome oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) catalyzes the concerted transfer of four electrons to molecular oxygen in an energy-yielding reaction coupled to proton translocation. In the last few years, progress has been made in determining the spatial relationships of the four metal ion centers intrinsic to the enzyme: the two heme iron atoms of Cyt *a* and a_3 and the two copper atoms, Cu_A and Cu_B. The Cu_B–a_3 distance has been shown to be approx. 4 Å by extended X-ray absorbance spectroscopy [1] and by EPR spectroscopy of the nitric oxide complex [2], which is consistent with the unusual magnetic properties of this tightly coupled pair [3]. The Cu_A-Cyt *a* distance in the CO-liganded compound has been found to be 8–13 Å by application of EPR saturation methods [4]. A distance of 10 Å was also estimated on the basis of EPR spectral lineshape considerations [5]. The Cyt *a*–Cyt a_3 distance in the NO-liganded compound was estimated as 12–16 Å by analysis of spin-relaxation of ferric Cyt *a* by ferrous Cyt a_3-NO [6] and 15 Å by EPR spectral line-shape changes [7]. In the present work, the distance

Abbreviation: Cyt, cytochrome; a_3, cytochrome a_3; *a*, cytochrome *a*.

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between Cyt a and a_3 is calculated from the dipolar spin relaxation of ferric Cyt a_3-azide by ferric Cyt a.

In a recent publication, evidence was presented that the g-values of ferric Cyt a_3-azide and Cyt a_3-cyanide reflect the oxidation state of Cyt a [8]. If Cyt a is close enough to Cyt a_3 to affect its spin relaxation, then the magnitude of this effect can be obtained simply by comparing the relaxation rate of Cyt a_3^{3+}-azide in the state in which Cyt a is ferric with that in which Cyt a is ferrous. In the present paper an effective spin–spin distance is obtained from the magnetic relaxation properties of the two species of Cyt a_3^{3+}-azide using a method of analysis similar to that previously described for the Cyt a–Cu_A distance [4]. The present calculation was simplified by the availability of orientation data: the orientations of the Cyt a and Cyt a_3 $\boldsymbol{g}$-tensors with respect to the mitochondrial membrane have been partially determined in oriented multilayers of membraneous cytochrome oxidase [9]. Furthermore, the anisotropy of the low-spin Cyt a_3^{3+}-azide signal permitted separate evaluation of the relaxation characteristics of the g_x and g_z unique orientations of the $\boldsymbol{g}$-tensors; this lent greater precision to the distance calculation without recourse to a more complex evaluation of data for non-unique (i.e., g_y) orientations as described by Goodman and Leigh [4] for the more isotropic Cu_A center.

Materials and Methods

Sample preparation. Cytochrome oxidase was isolated from frozen bovine heart mitochondria by the method of Yu, Yu and King [10]. Anaerobic, redox-buffered, oxidative redox titrations were performed as previously described [8]. Samples at a given redox potential were transferred anaerobically to EPR tubes. The tubes were frozen quickly in a solid CO_2 slurry of isopentane/methylcyclohexane (ratio, 5 : 1), then immersed immediately in liquid nitrogen, where they were stored until EPR measurements were taken.

Progressive microwave saturation. The microwave field strength H_1 was obtained by multiplying the incident power P by a cavity constant, as previously described [4]. In these experiments, $P = 1.0$ mW is equivalent to $H_1 = 1.0 \cdot 10^{-2}$ gauss. The uncorrected microwave saturation parameter $H'_{1/2}$ (or, equivalently, $P'_{1/2}$) was taken from a log–log plot of signal amplitude vs. H_1 (or P); $H'_{1/2}$ 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. The corrected saturation parameter $H_{1/2}$ is defined by $H^2_{1/2} = kH'^2_{1/2}$, where the correction factor k ($1 \leqslant k \leqslant 4$) is a function of the degree of inhomogeneous broadening, i.e., the ratio of lorentzian-to-gaussian line-widths, $\Delta H_L/\Delta H_G$ [11]. The lorentzian line-width at the measuring temperature was estimated by extrapolating from the line-width vs. temperature variation at higher temperature [12,4]. ΔH_G was taken from the inhomogeneous line-width at $T = 7$ K; the line-shape was assumed to be gaussian.

$H_{1/2}$ is related to the product of the spin-lattice and transverse relaxation times T_1T_2 according to $H^2_{1/2} = 1/\gamma^2 T_1T_2$, where γ is the gyromagnetic ratio. The dipolar component of T_1 was obtained by simple algebraic analysis of the Cyt a_3-N_3 T_1T_2 products in the spin-relaxed (ferric Cyt a) and unperturbed (ferrous Cyt a) states, using t_2 values from the line-width vs. temperature extrapolation. This method has been described in detail elsewhere [4].

Results and Discussion

Two distinct EPR resonances corresponding to Cyt a_3^{3+}-azide appear during potentiometric titration of cytochrome oxidase in the presence of azide. When Cyt a is in the ferrous state, Cyt a_3^{3+}-azide has g_z, g_y, and g_x values of 2.88, 2.19, and 1.64, respectively; oxidation of Cyt a is accompanied by conversion of this signal to one having principal g-values of 2.77, 2.18, and 1.74, respectively [8]. At pH 7.2 in the presence of 20 mM azide the midpoint potential (E_m) for the transition between these two species (i.e., for the reaction $a^{2+}a_3^{3+}$-$N_3Cu_B^+ \rightarrow a^{3+}a_3^{3+}$-$N_3Cu_B^+$) is 305 mV. At redox potentials (E_h values) close to the E_m, both species are present in approximately equal amounts. This can be seen in Fig. 1, where EPR signals at $t = 9.5$ K for the low-spin heme g_z region over a range of microwave power are shown for a sample at 319 mV. The $g = 2.88$ signal

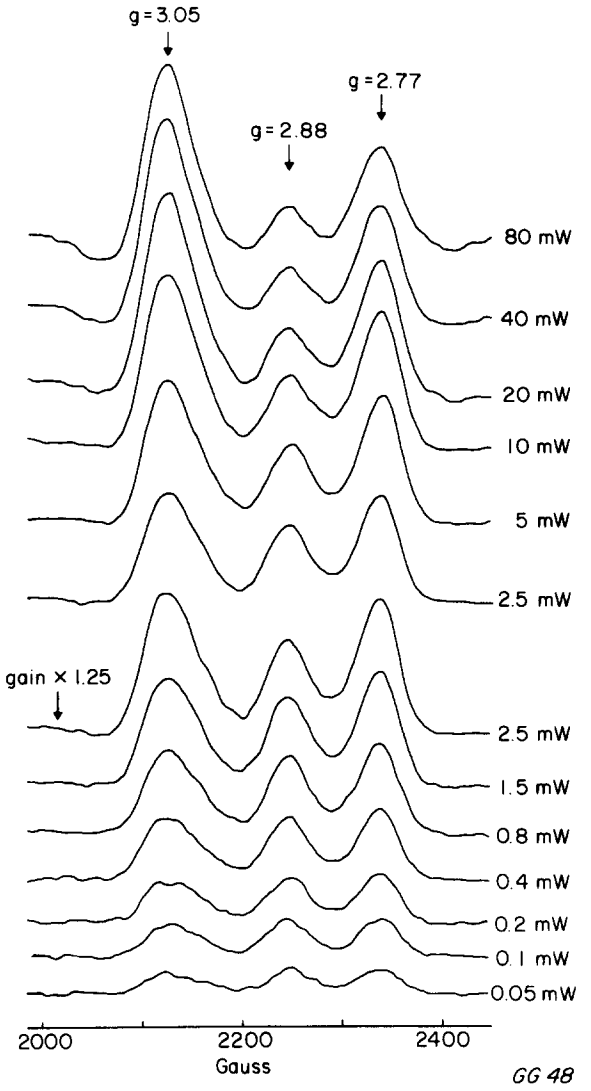

Fig. 1. X-band EPR signals of the $g = 3$ region of the azide complex of cytochrome oxidase as a function of microwave power. During redox titration of the enzyme at pH 7.2 in the presence of 20 mM azide, this sample, poised at 319 mV, was transferred to an EPR tube and frozen as described in the Materials and Methods section. The signals which appear are the g_z components of three low-spin heme signals: the low-potential ($g_z = 2.88$) and high-potential ($g_z = 2.77$) Cyt a_3^{3+}-N_3 signals, and the Cyt a^{3+} ($g_z = 3.00$, 3.05) signal. See text for details. EPR conditions: temperature, 9.5 K; modulation frequency, 100 kHz; modulation amplitude, 20 G; time constant, 0.25 s; scanning rate, 16.7 G/s; microwave frequency, 9.132 GHz; microwave power as shown, 0.05–80 mW.

becomes saturated at a lower microwave power than does the $g = 2.77$ species.

This effect can be quantitated by plotting log amplitude vs. log microwave power, with amplitude normalized in the linear (unsaturated) region. To eliminate overlapping spectral contributions, samples were taken at redox potentials at which only one of the two a_3^{3+}-azide species is present to any significant extent. At $E_h \approx 180$ mV, the $g = 2.88$ species is near maximal, Cyt a is more than 99% reduced, and the $g = 2.77$ species is therefore present at less than 1%. By contrast, at $E_h \gtrsim 400$ mV, the $g = 2.77$ signal is near maximal, and the $g = 2.88$ signal is negligible [8]. EPR spectra at $T = 9.8$ K for samples at 178 mV and 412 mV were taken over a range of microwave power; the resulting microwave power saturation curves for the $g_z = 2.88$ signal (from the 178 mV sample) and the $g_z = 2.77$ signal (from the 412 mV sample) are shown in Fig. 2A. The signals have clearly different relaxation characteristics. Under these conditions of microwave frequency and amplitude, the values of $P'_{1/2}$ are 1.7 and 4.8 mW for the $g_z = 2.88$ and $g_z = 2.77$ signals, respectively. Also shown are saturation curves for the g_x components of these two a_3^{3+}-azide species (Fig. 2B); the $P'_{1/2}$ values for the $g_x = 1.64$ and $g_x = 1.74$ components of these signals are, respectively, 2.1 and 6.2 mW. The g_x signals have poorer signal-to-noise ratios than their g_z counterparts; for this reason duplicate scans were made for particularly noisy signals.

It can be shown that $P_{1/2}$ for the individual signals is independent of the redox potential. At 282 mV, the $P'_{1/2}$ values for saturation of the $g_x = 1.64$ and $g_x = 1.74$ components are the same as they are at 178 mV and 412 mV, respectively (Fig. 2B). This is also true for both g_z components (data not shown). Thus, irrespective of redox potential, the low-potential signal is more easily saturated by application of microwave energy than is the high-potential signal. We know that Cyt a is low-spin ferric ($S = \frac{1}{2}$) when a_3^{3+}-azide is in its high-potential state, low-spin ferrous ($S = 0$) when a_3^{3+}-azide is in its low potential state. If this is the source of the enhanced relaxation of the high-potential signal, then an a–a_3 distance calculation could be based upon this effect. However, other possible sources of spin-relaxation must first be considered.

By following all the EPR signals of cytochrome oxidase which arise in the course of potentiomet-

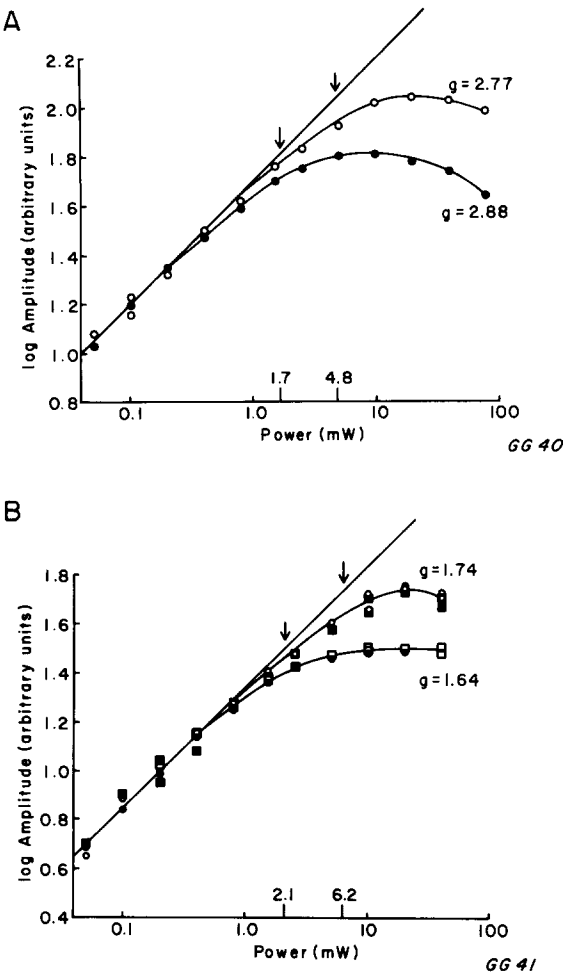

Fig. 2. Microwave power saturation of the low-potential and high-potential species of Cyt a_3^{3+}-N_3. During redox titration of the enzyme at pH 7.2 in the presence of 20 mM azide, samples poised at 178 mV, 282 mV, and 412 mV were prepared. Arrows indicate $P'_{1/2}$ values (see Materials and Methods). EPR conditions: temperature, 9.8 K; modulation frequency, 100 kHz; modulation amplitude, 20 G; time constant, 0.25 s, scanning rate, 16.7 G/s; microwave frequency, 9.13 GHz; microwave power as shown. Symbols: 178 mV (●), 282 mV (□, ■), 412 mV (○). Shown are saturation curves for the g_z components of the low-potential ($g_z = 2.88$) and high-potential ($g_z = 2.77$) a_3^{3+}-N_3 signals at 178 mV and 412 mV, respectively (A), and for the g_x components of the low-potential ($g_x = 1.64$) signal at 178 mV and 282 mV and the high-potential ($g_x = 1.74$) signal at 282 mV and 412 mV (B).

ric titration in the presence of azide, appearance of the two a_3^{3+}-azide species was found to be correlated with the redox behavior of Cyt *a*, but not Cu_A [8]. Even though the ratio of the two species of a_3^{3+}-azide is independent of the Cu_A oxidation state, this in itself does not preclude the possibility of spin-relaxation of Cyt a_3^{3+} by Cu_A^{2+}. If Cu_A^{2+} were effective in spin-relaxing a_3^{3+}-azide, then the degree of spin-relaxation would be a function of redox potential at potentials on either side of the midpoint of Cu_A^{2+}, which has $E_m = 240$ mV in the azide complex [8]. The fact that no redox potential dependence is observed for relaxation of either species of Cyt a_3^{3+}-azide is strong evidence that Cu_A^{2+} does not significantly spin-relax this heme, even when Cyt *a* is in the ferrous ($S = 0$) state.

The absence of magnetic interaction between Cu_A and Cyt a_3 is also indicated by the magnetic relaxation behavior of Cu_A. In the carbon monoxide complex at pH 8.6, the saturation parameter for the g_x–g_y region of the Cu_A signal at 15 K was found to have an approximately linear dependence on the fraction of ferric Cyt *a* in different samples [4]. A comparison has been made between the saturation parameter at $T = 15$ K for g_x of Cu_A at pH 8.2 in the presence of either 50 kPa carbon monoxide or 20 mM azide. Within the experimental error, the saturation parameter for Cu_A has the same dependence on Cyt *a* oxidation state in both the CO and N_3^- compounds (Fig. 3). The midpoint potential of Cyt a_3-CO is 300 mV at pH 8.2 in the presence of saturating CO [13], whereas that of Cyt *a* is 245 mV under these same

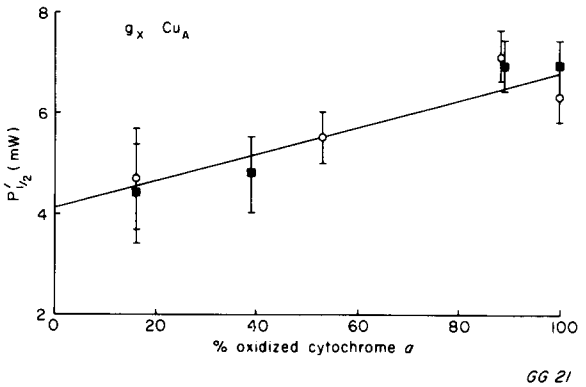

Fig. 3. $P'_{1/2}$ vs. % oxidized Cyt *a* for the g_x component of the Cu_A signal. $P_{1/2}$ was taken from a log–log plot of signal amplitude vs. microwave power for samples titrated oxidatively at pH 8.2 in the presence of either CO (■) or azide (○). EPR conditions: temperature, 15 K; modulation amplitude, 16 G; time constant, 0.16 s; other conditions as for Fig. 2.

conditions, implying that a_3-CO remains reduced during the course of Cyt a oxidation. In the presence of 20 mM azide at pH 8.2, a_3-azide is oxidized with $E_m < 100$ mV and Cyt a has $E_m \approx 300$ mV (Goodman, G., unpublished results); therefore, a_3-azide is in the ferric state throughout oxidation of Cyt a. Thus, the Cu_A saturation parameter appears to have the same dependence on Cyt a oxidation state whether Cyt a_3 is ferrous and liganded to CO, or ferric and liganded to N_3^-. That the Cu_A saturation parameter appears to be independent of the oxidation and ligation state of Cyt a_3 is consistent with the absence of a reciprocal effect of Cu_A on Cyt a_3 relaxation rate.

The data in Fig. 2 were obtained under non-adiabatic conditions of modulation frequency and amplitude. In order to relate a given value of $P_{1/2}$ to the product of spin-lattice and transverse relaxation times T_1T_2, adiabatic conditions of saturation must apply [11,14,4]. This means that the saturation experiment must satisfy the adiabatic condition: $\omega_m H_m \ll \gamma H_{1/2}^2$, where ω_m is the modulation frequency and H_m is the modulation amplitude. In Fig. 4 are adiabatic saturation curves for the g_z components of the low- and high-potential Cyt a_3^{3+}-azide signals ($g = 2.88$ and $g = 2.77$);

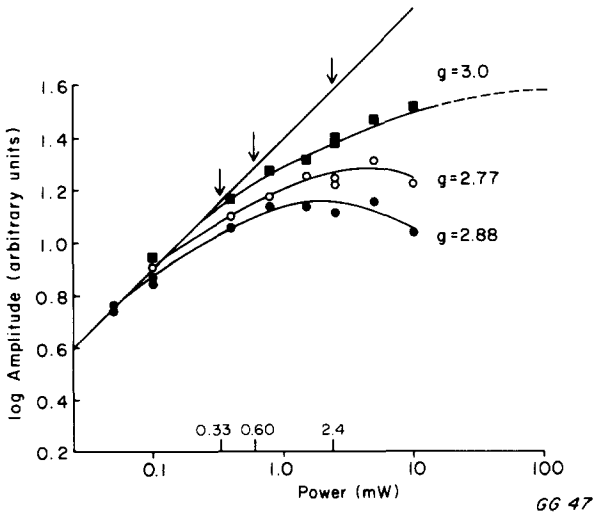

Fig. 4. Microwave power saturation of Cyt a^{3+} and the low-potential and high-potential species of Cyt a_3^{3+}-N_3 under adiabatic conditions. Samples prepared as in the Fig. 2 legend. EPR conditions: temperature 9.8 K; modulation frequency, 1 kHz; modulation amplitude, 16 G; microwave power as shown; other conditions as for Fig. 2. Symbols: low-potential a_3^{3+}-N_3 (●), high-potential a_3^{3+}-N_3 (○), Cyt a^{3+} (■).

the $P'_{1/2}$ values are 0.33 and 0.60, respectively. Note that the adiabatic $P'_{1/2}$ values are approx. a factor of 4 smaller than those obtained with 100 kHz modulation frequency and 20 G modulation amplitude, as observed previously for Cu_A signals [4]. Also shown in Fig. 4 is an adiabatic saturation curve for the $g_z = 3.0$ signal of Cyt a, which has $P'_{1/2} = 2.4$ mW. From the uncorrected saturation parameter $H'_{1/2}$ corresponding to each of these adiabatic $P'_{1/2}$ values, the T_1T_2 product was calculated: T_1T_2 is equal to $0.52 \cdot 10^{-10}$, $0.28 \cdot 10^{-10}$ and $0.60 \cdot 10^{-11}$ s^2 for the $g = 2.88$, $g = 2.77$ and $g = 3.0$ signals, respectively.

The transverse relaxation time T_2 can be obtained independently of the T_1T_2 product, according to the relationship between T_2 and the lorentzian line-width: $T_2 = 2/\gamma\Delta H_L$. In fig. 5A the temperature dependence of the total line-width ΔH_T of the $g = 2.88$ signal is shown in the range 27–65 K. Above 45 K the variation in line-width as a function of temperature is sufficiently large that a meaningful determination of the lorentzian line-width ΔH_L can be made (see Fig. 5 legend). The line of least squares through the values of ΔH_L so determined has slope 3.82, and intersects $T = 9.8$ K at $\Delta H_L = 0.11 \pm 0.01$ G, corresponding to $T_2 = 2/\gamma\Delta H_L = (7.3 \pm 0.7) \cdot 10^{-7}$ s. The line width vs. temperature for the $g = 2.77$ signal was similarly determined, extrapolating to $\Delta H_L = 0.16 \pm 0.01$ G, which gives $T_2 = (5.1 \pm 0.3) \cdot 10^{-7}$ s at 9.8 K (Fig. 5B). T_2 for Cyt a^{3+} in the azide complex was likewise obtained by constructing a line-width vs. temperature plot for a sample poised at 412 mV in the presence of 20 mM azide at pH 7.2. Linear regression gave a line with slope = 1.54 and correlation coefficient 0.965 (data not shown). Extrapolation to 9.8 K yielded $\Delta H_L = 7.5 \pm 2.0$ G, which corresponds to $T_2 = (1.1 \pm 0.3) \cdot 10^{-8}$ s.

The observed gaussian line width ΔH_G is 42 G for both the $g = 2.77$ and 2.88 signals [8]; the corresponding $\Delta H_L/\Delta H_G$ ratios have values of 0.0035 ± 0.0005. This degree of inhomogeneous broadening suggests that $k \approx 1.2$ [11]; therefore we have assumed $H_{1/2} = 1.2\ H'_{1/2}$. The corrected T_1T_2 products are then $0.43 \cdot 10^{-10}$ and $0.23 \cdot 10^{-10}$ s^2 for the $g = 2.88$ and $g = 2.77$ signals, respectively. For the Cyt a g_z signal, the gaussian line-width is also 42 G, implying that $\Delta H_L/\Delta H_G = 0.02$. A correction factor of $k = 2$ was assumed;

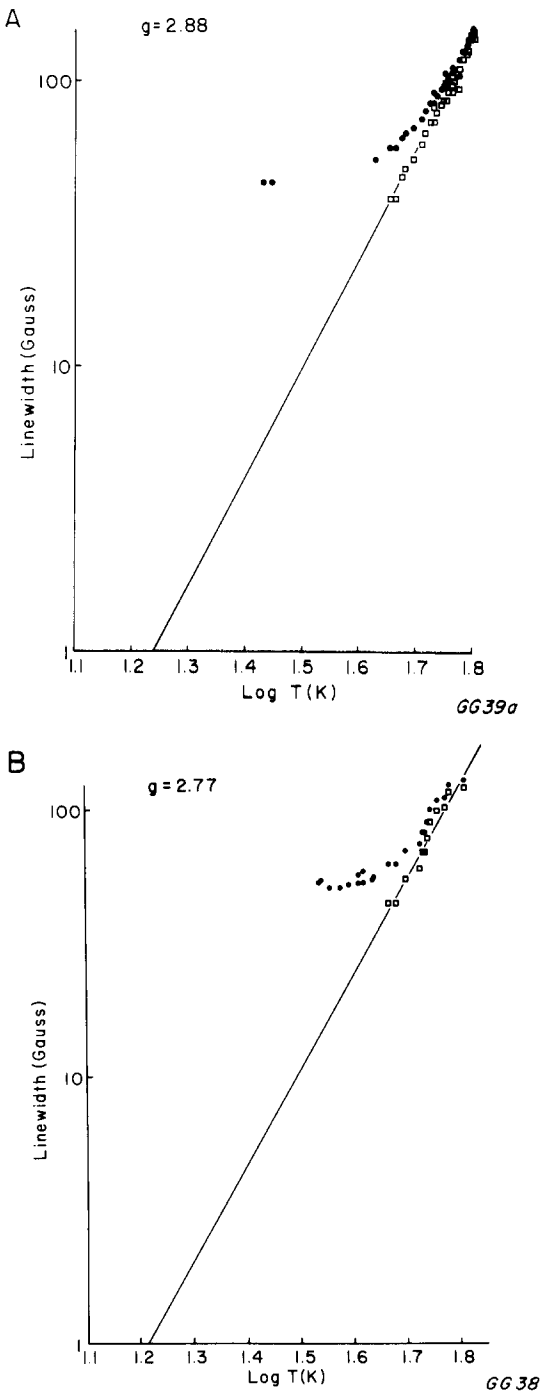

Fig. 5. Log–log plot of line-width vs. temperature for the low-potential and high-potential species of cytochrome a_3^{3+}-N_3. The 178 mV (A) and 370 mV (B) samples were those used to obtain the data of Fig. 4. Measured line-width ΔH_T (●); lorentzian contribution to the line-width ΔH_L (□), calculated 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 ΔH_L points above 40 K. EPR conditions: microwave power, 20 mW; other conditions as given in the legend for Fig. 4.

the corrected T_1T_2 product is then $0.30 \cdot 10^{-11}$ s^2.

From a given T_1T_2 product and the corresponding T_2, T_1 can be deduced: $T_1 = (6.1 \pm 0.6) \cdot 10^{-5}$, $(4.6 \pm 0.3) \cdot 10^{-5}$, and $(3.0 \pm 0.8) \cdot 10^{-4}$ s for the $g = 2.88$, $g = 2.77$, and $g = 3.01$ signals, respectively.

Distance calculation

We assume a dipolar mechanism for the effect of Cyt *a* on the spin relaxation of Cyt a_3. The dipolar contribution $(1/T_1)_D$ to the total spin-lattice relaxation rate $(1/T_1)_T$ is defined operationally as

$$\left(\frac{1}{T_1}\right)_D = \left(\frac{1}{T_1}\right)_T - \left(\frac{1}{T_1}\right)_I \quad (1)$$

where $(1/T_1)_I$ is the intrinsic spin–lattice relaxation rate, i.e., the rate which would be observed in the absence of paramagnetic interaction. In the case of Cyt a_3-N_3 the intrinsic rate can be obtained by measurements on the low-potential a_3^{3+}-N_3 signal, since Cyt *a* is ferrous in all molecules in which this signal appears. The high-potential a_3^{3+}-N_3 signal appears only in those molecules in which Cyt *a* is in the ferric ($S = \frac{1}{2}$) state; hence, measurements yield the combined dipolar and intrinsic relaxation rates.

For the g_z components of the Cyt a_3^{3+}-N_3 signals, application of Eqn. 1 results in $(1/T_1)_{D,z} = 5.4 \cdot 10^3$ s^{-1} (range $(2.2\text{–}8.1) \cdot 10^3$ s^{-1}). Because of poorer signal-to-noise ratios, saturation of the g_x components were not measured under adiabatic conditions. However, the T_1T_2 products calculated from the $P'_{1/2}$ values for g_x and g_z obtained under non-adiabatic conditions (Fig. 2) were compared: $(T_1T_2)_z/(T_1T_2)_x = 0.55$ for the low-potential signal and 0.67 for the high-potential signal. The assumption was made that the dipolar contribution to T_1 has a similar dependence on the *g*-value. Thus, for a given value of $(1/T_1)_{D,z}$, a range of possible values of $(1/T_1)_{D,x}$ can be predicted. This expected relationship between $(1/T_1)_D$ values for the g_x and g_z directions of Cyt a_3-azide was used

in the distance calculation to define the allowed orientations of the spin systems of Cyt a and a_3 (see below).

If Cyt a exerts its effect upon the spin–lattice relaxation rate of Cyt a_3-azide by a dipolar mechanism, then the temperature dependence of the effect should parallel the temperature dependence of $1/T_1$ of Cyt a. Microwave power saturation at 15 K was measured as for experiments at 9.8 K. The dipolar relaxation rate $(1/T_1)_D$ of the g_z component of a_3^{3+}-azide at 15 K was derived and a power law dependence on temperature was assumed: $(1/T_1)_D = bT^m$, where m and b are unknown constants. Simultaneous equations with $T = 15$ K and 9.8 K were solved for m, yielding $m = 6.8 \pm 2$. Solution of the analogous equations for $1/T_1$ of Cyt a yielded $m = 7.9 \pm 2$. This indicates that it is reasonable to assume a dipolar mechanism for relaxation of Cyt a_3-N_3 by cytochrome a.

In order to compute the spin–spin distance between Cyt a and a_3 based on a dipolar relaxation model, the relative orientation of the two $\boldsymbol{g}$-tensors must be determined, or else all possible orientations would have to be considered. Membraneous cytochrome oxidase provides a convenient system in which to study the orientation of the metal ion centers. The spectral similarities between isolated and membraneous preparations leads us to assume that the relative orientations are maintained upon disruption of the membrane with detergents [4]. In Cyt a and a_3, the planar porphyrin heme is perpendicular to the g_z axis of the $\boldsymbol{g}$-tensor [15]. The two cytochromes are situated in the mitochondrial inner membrane in fixed orientations: the plane of the membrane is perpendicular to the heme planes, which means that the g_z axis lies in the membrane plane. The g_x and g_y axes of Cyt a form angles of 60° and 30° to the membrane normal [16]. In Cyt a_3-azide the g_x axis lies in the membrane plane along with the g_z axis; thus, the g_y axis points along the normal to the membrane [9]. Of the three angles necessary to specify the relative orientation of the $\boldsymbol{g}$ tensors of Cyt a and a_3, two can be deduced from the results cited above. A third angle, α, which lies between the g_z axes of the two cytochromes, remains undetermined.

The dipolar component $(1/T_1)_{D,z}$ of the spin–lattice relaxation time of Cyt a_3^{3+}-azide due to spin relaxation by Cyt a was solved for the spin–spin distance r as a function of the dipolar angle θ according to the method described previously [4]. The calculation was performed at 3° intervals for values of α between 0° and 90°. The orientation of the magnetic field in the $\boldsymbol{g}$ tensor coordinate system for Cyt a_3-azide is described by the usual polar coordinates (θ_g, ϕ_g). The dipolar rate equation for the g_z component of a_3-azide was solved for r at each α for ten values of θ_g at each of ten values of ϕ_g taken at equally spaced intervals over the surface of a sphere.

When solving for r as a function of $(1/T_1)_{D,z}$, an angular transformation relating the g_z axis of a_3-azide to the Cyt a $\boldsymbol{g}$-tensor was employed. For each $(\alpha, \theta_g, \phi_g)$ point, a value of $(1/T_1)_{D,x}$ was computed from the dipolar rate equation using a transformation matrix appropriate for the g_x axis of a_3-azide. The computed $(1/T_1)_{D,x}$ was compared with the experimental value of $(1/T_1)_{D,z}$ upon which the computation was based. If the ratio of these two relaxation rates was found to fall within the limits discussed above, then this $(\alpha, \theta_g, \phi_g)$ point was used to compute the distance r.

All $(\alpha, \theta_g, \phi_g)$ sets which met this criterion were found to have values of the angle α (formed by the g_z axes of the two cytochromes) such that $0° \leqslant \alpha \leqslant 18°$. The cytochrome a–Cyt a_3 distance thus computed is $r = 19 \pm 8$ Å. This distance range overlaps the value of 15 Å reported by Mascarenhas et al. [7] and the range 12–16 Å reported by Ohnishi et al. [6]. It should be noted that the other two studies do not allow for all possible dipolar angles and relative orientations of the $\boldsymbol{g}$-tensors. The calculation described herein is thus more rigorous, though the computed value for r is less precise. As discussed previously, Hopfield's model of the distance dependence of tunnelling [17], when applied to the 605 nm (cytochrome α band) transition energy of 2.05 eV, predicts that $r \geqslant 12.6$ Å [4], which is consistent with our results. We conclude from the work reported here that larger values of r (> 20 Å) can in principle give rise to the amount of spin relaxation observed in these experiments. Greater precision in the distance calculation must await better orientation information or application of more sophisticated techniques.

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