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DUAL-MODE EPR SPECTROMETRY OF O_2-PULSED CYTOCHROME *c* OXIDASE

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O_2-activated bovine heart cytochrome *c* oxidase has been examined by dual-mode EPR spectrometry. Resonances have been observed at $g = 10$ and 4.5 in the parallel mode and at $g = 10$, 5, 1.8 and 1.7 in the normal mode. The bulk of these signals are interpreted to come from a stoichiometric $S = 2$ system with $|a| = 0.17$ cm^{-1}, $D = +2.1$ cm^{-1}, $|E| = 0.026$ cm^{-1}, $g = 2$. Exchange coupling between cytochrome a_3 and Cu_B is not indicated.

The redox centers in cytochrome *c* oxidase have been extensively studied by EPR spectrometry for over 25 years [1], which has resulted in a voluminous data set but not in a unifying interpretation. Of the four potentially EPR exhibiting metal centers, cytochrome *a*, Cu_A, cytochrome a_3, Cu_B, only the first two are currently identified with EPR signals from noninteracting $S = 1/2$ systems [2,3]. The other two centers have long been considered EPR undetectable in the oxidized enzyme as a result of strong exchange coupling into an integer spin system [4]. A weak EPR signal from resting cytochrome oxidase at $g = 12$ was recently characterized by quantitative parallel mode EPR as arising from a stoichiometric $S = 2$ system with a relatively small zero-field interaction and no detectable hyperfine interaction [5]. This proposal supports the model [6] of tetravalent high-spin iron in cytochrome a_3, rather than the alternative model of an antiferromagnetically coupled Fe-Cu pair. Recent Mössbauer data have been interpreted in favor of this latter, heteronuclear-cluster model [7]. Activated cytochrome *c* oxidase is obtained by reoxidation of the fully reduced enzyme with a pulse of molecular oxygen. This pulsed oxidase is also named 'the $g = 5$ species' after its EPR signal with the unusual *g* values of 5, 1.8, 1.7 [8]. The signal's dependence on microwave frequency is consistent with any integer spin $S \geq 1$ [9]. Below, we describe detection of new EPR resonances from pulsed oxidase, and we propose that these and the previously reported lines [8] can be interpreted as the components of a quintet spectrum.

For a magnetically isolated $S = 2$ octahedral system with tetragonal and rhombic distortions, the spin Hamiltonian in the notation of Abragam and Bleaney is:

$$H = \frac{a}{120}\left(O_4^0 + 5O_4^4\right) + \frac{D}{3}O_2^0 + EO_2^2 + \beta B \cdot g \cdot S$$

in which the O_j^i's are spin operators; *a* is the coefficient of the fourth degree operator for cubic symmetry [10]. Additional 3rd and 4th order terms, allowed by symmetry, were found to be unnecessary to describe the resonances. A schematic view of the energy levels in zero magnetic field is given in Fig. 1. Depending on the magnitude of the parameters *a*, *D* and *E* versus that of the applied microwave quantum, from zero to ten transitions

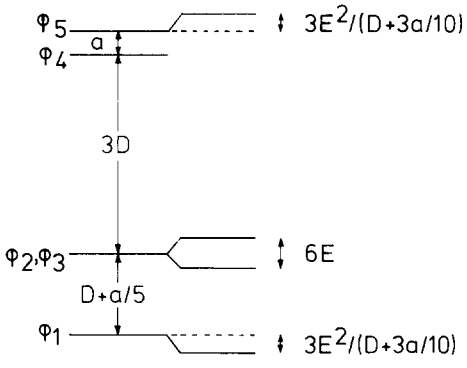

Fig. 1. Approximate relative energies within a spin quintuplet described by Eqn. 1 for $B = 0$. The expressions are correct up to second order in perturbation theory. When any one of the ten splittings is comparable in magnitude to the microwave energy, accurate spectral synthesis requires computation of the splittings by matrix-diagonalization methods.

are possible. Each transition gives rise to a subspectrum characterized by three effective g values. For example, in the resting enzyme only the transition between the highest two levels has been observed with approximate g values of 12, 0, 0 in the X band and 9, 0, 0 in the P band [5,9].

Bovine-heart cytochrome c oxidase was isolated following Hartzell and Beinert [11]. The activated enzyme was prepared by rapid mixing with 5 mM tricine cacodylate buffer (pH 7.2) saturated with O_2, and rapid freezing in cold isopentane [8]. Data interpretation is based on powder-spectral synthesis. If the zero-field splittings are large compared to the microwave energy, perturbation theory may be applicable; in general, however, exact solutions must be obtained by numerical diagonalization of the energy matrix. The FORTRAN 77 simulation program runs through the solid angle, through a normal distribution in zero-field parameters, and through the d.c. field. For every step, it computes the ten transition energies from the eigenvalues obtained by matrix diagonalization, the Boltzmann weighting, the eigenvectors, and the transition probabilities for two configurations of the microwave magnetic field B_1, namely, parallel or perpendicular to the external field B_0. The stick spectrum is convolved with a Gaussian in frequency space.

Fig. 2 shows the dual-mode X-band spectrum of pulsed cytochrome oxidase. For the microwave magnetic component parallel to the static field,

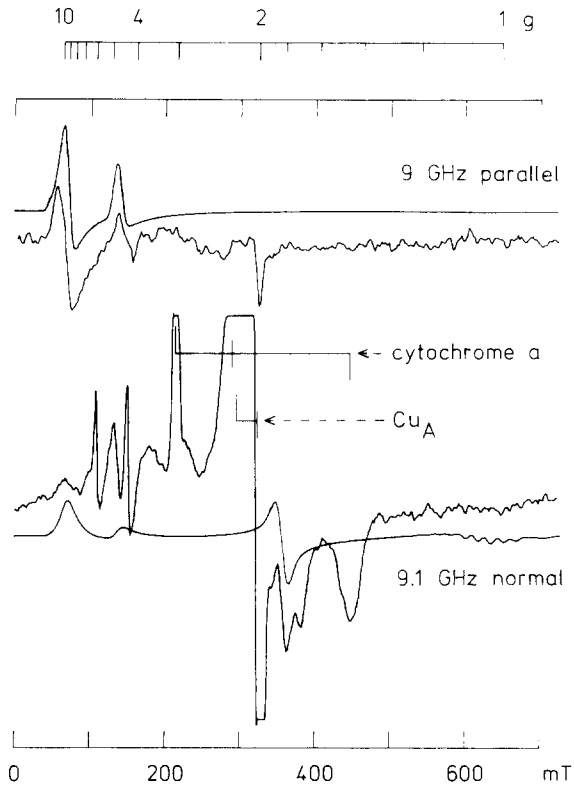

Fig. 2. Two X-band EPR spectra of 0.46 mM O_2-pulsed bovine heart cytochrome c oxidase recorded under identical conditions except that $B_1 \| B_0$ for the upper trace and $B_1 \perp B_0$ for the lower trace. The two noiseless traces simulate an $S = 2$ spectrum with $|a| = 0.17 \pm 0.04\ cm^{-1}$ $D = +2.1 \pm 0.2\ cm^{-1}$, $|E| = 0.026 \pm 0.002\ cm^{-1}$, $g = 2$ and a linewidth of 0.006 $h\nu$. The zero-field parameters are spread over a 7-point sampling of a Gaussian distribution. The line at $g = 10$ is from the $\phi_5 \leftarrow \phi_4$ transition; the lines at $g = 4.5$, 1.8 and 1 are from the $\phi_3 \leftarrow \phi_2$ transition. The data were taken on a Varian E-9 spectrometer with an E-236 bimodal cavity: microwave frequencies, 9085 MHz parallel, and 9138 MHz perpendicular; microwave power, 400 mW; modulation amplitude, 2.5 mT; modulation frequency, 100 kHz; temperature, 19 K.

$B_1 \| B_0$, the $\Delta m = 1$ transitions are several 100-fold attenuated. Thus, a weak Cu_A signal is still detectable in the $B_1 \| B_0$ spectrum. In addition to the $B_1 \perp B_0$ signals from low-spin ferric cytochrome a, from Cu_A(II) and from trace contaminants ($g = 6$; 4.3) there are several other resonances, notably at $g = 10$ and 4.5 in the parallel mode and at $g = 10$, 5, 1.8, 1.7 in the normal mode. The bulk of these signals can be simulated as arising from the $\phi_3 \leftarrow \phi_2$ and $\phi_5 \leftarrow \phi_4$ transitions within two non-Kramers doublets of an $S = 2$ system with $|a| = 0.17\ cm^{-1}$,

$D = +2.1$ cm^{-1}, $|E| = 0.026$ cm^{-1}, $g = 2$. In a previous analysis *, an integer spin system was also proposed but with different zero-field parameters [9]; however, higher-order terms in S, parallel-mode data, and powder shapes were not considered at that time. Although the simulation of the normal-mode spectrum shows a weak signal at $g = 1$, the quality of the experimental data does not allow this prediction to be conclusively tested at present. Ripples in the simulated peak at $g = 1$ reflect the 7-point distribution in zero-field parameters. The simulation does not account for $B_1 \perp B_0$ lines at $g = 5$ and 1.7; we have no explanation for these discrepancies. When extrapolated to a microwave frequency of 15 GHz, the simulation of the normal-mode spectrum shows that all lines lose intensity relative to the $g = 1.8$ line and that line positions shift only marginally (not shown). This alternative to a previously proposed interpretation of the *P*-band spectrum [9] explains observation of a line at $g = 1.8$ and the apparent absence of lines at $g = 10$, 4.5, 1. Again, some other lines, notably one at $g = 2.55$ [9], are not accounted for.

An independent check on the simulation of the dual-mode spectrum is made by measuring the temperature dependence of the $g = 10$ line, the $\Delta m = 4$ transition within the upper two levels of the spin quintet. Since the shape of the $g = 10$ signal does not change at least up to $T = 20$ K, the peak-to-peak amplitude times the temperature should be proportional to the fractional population of the non-Kramers doublet. Assuming $a/5 \ll D$, the axial zero-field splittings are approx. D and $3D$, and we can use our previous Eqns. 14 and 14a in Ref. 5 to describe the temperature dependence of the $\Delta m = 4$ transitions. Fig. 3A shows the fractional population data versus reciprocal temperature together with a best fit to a Boltzmann-population distribution (i.e., the solid trace and the units on the ordinate axis). The result of the minimization, $D = +2.2$ cm^{-1} (range 1.3–3.1 cm^{-1}), is consistent with the value obtained from spectral synthesis.

A perturbation-theory approach to simulate the

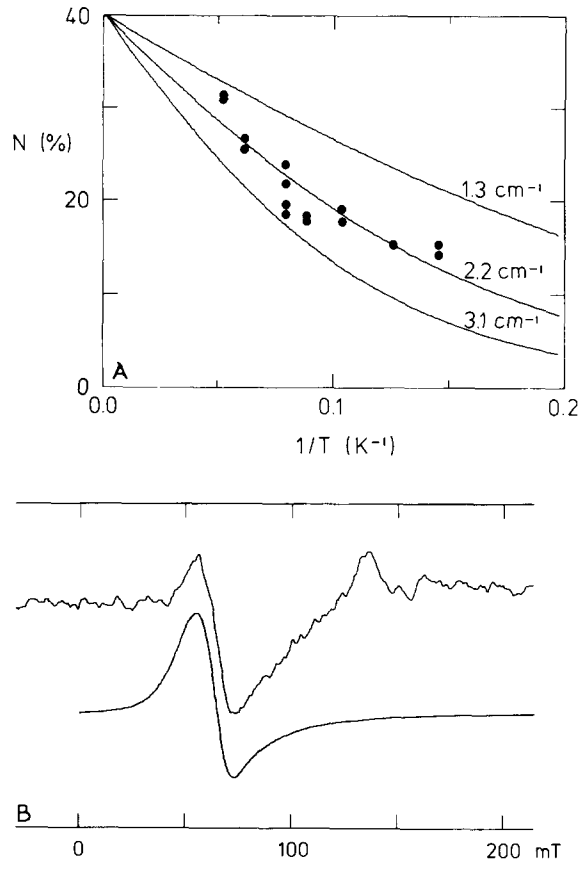

Fig. 3. (A) Fractional population of the second non-Kramers doublet in the quintet ground state of a paramagnet in O_2-pulsed cytochrome *c* oxidase. The product of sample temperature and peak-to-peak amplitude around $g = 10$, when fitted to a Boltzmann distribution, gives $D = +2.2 \pm 0.9$ cm^{-1}. (B) Perturbation theory fit to the parallel-mode $g = 10$ line. The simulation parameters are $g = 2$; linewidth $b_0 = 0.8$ mT; rhombic shift $b_r = 22$ mT (b_0 and b_r are defined in Ref. 5, Eqns. 6 and 7). Quantitation on the basis of this simulation gives approx. 1.2 $S = 2$ systems per enzyme molecule.

$\Delta m = 4$ transition in weak field Zeeman systems was previously described in Ref. 5. Quantitation is accomplished by comparison to a simulation of the spectrum of an $S = 2$ model compound of known concentration. The perturbation-theory simulation of the parallel-mode $g = 10$ signal is given in Fig. 3B. The spectral shape is not faithfully reproduced, possibly indicating the crudeness of the assumption of isotropic g strain. A similar conclusion was drawn for the $g = 12$ signal in resting and partially reduced oxidase [5]. Compari-

* We would like to correct a statement made in this previous work [9] that S.I. Chan and his coworkers had chosen an interpretation for the $g = 5$ resonance when, in fact, they had not [12].

son of Fig. 3B with the equivalent traces from a 10 mM solution of $FeSO_4$ (cf. Ref. 5) gives an $S = 2$ concentration of 0.55 mM, assuming $D = 2.1\ cm^{-1}$. Since the enzyme concentration, determined from the optical spectrum of the original preparation and corrected for dilution in the rapid-mixing apparatus, is 0.46 mM, we tentatively conclude that the $g = 10$ signal represents one $S = 2$ system per molecule of cytochrome aa_3.

Three models have been proposed [4,6,13] to account for a net $S = 2$ system in oxidized cytochrome oxidase:

(a) Fe(III)($S = 5/2$) – Cu(II)($S = 1/2$)

(b) Fe(IV)($S = 2$)

(c) Fe(III)($S = 3/2$) – Cu(II)($S = 1/2$)

Since D appears too small for heme Fe(III) ($S = 5/2$), since no Cu hyperfine is observed, and since heme Fe(III) ($S = 3/2$) is unprecedented but heme Fe(IV) ($S = 2$) with a relatively low reduction potential has been reported to exist [14], it seems to us that the attribution of a formal charge of 4 + to the heme of O_2-pulsed cytochrome a_3 (i.e., no coupling to Cu) cannot be ruled out as a viable alternative. We emphasize that the description of the heme as having an Fe(IV) ion rather than a heme with an Fe(III) ion with cationic porphyrin may be a semantic choice. More extensive studies of the cytochrome a_3 site and of model compounds will be necessary to clarify the distinction.

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