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# ELECTRON-ELECTRON DOUBLE RESONANCE MEASUREMENTS ON XANTHINE OXIDASE

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## Summary

Electron-electron double resonance measurements were carried out on milk xanthine oxidase (xanthine:oxygen oxidoreductase EC 1.2.3.2) and the spectra obtained supported a previous model, based on EPR data, proposing a spin-spin interaction between unpaired electrons associated with Fe-S and Mo. The technique demonstrated that the additional apparently isotropic, splitting in the Mo EPR spectra observed at low temperature is produced by a single site giving two spectra interconverting at a rate consistent with the Fe-S spin lattice relaxation time. Other data concerning the model and the relaxation behaviour of the species are discussed.

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Xanthine oxidase from bovine milk has been widely studied using physical techniques including electron paramagnetic resonance (EPR) [1]. Lowe, Lynden-Bell and Bray [2] described a change in form of the molybdenum EPR spectrum with temperature. This was interpreted as an isotropic coupling to one of the iron-sulphur systems [with a $g_{av}$ of 1.95] within the molecule that was averaged out at higher temperatures because of rapid iron spin-lattice relaxation. This work has been extended (see summary by Bray [3]) to a total of four systems, two in xanthine oxidase and two in the related bacterial enzyme, xanthine dehydrogenase. A possible explanation to account for the absence of dipolar interaction between the iron-sulphur and molybdenum proposed by Lowe et al. [2] is that the paramagnetic centers are separated by a distance of the order of 40 Å, but this seemed improbably high. (The distance has been revised [3] and a value of the order of 30 Å is a more recent estimate.) We now report electron-electron double resonance

(ELDOR) investigations that were undertaken with a view to contributing to an understanding of this rather complex spin system.

ELDOR in organic systems was first employed by Hyde, Chien and Freed [4]. ELDOR in frozen solutions was studied by Hyde, Sneed and Rist [5] and this paper has been useful in interpretation of spectra obtained in the present work. Access to the ELDOR literature is given in a review by Hyde [6].

The technique involves irradiating one portion of the EPR spectrum with a first intense pumping microwave source and observing the effect on another portion with a second microwave source. ELDOR signals may arise from any one of a variety of cross-communication mechanisms. The fractional change in the EPR signal intensity is approximately the ratio of the spin-lattice relaxation probability to the cross-communication rate.

The present experiments were performed using a standard Varian E-9 EPR spectrometer equipped with an E-800 ELDOR accessory, and a liquid helium 'boil-off' apparatus described by Ranon and Hyde [7]. The temperature range was 15 to 40 °K. All spectra were obtained using 100 kHz field modulation frequency.

Enzyme preparation and manipulation methods were as described by Swann and Bray [8]. Cyanide-treated enzyme was prepared according to Komai et al. [9] and the anaerobically reduced allopurinol sample (no EPR active Mo or flavin) similarly to Massey et al. [10].

Fig. 1 shows the low temperature form of the 'Slow' (see Ref. 1) Mo spectrum prepared in $^1H_2O$ buffer. ELDOR spectra from this sample were obtained at points marked A—D in Fig. 1 and are shown in Fig. 2. As discussed in the appendix of the paper by Hyde et al. [5], inhomogeneously broadened

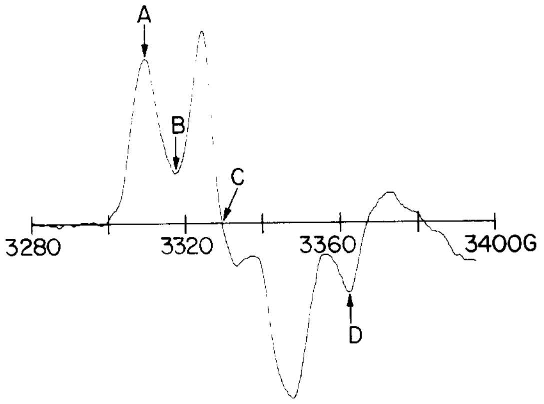

Fig.1. Low temperature form of the 'Slow' $Mo^V$ signal from xanthine oxidase: 30 °K, 10 mW microwave power, 9.2 GHz, 2 G field modulation at 100 kHz. The sample [0.5 mM] was of cyanide inactivated xanthine oxidase and was reduced anaerobically with 20 mol dithionite per mol enzyme for 20 min at pH 8.2 in $^1H_2O$ buffer. ELDOR traces were obtained while observing points A—C with $\nu_P < \nu_O$ and at D with $\nu_P > \nu_O$.

EPR spectra are expected to yield ELDOR lines that are a composite of a derivative lineshape and a pure absorption lineshape. The composition can vary from one extreme to the other depending on the setting of the observing resonant condition and the amount of overlap in the powder EPR spectrum. Assuming the validity of the theoretical model, it is not difficult to estimate the true line center from such a composite. The line at 14 MHz, corresponding to the Zeeman frequency of the free proton, arises from weak coupling to protons in the immediate environment of the molybdenum [5]. Its lineshape is sensitive to temperature and also to the observing and pumping power. It generally appears as a rather symmetrical ‘first derivative type’ line. A sample prepared in $^2H_2O$ buffer also showed this line with a similar intensity, implying that few of the protons in the neighbourhood of the molybdenum are exchangeable. A line at 28 MHz (see Fig. 2B), which was only observed at the highest pump powers, is believed to arise from double proton spin flips.

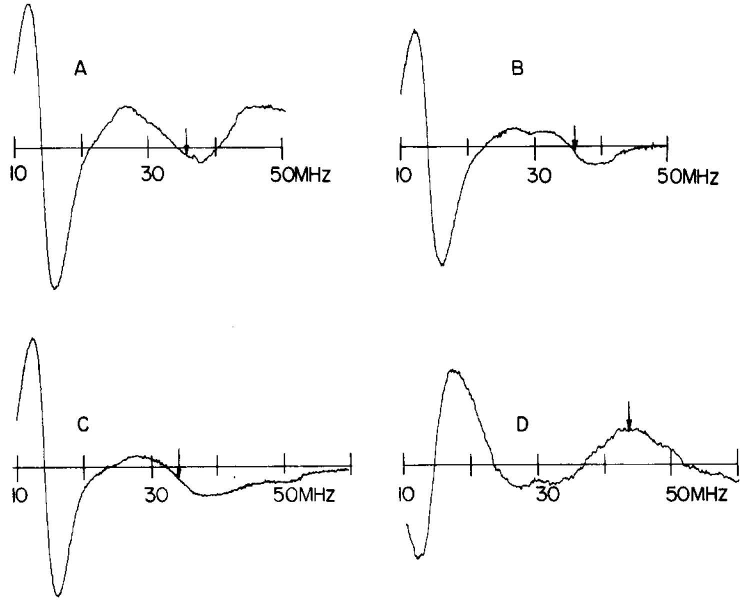

**Fig.2. ELDOR spectra: observing power 10 mW, pump power attenuation 0 dB, except C which was 5 dB, 30 °K, 1 G field modulation amplitude at 100 kHz. Spectra correspond to points A—D of Fig. 1. Spectrum D is reversed in sense compared with spectra A—C because of the change from $\nu_P < \nu_O$ to $\nu_P > \nu_O$. The arrows show the values taken for the position of the line discussed in the text.**

There were weak indications of the 16 G proton splitting [11] at 18 °K.

The line at about 35 MHz (see Fig. 2) was present in samples prepared with both $^1H_2O$ and $^2H_2O$ buffer and correlates with the low temperature splitting of Lowe et al [2], which they reported for this system to be 11 G (31 MHz). The presence of this ELDOR line shows that the low-temperature Mo EPR spectrum is given by one site converting between two spectral forms. As the temperature is varied from 18 to 35 °K, there is very little change in the intensity of this signal, although there is some indication of broadening at 45 °K. There is no detectable shift in the position of this line with temperature. The line occurs at different positions when the observing frequency is set to different points of the Mo spectrum and varies from 35 MHz at the low field peak (Point A of Fig. 1) to 44 MHz at the High field peak (Point D of Fig. 1). (Our estimates of the correct line centres are indicated by arrows in Fig. 2. These were made, as discussed above, from a consideration of the contributions of first derivative and absorption type lineshapes as judged particularly by the overall shape and the position of the baseline at high frequencies: for example,C and D have high proportions of first derivative and absorption lineshapes respectively.)

This is not consistent with the 1—2G [3—6 MHz] anisotropy observed by Lowe et al. [2] and although it is possible that this inconsistency can be accomodated within the uncertainties of the two experiments, we, nevertheless, prefer not to suppress it. The central question is: Why is there no apparent indication of dipolar interaction between the iron-sulphur system and the Mo system in the EPR spectrum? While Lowe et al. [2,3] have proposed a separation of 30 Å to explain this absence, the approximately two-fold greater anisotropy of the interaction obtained here by ELDOR suggests that the question whether or not there is a considerable dipolar interaction may still be open.

The Lowe et al. [2] model implies that the two Mo spectra should interconvert at the rate of iron-sulphur spin-lattice relaxation. While pulse measurements of spin-lattice relaxation times are not available, progressive saturation experiments indicate that the iron-sulphur spin-lattice relaxation time is always less than that of the Mo. Thus, the interconversion rate is more rapid than the Mo spin-lattice relaxation rate and very strong ELDOR signals are expected. The observed ELDOR behaviour is consistent with this understanding. The 35 MHz ELDOR line is approximately 1/3 for the peak intensity of the EPR derivative display. Precise ELDOR reductions extrapolated to infinite pump powers were not obtained and, in addition, the observing power was at a partial saturation level which also complicates quantitative determination of the reduction. However, the qualitative result is that the ELDOR effect is large.

The intensity of the line is dependent on the observation frequency, and the width of the narrowest line observed (see Figs 2B and 2C) is 10 MHz (peak to peak of first derivative). This was the value with the observing frequency set at the first minimum (Point B in Fig. 1) and also the crossover (Point C in Fig. 1). The crossover setting also gave the largest intensity of this 35 MHz ELDOR effect. The width of the line did not decrease further with decreases in field modulation amplitude. The observed width and shape of the ELDOR line [note the long tails] is one of the new pieces of information obtained in the present work that must be accommodated by a fully satisfactory model of this spin system.

When the signal was detected with a reference 90° out-of-phase with respect to the modulating magnetic field, both the 14 and 35 MHz lines were still present, although of different relative intensities suggesting that passage effects are complicating interpretation of the spectra.

Since it is more difficult to saturate the Mo EPR when Fe-S reduced than when it is not (Lowe, D.J., and Bray, R.C., unpublished), the presence of paramagnetic iron has the effect of shortening the spin-lattice relaxation time of Mo. Modulation of the dipolar interaction because of Fe-S ($g_{av}$ = 1.95) relaxation (Abragam [12] p. 380, Eqn 40) is a possible mechanisms. A paradox, however, is that a distance of the order of 30 Å is required to reduce dipolar interaction to the degree observed in the EPR and ELDOR spectra, while a distance of only 2 Å is required to obtain the effect on Mo relaxation. (A ratio of $T_1$ [Fe-S]/$T_1$ [Mo] of 10 has been used in the calculation. Note that this mechanism is relatively ineffective since the Fe-S ($g_{av}$ = 1.95) relaxation is at a rate much less than the resonance frequency of Mo). One might invoke dipolar coupling of the Mo to the $g_{av}$ = 2.01 Fe-S system (which may be closer to the Mo, has a much shorter $T_1$, and is always present in our samples when $g_{av}$ = 1.95 Fe-S is observable) to explain the enhanced relaxation. Additionally, one might invoke a cross-relaxation mechanism between either Fe-S and Mo. Once again, to reiterate the central and unanswered question: Is the dipolar coupling between $g_{av}$ = 1.95 Fe-S and Mo truly small or might there be some reason that makes it unobservable in the ordinary EPR spectra?

We attempted to demonstrate an iron-sulphur to molybdenum interaction directly by observing the low field Mo EPR peak [Point A in Fig. 1] and sweeping the pump frequency through the lower field portion of the iron-sulphur spectrum, and also by observing at the high field peak [Point D in Fig. 1] and sweeping the pump frequency through the derivative line of the iron-sulphur signal. No effects were observed. This should not be regarded as definitive, however, since lower temperatures or higher pump powers may be necessary to observe incipient disappearance of the Mo low-temperature splitting as the correlation time is shortened by pumping the iron-sulphur in the same molecule.

In this paper we have obtained results consistent with previous models and demonstrated conclusively that only one site gives rise to the low-temperature split Mo spectrum. There is also information in the ELDOR lineshapes, temperature dependence, and the effect of rapid passage that will probably be sensitive to physical models of the system.

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