Electron nuclear double-resonance (ENDOR) spectroscopy of amine oxidase from pig plasma

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Electron nuclear double-resonance ('ENDOR') spectroscopic studies on pig plasma amine oxidase have been carried out at 15 K. Deuterium-exchange studies show the presence of two sets of exchangeable protons, probably from two water molecules; from the magnitude of their hyperfine couplings, one is assigned to be equatorially, and the other axially, co-ordinated. Only one ^{14}N hyperfine coupling is observed, suggesting that the bonding of all amino acid (histidine) or organic cofactor ligands is similar. Upon addition of azide, a further hyperfine coupling to nitrogen is observed which is smaller than that observed for the native enzyme; the hyperfine couplings to the remaining nitrogens are slightly altered.

INTRODUCTION

Amine oxidase (EC 1.4.3.6) from pig plasma has been purified to homogeneity and is known to contain bound Cu^{2+} ions necessary for catalytic activity (Barker *et al.*, 1979). E.p.r. and water proton relaxation studies indicate that the copper sites have a geometry close to square pyramidal with three to four nitrogens and two or one oxygens (probably from water) as first-shell ligand atoms. These studies further suggest that the inhibitors azide and cyanide displace equatorial, but not axial, water co-ordinated to the copper. A tightly bound organic cofactor is also necessary for enzyme activity; early reports (Blaschko & Buffoni, 1965) indicated that this was pyridoxal phosphate, but recently evidence has been presented for the ox plasma enzyme that the cofactor is pyrroloquinoline quinone (Ameyama *et al.*, 1984; Lobenstein-Verbeek *et al.*, 1984). It is not known with certainty whether the organic cofactor is co-ordinated to copper, though available evidence indicates that this might be the case (Finnazzi-Agro *et al.*, 1977; Suzuki *et al.*, 1983).

ENDOR spectroscopy is a magnetic-resonance technique well suited to characterization of the ligands co-ordinated to paramagnetic metal-ion centres in proteins (Eachus & Olm, 1985). In the present paper we describe results from ENDOR studies on pig plasma amine oxidase which provide further support for the copper centres having co-ordinated nitrogen atoms (e.g. from histidine) and two exchangeable water molecules as ligands.

MATERIALS AND METHODS

All chemicals (reagent grade) were purchased from BDH (Poole, Dorset, U.K.), except for Dowex chelating resin, which was obtained from Sigma (Poole, Dorset, U.K.). Amine oxidase was purified from pig plasma and its homogeneity characterized as described by Rius *et al.* (1984). The enzyme was stored at liquid- N_2 temperatures

as a concentrated solution (20 mg/ml) in 0.05 M-potassium phosphate buffer, pH 7.0. Samples of enzyme for the ENDOR studies were prepared by dialysis against 5 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-disodium EDTA (two changes of buffer, 2000 vol. excess). The dialysed enzyme was concentrated by using an Ultrafiltration apparatus (Sartorius, Göttingen, Germany) to a final protein concentration of 75 mg/ml corresponding to a Cu^{2+} content of 0.8 mM. The azide derivative was prepared by addition of 0.025 ml of Dowex-chelating-resin-treated NaN_3 (1.0 mM) to 0.5 ml of the concentrated enzyme; the azide concentration was chosen to convert the enzyme completely into the inhibited complex (Barker *et al.*, 1979).

ENDOR spectra were recorded on a Bruker ER200 e.s.r. spectrometer with a Bruker ER 250 computer-controlled triple-resonance system. Radio frequencies in the range of 0.5–30 MHz were generated and amplified to 100 W. Spectra were recorded at 15 K with an Oxford Instruments ESR900 liquid-helium cryostat. Single-crystal-like ENDOR spectra were obtained by making measurements at the extreme field limits of the e.p.r. spectrum, namely on the M_I (quantum number) = $-3/2$ copper parallel line and two-dimensional ENDOR spectra by measurements on the copper perpendicular feature.

RESULTS

The ENDOR spectrum of pig plasma amine oxidase in the parallel region shows six pairs of lines (a–f in Fig. 1a) centred on the free proton nuclear frequency (ν_{H}) of 11.60 MHz. The separation between pairs of lines (a–a etc.) gives the proton hyperfine coupling. Six distinct types of inequivalent proton are observed, with hyperfine couplings of 6.15, 3.93, 1.34, 0.92, 0.66 and 0.24 MHz (aa–ff respectively). The two larger couplings are very broad and show that the protons are in ill-defined sites and probably liberating, in contrast with the narrow lines

Abbreviations used: ENDOR, electron nuclear double resonance; Im, imidazole.

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Fig. 1. ENDOR spectrum of native pig plasma amine oxidase in (a) $^1\text{H}_2\text{O}$ and (b) $^2\text{H}_2\text{O}$

The magnetic field is set on the $M_I = -3/2$ feature of $g^\parallel$.

from the other four protons, which indicate firmly bound and clearly defined sites. The spectrum of the enzyme in $^2\text{H}_2\text{O}$ is quite different (Fig. 1b); the position of line c (Figs. 1a and 1b) is unchanged, but those labelled d–f have been replaced by a strong broad line centred on the free proton frequency. From our experience we know that the presence of $^2\text{H}_2\text{O}$ can broaden proton ENDOR lines generally, presumably through its influence on relaxation times. The low-frequency pair of lines a and b have disappeared completely, but there is only a partial reduction in the height of the higher frequency pair a and b, suggesting that there are underlying lines arising from other nuclei. The couplings to protons a and b almost certainly arise from two different types of co-ordinated water molecules: that with a coupling of 6.15 MHz arising from water molecules in axial positions and that with coupling of 3.93 MHz from water molecules in equatorial positions. Evidence for this, besides the broad linewidths and effects of deuteration, is their magnitude and general similarity to couplings found in the ENDOR spectrum of $[\text{Cu}(\text{H}_2\text{O})_6]^{2+}$ in Tuttins salt (Atherton & Horsewill, 1979).

The ENDOR spectrum recorded in the perpendicular region (Fig. 2a) also shows six pairs of lines. These cannot be correlated directly with those from the parallel region, and meaningful hyperfine couplings cannot be deduced nor any estimates made of the number of different protons contributing to the spectrum; the main reason is because the principal directions of each proton tensor are not along the molecular axes x , y or z , and so, in this region, the complicated angular dependence of the proton tensors means that at least two pairs of ENDOR lines, and in some cases many more, can be generated for each proton. The spectra nevertheless serve as a useful fingerprint. In the perpendicular region for the deuterated sample (Fig. 2b), the spectra show a marked reduction in

Fig. 2. ENDOR spectrum of native pig plasma amine oxidase in (a) $^1\text{H}_2\text{O}$ and (b) $^2\text{H}_2\text{O}$

The magnetic field is set on the $g^\perp$ feature.

the intensities of the proton lines marked with an asterisk, and these could be related to one of the lines a or b in the parallel region whose proton has been deuterated. The weak lines shown in Fig. 2(a) under high-gain instrument settings are remnants of parallel ENDOR features, because the magnetic field at which the ENDOR spectrum is recorded in the perpendicular region contains some element of the copper $M_I = +3/2$ parallel feature. Lines centred on the deuterium nuclear frequency [2.16 MHz at 0.3310 T (3310 G)] can be seen at low frequencies (Fig. 3), but again cannot be simply interpreted because they were measured on the copper perpendicular feature.

The only ENDOR lines attributable to ^{14}N in the parallel region are those at 13.5 and 14.6 MHz (the higher-frequency pair a and b in Fig. 1a). Their separation of 1.1 MHz is close to that expected for a ^{14}N nucleus, and they are centred at 14.05 MHz, which is half the hyperfine coupling, since $A_N > \nu_N$; thus the hyperfine coupling is 28.1 MHz [1 mT (10 G)]. These lines occur at the same positions in $^2\text{H}_2\text{O}$ and in the azide-bound form (see below) in which they are marginally shifted to higher frequency by 0.1 mHz. The lineshape and separation are perturbed by a large quadrupole term. The outer pair of the four expected split lines of ^{14}N will have a very different relaxation rate compared with the inner pair. If quadrupolar split lines are not resolved, then the separation can be greater or less than $2\nu_N$, depending upon whether the outer pair of quadrupole split lines are stronger or weaker than the inner pair. In this case, separations less than $2\nu_N$ are found. This effect has not hitherto been observed in powder spectra, largely because of the poor resolution of earlier spectrometers.

Hyperfine coupling to nitrogen is observed in the perpendicular region where the intensity of the observed features is much greater, and lines assigned to ^{14}N are

Fig. 3. Low-frequency ENDOR spectrum of amine oxidase in $^2\text{H}_2\text{O}$

The magnetic field is set on the $g^\perp$ feature.

Fig. 4. High-frequency ENDOR spectrum of amine oxidase in (a) $^1\text{H}_2\text{O}$ and (b) in $^1\text{H}_2\text{O}$ with azide added

The magnetic field is set on the $g^\perp$ feature.

observed at 2.2, 4.0 (Fig. 3), 21.7, 24.0, 26.7 and 29.6 mHz (Fig. 4a). The two low-frequency lines are at positions very similar to those of the non-co-ordinated imidazole nitrogens in $\text{Cu}(\text{Im})_4^{2+}$ (van Camp *et al.*, 1981). Although there are four well-resolved lines in the 20–30 MHz region almost certainly arising from nitrogen coupling, it cannot be deduced how many nitrogens, whether equivalent or non-equivalent, are contributing. Their hyperfine coupling must be about 50 MHz, which is typical for a nitrogen bonded directly to copper.

Upon addition of azide to the enzyme, the ENDOR spectrum in the parallel region (Fig. 5) comprises a group of proton lines (pqpp) and two pairs of lines r–r and s–s. There are no proton lines in the region 8–10 MHz, as found for the native enzyme (Fig. 1a). This suggests that

Fig. 5. ENDOR spectrum of amine oxidase in $^1\text{H}_2\text{O}$ after addition of azide

The magnetic field is set on the $M_I = -3/2$ feature of $g^\parallel$.

Fig. 6. Model of the copper centre(s) in pig plasma amine oxidase

The broken lines indicate the weaker bonding in the axial direction; the oxygen atom in the sixth co-ordination position could come from water or the organic cofactor. The nitrogen atoms are from protein histidine and possibly from the organic cofactor.

the co-ordinated water molecules have been displaced by the azide or at least weakened so that their interaction is minimal. The higher-frequency line overlaps with the high-frequency p line, but is undoubtedly present when their relative intensities are considered. These lines (r–r) are not centred on ν_H and are attributed to ^{14}N . They occur at 10.9 and 12.4 MHz and represent a hyperfine coupling of 23.3 MHz [84 mT (8.4 G)]. They do not appear in the spectrum of the native enzyme and are assigned to coupling to the co-ordinated azide nitrogen. The other pair of lines (s–s) is at a similar frequency to the nitrogen in the native enzyme, as has already been discussed.

The proton spectrum in the perpendicular region does not change significantly, but there is a dramatic change in the nitrogen ENDOR spectrum in the region 18–30 MHz (Fig. 4b). This suggests a major structural change associated with different hyperfine couplings to the azide and possibly to the co-ordinated histidines.

DISCUSSION

The results show that the native enzyme has nitrogen ligands co-ordinated to the copper, and the hyperfine couplings are consistent with the nitrogens being roughly equivalent. The ^{14}N ENDOR lines are broad and could hide unresolved structure from different nitrogen atoms with slightly different hyperfine couplings. There are two large hyperfine couplings from protons which are

exchangeable with deuterium. The magnitudes of these proton couplings suggest that they arise from a tightly bound equatorial water molecule and one or more equivalent and weakly bound axial water molecule(s). The remaining proton couplings are small in magnitude, not deuterium-exchangeable and narrow in width. This suggests that they arise from co-ordinated histidines; the data do not exclude co-ordination of the organic cofactor to copper, however. Addition of azide results in a further ^{14}N coupling, a minor change to the other ^{14}N couplings, the loss of the large hyperfine couplings to the equatorial and axial water molecules and changes to the pattern of lines from non-exchangeable protons.

We propose a structure for the copper centres in pig plasma amine oxidase in which the ENDOR data are consistent with the e.s.r. data and also with the changes resulting from addition of azide (Fig. 6). Further evidence in support of the structure is that the e.p.r. spectrum becomes more axial upon addition of azide (Barker *et al.*, 1979). This suggests that there are three co-ordinating equatorial nitrogens and one equatorial oxygen in the native enzyme, changing to the higher symmetry arrangement of four equatorial co-ordinating nitrogens in the azide-bound form. Also the copper parallel hyperfine lines are broader with azide present, indicating further unresolved hyperfine coupling. The reason for the nitrogens in the native enzyme apparently exhibiting the same hyperfine couplings in the ENDOR spectrum is because they are chemically similar as well as all being co-ordinated in the copper equatorial plane. It may further be noted that histidine, pyridoxal phosphate and pyrroloquinoline quinone (the latter two being proposals for the organic cofactor) are similar in being aromatic with sp_2 lone pairs of electrons; consequently the ENDOR spectrum would not distinguish between them.

The hyperfine couplings we observe are more anisotropic than that found in the model complex $\text{Cu}(\text{Im})_4^{2+}$ (van Camp *et al.*, 1981). This is not surprising, since the

model complex has a higher symmetry. Furthermore, for tetragonal stereochemistry such as is present in the enzyme, the principal directions of the hyperfine tensor is along the Cu–N bond. Thus on $g^{\parallel}$ the hyperfine coupling to nitrogen is $A^{\perp}$, which is always less than $A^{\parallel}$, as indeed has been found in the present study.

Finally, it should be noted that pig plasma amine oxidase in the native form has two bound coppers (Barker *et al.*, 1979). The evidence presented herein fails to reveal any difference between the structures of these two copper centres.

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