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Articles

Proton Electron–Nuclear Double-Resonance Spectra of Molybdenum(V) in Different Reduced Forms of Xanthine Oxidase†

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ABSTRACT: Electron–nuclear double-resonance (ENDOR) spectra of protons coupled to molybdenum(V) in reduced xanthine oxidase samples have been recorded. Under appropriate conditions these protons may be studied without interference from protons coupled to reduced iron–sulfur centers. Spectra have been obtained for the molybdenum(V) species known as Rapid, Slow, Inhibited, and Desulfo Inhibited. Resonances corresponding to at least nine protons or sets of protons are observed for all four species, with coupling constants in the range 0.08–4 MHz. Most of these protons do not exchange when ^2H_2O is used as solvent. Additional protons giving couplings up to 40 MHz are also detected. These correspond to EPR-detectable protons studied in earlier work. The strongly coupled protons may be replaced by ^2H, through appropriate use of ^2H_2O or of ^2H-substituted substrates, with consequent disappearance of the ^1H resonances. In most cases the corresponding ^2H ENDOR features have also been observed. The nature of the various coupled protons is briefly discussed. Results permit specific conclusions to be drawn about the structures of the Inhibited and Desulfo Inhibited species. In particular, the data indicate that the aldehyde residue of the Inhibited species has been oxidized and that the four protons derived from the ethylene glycol molecule in the Desulfo Inhibited species are not all equivalent. Recent assignments [Edmondson, D. E., & D'Ardenne, S. C. (1989) *Biochemistry 28*, 5924–5930] of the weakly coupled protons in the latter species appear not to be soundly based. The possibility of obtaining more detailed structural information from the spectra is briefly considered. In agreement with the above workers, ^{14}N ENDOR was not detected, indicating the absence of a nitrogen ligand of molybdenum in the enzyme.

A variety of spectroscopic methods is available for the determination of local structural information on the environment of paramagnetic centers in metalloenzymes. Thus, in the case of molybdenum enzymes knowledge of the environment of the metal comes almost exclusively from EPR and EXAFS spectroscopy. In the particular case of the molybdenum iron–sulfur flavoprotein xanthine oxidase, extensive studies by these methods [see Bray (1988) for review] have provided quite detailed information on the structure of the molybdenum center and the nature of the catalytic reaction. A prime feature of these EPR results is the finding that the molybdenum center of the enzyme, at the molybdenum(V) level, can exist in a number of clearly defined states of ligation of the metal. Each

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state gives rise to its own characteristic EPR signal, distinguished by names such as Rapid, Slow, Inhibited, etc. From a combination of isotopic substitution experiments using EPR spectroscopy and EXAFS data, considerable information is now available about the ligation of the molybdenum for each of these species.

A spectroscopic method available for many years, that is now finding increasing application in the metalloenzyme field, is ENDOR [electron–nuclear double resonance; see Hüttermann and Kappl (1987) for review]. The method in effect uses EPR to detect NMR transitions. Its particular advantage over EPR is its ability to detect and provide information on nuclei up to about 10 Å from the central metal atom and too weakly coupled to it for hyperfine splittings to be resolved in EPR spectra. Both qualitative and, increasingly in recent years, quantitative information may be obtained from analysis of ENDOR spectra.

A study of the molybdenum center of xanthine oxidase by ENDOR not only should serve to further deepen knowledge of the structure of this center but also might provide a stimulus toward perfecting techniques for analyzing quantitatively ENDOR spectra from other metalloenzymes. We now summarize ENDOR results on xanthine oxidase obtained over a number of years and comment on the recent related, but less extensive, work by Edmondson and D'Ardenne (1989). Parts of our work have appeared in preliminary form in symposium proceedings (Pinhal et al., 1989; Bray et al., 1990).

Materials and Methods

Xanthine Oxidase Samples. Xanthine oxidase was prepared from buttermilk as described previously [procedure H1,2 of Ventom et al. (1988)]. Individual molybdenum(V) signal giving species were generated generally as indicated by Bray and Gutteridge (1982) or, for the Rapid species, as indicated by Morpeth et al. (1984). In all cases 50 mM Na^+-Bicine buffer, pH 8.2, was employed. Enzyme samples in ^2H_2O were prepared by freeze-drying and redissolving, the procedure being repeated to reduce contamination with ^1H_2O. [$^2H_4$]Ethylene glycol (98% enrichment) was obtained from MSD Isotopes. Rather concentrated enzyme samples were required to obtain good ENDOR spectra, and molybdenum(V) concentrations in the range of 0.2–0.7 mM were generally used. In most experiments, no attempts were made to remove excesses of substrates or other reducing agents. Iron–sulfur centers were thus at least partly in the reduced state in all samples, unless otherwise stated.

ENDOR Spectroscopy. Preliminary ENDOR experiments were carried out in Stockholm and in Leicester, modified Varian and Bruker equipment being used, respectively. ENDOR spectra presented were recorded in the Nitrogen Fixation Laboratory, University of Sussex, on an updated Bruker ER 200D-SRC spectrometer, equipped with a radio-frequency amplifier of 100-W nominal output power and an Oxford Instruments ESR 900 cryostat. Recording conditions were generally as follows: temperature, 25 or 30 K; microwave frequency, 9.42–9.48 GHz; microwave power, 0.5–2 mW; radio-frequency power, 1-dB attenuation; modulation depth, 50 kHz. Scan rates, response times, and numbers of points were generally such that resolution was limited by modulation depth. Any deviations from these conditions are noted in the figure legends. Accumulation times of 1–16 h were used for the spectra illustrated. Note that at the temperatures used for samples prepared without removal of excess reducing agent, all the molybdenum EPR spectra would be split by coupling between molybdenum(V) and reduced iron–sulfur centers (Lowe et al., 1972; Lowe & Bray, 1978).

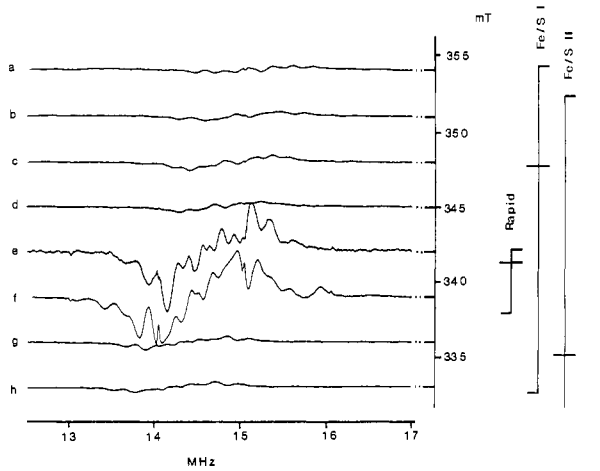


FIGURE 1: Proton ENDOR spectra from xanthine oxidase at different field settings. Spectra a–h were recorded on a reduced xanthine oxidase sample at different field settings, but under otherwise identical conditions. They are shown all plotted at the same amplification. The field values are indicated by markers on the vertical scale, and the stick diagrams correspond to the principal g values of the Rapid Mo(V) EPR signal and of reduced iron–sulfur centers, Fe/S I and Fe/S II (g_2 and g_3 only). The sample used was that of Figure 5b.

Results

General Features of ENDOR Spectra from Xanthine Oxidase. Protons are abundant in proteins, and those relatively close to paramagnetic metal centers in metalloproteins would be expected to show various degrees of hyperfine coupling to the central metal ion. It is therefore not surprising that proton ENDOR spectra, generally showing couplings from a number of rather weakly coupled protons, have been observed and studied in, for example, heme proteins (Höhn et al., 1983) iron–sulfur proteins (Fritz et al., 1971), copper proteins (Rist et al., 1970), and also flavoproteins (Eriksson et al., 1970). As is demonstrated below, xanthine oxidase fits into the same pattern, yielding molybdenum(V) proton ENDOR spectra containing a wealth of detail.

Reduced xanthine oxidase samples in general show not only molybdenum(V) EPR signals but also those from the reduced iron–sulfur centers, Fe/S I and Fe/S II, and sometimes also that from the FADH• semiquinone. Care therefore needs to be taken to distinguish molybdenum ENDOR from that of the other constituents. Under appropriate recording conditions it was found by G. Anger, A. Ehrenberg, and R. C. Bray (unpublished work) that ENDOR from the iron–sulfur centers of xanthine oxidase is readily observable. However, the relatively high temperature and low microwave power used in the present work (see Materials and Methods) would tend to minimize any interference from iron–sulfur centers, since their EPR signals are relatively difficult to saturate (Bray, 1975). Only negligibly small FADH• EPR signals were present in any of the samples we examined, so that interference from flavin ENDOR should not be a complication.

Figure 1 shows ENDOR spectra recorded at a series of different field positions across the EPR spectrum from a xanthine oxidase sample reduced with formamide (Morpeth et al., 1984) and showing the Rapid molybdenum(V) and the Fe/S I and Fe/S II signals. Field positions used for spectra a–h are indicated on the vertical scale, which is accompanied by stick diagrams corresponding to the principal features of the different EPR signals. ENDOR spectra e and f are clearly primarily from the molybdenum. The origin of the much weaker spectra, spectra a–d, g, and h, is less certain. A significant contribution from the 25% of ^{95}Mo and ^{97}Mo isotopes will certainly be present. EPR spectra (George & Bray, 1988)

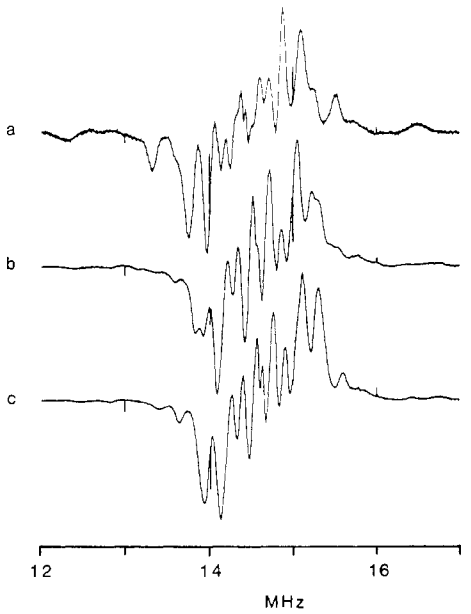


FIGURE 2: ENDOR spectra corresponding to the Rapid EPR signal, recorded at the three principal **g** values. (a–c) Excitation at the g_1, g_2, and g_3 positions, respectively, of the Rapid signal, generated with formamide.

from these isotopes extend for 10–15 mT on either side of the features from the 75% of $I = 0$ isotopes. Some small contribution from Fe/S I is also possible. However, whatever the origin of the weak features, it is clear that molybdenum ENDOR can be observed in the presence of reduced iron–sulfur centers, without serious interference by them. Further direct confirmation of this point is given in a subsequent section of this paper.

Orientation Dependence of ENDOR Spectra and the Number of Coupled Protons. Spectra a–c of Figure 2 show proton ENDOR spectra for the Rapid molybdenum(V) EPR signal from xanthine oxidase, stimulated respectively at the g_1, g_2, and g_3 positions. All spectra are approximately symmetrical and centered on the free proton frequency, ν_n, of approximately 14.5 MHz for the field values employed. This is in accord with the normal resonance condition for protons such that a pair of peaks corresponding to a particular proton are centered on ν_n and split by the hyperfine coupling constant. The spectra reveal protons coupled to molybdenum with hyperfine couplings in the approximate range of 0.08–4.0 MHz. For the species of interest here, which have rhombic **g** tensors, a single proton gives rise in the ENDOR spectrum to a single pair of lines, only when the magnetic field is set at either g_1 or g_3 in the EPR spectrum (Hoffman et al., 1985). At other fields, a considerably larger number of features is expected. Also, in general, the axes of the hyperfine coupling will not coincide with the **g** axes, and furthermore, the hyperfine axes will be different for each proton. The forms of the experimental spectra observed at different **g** values reflect these expectations and differ substantially from one another. A further consequence is that the apparently simple matter of estimating the number (or more strictly the number of sets) of coupled protons by counting pairs of features in the ENDOR spectra is not a straightforward one. Thus, from inspection of the spectra we can do no more than say that they indicate coupling to molybdenum of probably at least nine protons, or sets of equivalent protons.

ENDOR of Different Molybdenum(V) Species from Xanthine Oxidase. In Figure 3 the ENDOR spectrum for the Rapid signal is compared with that from the Slow, Inhibited, and Desulfo Inhibited signals (spectra a–d, respectively). In

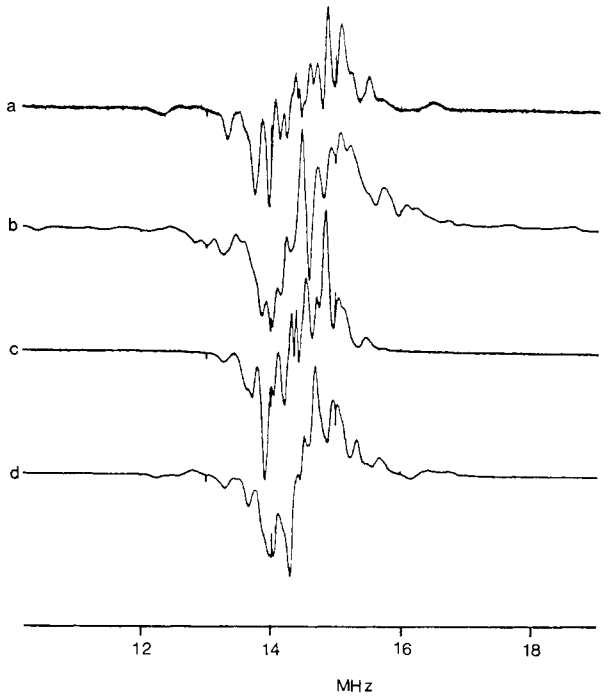


FIGURE 3: ENDOR spectra for different molybdenum(V) EPR signal-giving species. (a–d) Rapid, Slow, formaldehyde Inhibited, and Desulfo Inhibited molybdenum(V) species, respectively. All spectra were stimulated at the g_1 feature of the EPR spectrum. The recording temperature of spectrum b was 10 K.

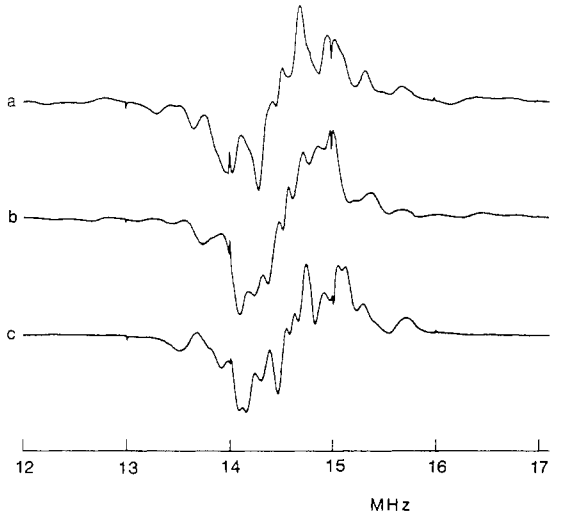


FIGURE 4: ENDOR spectra corresponding to the Desulfo Inhibited EPR signal, recorded at the three principal **g** values. (a–c) Excitation at the g_1, g_2, and g_3 positions, respectively, of the Desulfo Inhibited signal.

all cases the ENDOR was stimulated at the g_1 feature of the EPR spectrum. While clearly there are differences among the spectra, particularly relating to protons with coupling constants greater than about 2 MHz, there are also similarities, at least with regard to the number of features in the central region and their line widths. When the ENDOR was stimulated at the g_2 or g_3 features of the EPR spectrum instead of at the g_1 position, this gave changes in all cases analogous to those illustrated for the Rapid signal in Figure 2. This is illustrated for the Desulfo Inhibited signal in Figure 4.

ENDOR from Exchangeable EPR-Detectable Protons. It has long been known that the Rapid and Slow EPR spectra of xanthine oxidase show protons coupled to molybdenum that are exchangeable with the solvent water. In the former (formamide Rapid type 1 signal; Morpeth et al., 1984), there is a strongly coupled proton (A_{av} = 35 MHz; coupling ap-

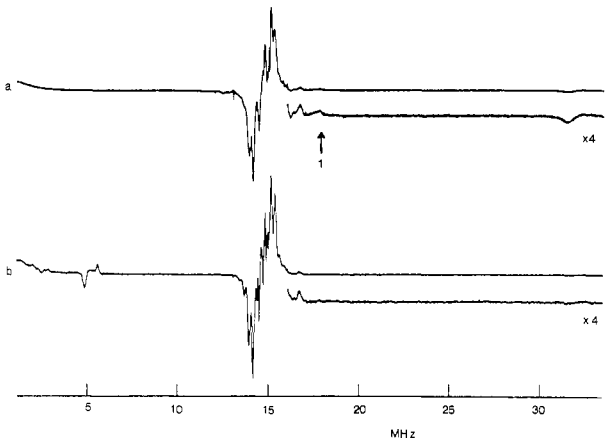


FIGURE 5: ENDOR spectra for the Rapid signal in (a) ^1H_2O and (b) ^2H_2O, plotted over a wide frequency range. ENDOR was stimulated at the g_2 position of the EPR spectrum, and formamide was used to reduce the sample. Parts of the spectra are shown replotted with the amplification increased by the factors indicated. (For the meaning of the arrow, see the text.)

parently mainly isotropic) and a more weakly coupled proton (A_{av} = 7 MHz; coupling showing substantial anisotropy). For the latter (Slow signal in Bicine buffer; Gutteridge et al., 1978), there is a strongly coupled proton (A_{av} = 45 MHz) and apparently also a more weakly coupled one (A_{av} = 4 MHz). Clearly, it was of interest to see if these protons could be detected by ENDOR.

Figure 5 shows ENDOR spectra for the Rapid signal over a wider frequency range than that illustrated in Figures 1–4. Spectra are shown for samples (a) in ^1H_2O and (b) in ^2H_2O. The most striking difference between the spectra is the feature at 31 MHz in (a) replaced by a feature at 5 MHz in (b). For proton couplings greater than the free proton frequency of 14.5 MHz, the expected form of ENDOR spectra is not that discussed previously. Instead, the normal resonance condition is changed, so that a pair of peaks is expected, separated by twice the proton frequency and centered at a frequency equal to half the hyperfine coupling constant. The strongly coupled proton at the Rapid signal is therefore expected to show features at 3 and 32 MHz, respectively. Thus, the feature at 31 MHz in Figure 5a is in the expected position for this proton (the slight discrepancy being no doubt related to the anisotropic component of the coupling). The feature predicted for 3 MHz is apparently too weak to be detected. For ^2H ENDOR, the frequency for the free nucleus is 6.514 times smaller than that for ^1H ENDOR, and the magnitude of hyperfine splittings will be smaller by this same factor than the corresponding values from ^1H ENDOR. The feature in Figure 5b at 5 MHz is therefore the expected one for the strongly coupled ^2H nucleus. Figure 5a also shows evidence (arrow 1) for a proton with a coupling of about 6 MHz, which is not present in the corresponding sample in ^2H_2O (Figure 5b). This is clearly the weakly coupled proton of the Rapid type 1 species, seen in EPR spectra. Closer to the free proton frequency (of about 14.5 MHz) there seem to be additional, but less striking, differences between the sample in ^1H_2O and that in ^2H_2O. However, these are not readily apparent from the spectra as presented in Figure 5. The question of exchangeability of one or more of the weakly coupled protons is taken up again below.

ENDOR spectra (not shown) for the Slow signal showed results analogous to those for the Rapid signal. Thus, a feature at 34 MHz in the sample in ^1H_2O was replaced in ^2H_2O by one at 5 MHz. These features clearly correspond to the strongly coupled proton of the Slow signal. Additionally, features centered on the free proton frequency, corresponding

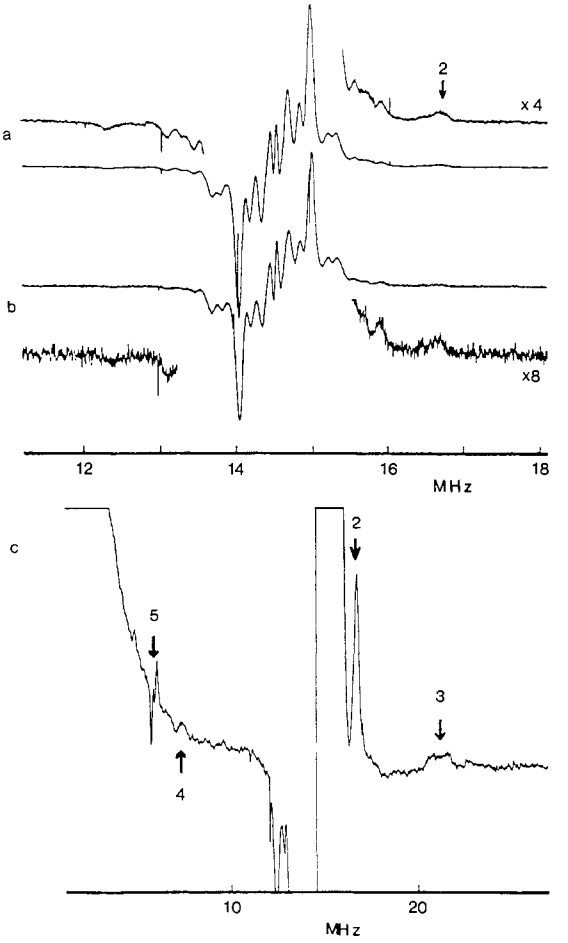


FIGURE 6: ENDOR spectra from the Inhibited EPR signal. In spectra a and c, $^1HC^1HO$ was used as substrate and in spectrum b $^2HC^2HO$. ENDOR was stimulated at the g_2 position. Parts of spectra a and b are shown replotted with the amplication increased by the factors indicated. In spectrum c, the small feature, centered at about 5.9 MHz (arrow 5), is due to ^{31}P ENDOR; the peak marked by arrow 2 in spectrum c corresponds to the one similarly marked in spectrum a. (For the meaning of the other arrows, see the text.)

to a splitting of about 2.5 MHz, disappeared in the sample in ^2H_2O and no doubt correspond to a weakly coupled exchangeable proton, presumably that seen in the EPR.

ENDOR from Substrate-Derived Protons, Not Exchangeable with Solvent Protons. It is known (Pick et al., 1971) that when the Inhibited signal giving species [the product of a side reaction during turnover by the enzyme of several aldehyde substrates; see Morpeth and Bray (1984)] is generated by using formaldehyde, then the EPR spectrum shows coupling from a single proton, having (Tanner et al., 1978) A_{av} = 13 MHz. This proton is not exchangeable with ^2H_2O. That it is derived from the formaldehyde molecule is shown (Pick et al., 1971) by the disappearance of the splitting when $^2HC^2HO$ replaces the $^1HC^1HO$.

The ENDOR spectrum, stimulated in the g_2 position, for the Inhibited signal generated with normal and deuterium-substituted formaldehyde is illustrated in Figure 6. No difference between the samples is detectable in the central region, for protons showing couplings of up to about 4 MHz (Figure 6a,b). In Figure 6c the spectrum from the sample obtained with unlabeled formaldehyde is shown, over an expanded frequency range, to include features from the EPR-detectable proton (A = 14 MHz; see arrows 3 and 4. These disappeared when $^2HC^2HO$ was used (data not shown). An additional feature in the spectrum of Figure 6c (arrow 5) is considered in a subsequent section.

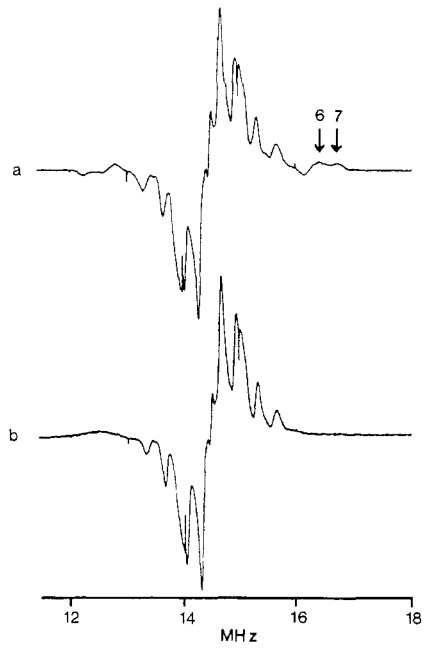


FIGURE 7: ENDOR spectra from the Desulfo Inhibited EPR signal. In spectrum a [$^1H_4$]ethylene glycol was used and in spectrum b [$^2H_4$]ethylene glycol. ENDOR was stimulated at the g_1 position. (For the meaning of the arrows, see the text.)

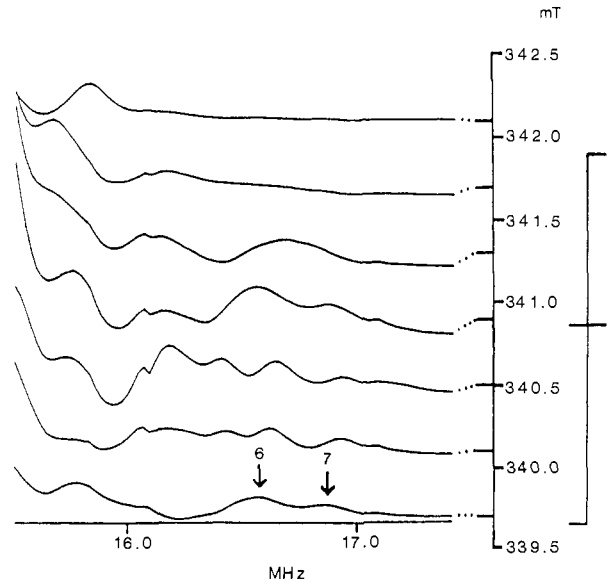


FIGURE 8: ENDOR spectra from the Desulfo Inhibited signal, recorded at different field settings. Only the high-frequency part of the spectrum is shown; [$^1H_4$]ethylene glycol was used to generate the signal. The field values are indicated by markers on the vertical scale, and the stick diagram corresponds to the principal **g** values of the Desulfo Inhibited species. (For the meaning of the arrows, see the text.)

We also used a deuterium-substituted substrate in investigating the structure of the Desulfo Inhibited signal giving species. This species is obtained (Lowe et al., 1976) by treatment with ethylene glycol of the reduced desulfo modification of xanthine oxidase (in which the sulfido ligand of the functional enzyme has been replaced by an oxo ligand). Figure 7 shows ENDOR spectra generated with (a) ordinary glycol and (b) deuterium-substituted glycol. Features (arrows 6 and 7) corresponding to A of about 4 MHz are seen only when ordinary glycol is used and are thus derived from the protons of the ethylene glycol residue. [Indeed, their detection in the ENDOR spectra was foreshadowed by the observation (George, 1985) of proton spin-flip transitions in the EPR spectrum of Desulfo Inhibited species, when recorded at high microwave powers.]

As for all the spectra so far presented, iron–sulfur centers were reduced in the samples of Figure 7. The Desulfo Inhibited species is a very stable one, so that without loss of the signal samples showing it may be gel filtered, thus removing excess dithionite and glycol and permitting the iron–sulfur centers to become reoxidized. We therefore also recorded ENDOR spectra for samples corresponding to those in Figure 7, but with the iron–sulfur centers oxidized. This allowed us to check further the conclusions reached above that, under the recording conditions used, iron–sulfur ENDOR was not interfering with molybdenum ENDOR. In confirmation that this is indeed the case, the spectra so obtained (data not shown) were essentially indistinguishable from spectra a and b of Figure 7, respectively. In subsequent work on the Desulfo Inhibited species we employed gel-filtered samples, in which the iron–sulfur centers are oxidized.

Figure 8 shows the high-frequency part only of the ENDOR spectrum from Desulfo Inhibited generated with ordinary glycol, recorded at a series of different field values corresponding to different points in the EPR spectrum. The arrows (numbered 6 and 7) indicate features corresponding to those from the glycol residue, as marked in Figure 7. Clearly, there are complex movements of the peaks as the exciting point in the EPR spectrum is shifted.

ENDOR from Weakly Coupled Protons. As already stated, all the four xanthine oxidase species we have studied show features from a number of weakly coupled protons, giving splittings up to about 3 MHz. As noted from Figure 5, changes in this spectral region on changing the solvent to ^2H_2O are relatively small. To obtain more information on this point, we recorded the ENDOR spectrum of the Desulfo Inhibited species, generated with ordinary glycol, in ^1H_2O and in ^2H_2O (Figure 9a,b). There are small but reproducible differences between the two spectra whose origin we are still investigating. However, in confirmation of the presence of a weakly coupled exchangeable proton, the sample in ^2H_2O shows clear evidence (Figure 9c) for ^2H ENDOR centered at 2.2 MHz and corresponding to a coupling of about 0.3 MHz. Nevertheless, from these data, from Figure 5, and from additional data (not shown) on the other signal-giving species, it is clear that the majority of the weakly coupled protons are not exchangeable with the solvent.

Additional Features in the ENDOR Spectra. The features indicated by arrow 5 in the spectrum of the Desulfo Inhibited species (Figure 9c), and similarly in that of the Inhibited species (Figure 6c), call for comment. They are centered at 5.9 MHz and are due to ^{31}P ENDOR, the first example of this from a metalloenzyme; details will be presented elsewhere (B. D. Howes, B. Bennett, D. J. Lowe, and R. C. Bray, unpublished work; Pinhal et al., 1989; Bray et al., 1990).

The absence in the spectrum of Figure 9c, from the Desulfo Inhibited species in ^2H_2O, of any feature at 4.9–5.0 MHz is taken up under Discussion.

At no point in our work have we observed resonances that could be attributed to ^{14}N ENDOR. Since resolved hyperfine coupling to ^{14}N nuclei is not seen in the EPR, only relatively weakly coupled nitrogens could be present. These would give pairs of lines, either centered on 1.0 MHz and separated by the hyperfine coupling or at higher frequencies (to, say, 10 MHz) and separated by 2.0 MHz. Both the expected splitting and the line shape might be modified by nuclear quadrupole coupling. As is indicated, e.g., in Figures 5a and 9c, no such features were apparent in any of our samples. In the case of the Desulfo Inhibited species we also looked specifically,

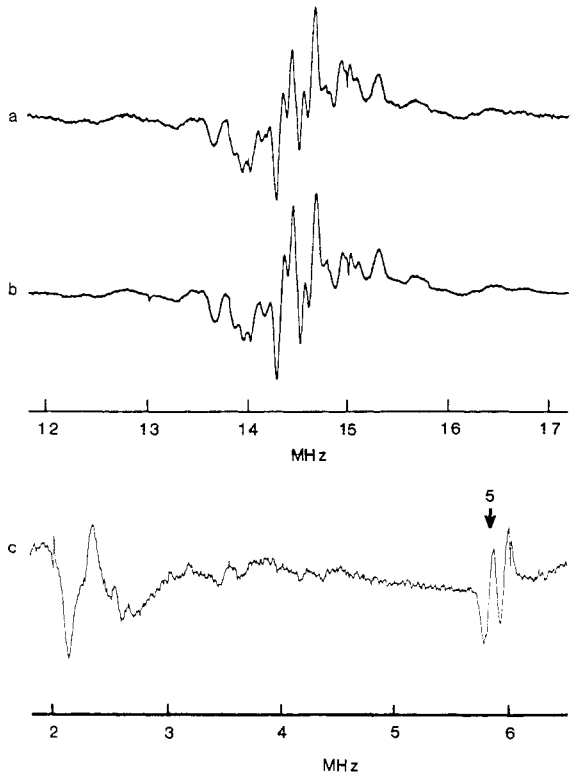
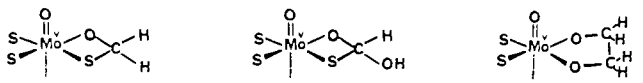


FIGURE 9: ENDOR from the Desulfo Inhibited signal in (a) ^1H_2O and in (b and c) ^2H_2O. ENDOR was stimulated at the g_1 position. A modulation depth of 20 kHz was used in spectra a and b and of 50 kHz in spectrum c. In spectrum c, the spectrum shown has been corrected by subtracting both a cubic curve and a background signal. The latter, which was obtained by stimulating 20 mT away from the features of the EPR spectrum, showed a number of relatively weak features (including some in the region of 5 MHz).

Chart I. Proposed Structures of Different Species from Xanthine Oxidase[a]



a b c

[a] (a and b) Alternative structures for the Inhibited species, with formaldehyde as the inhibiting aldehyde; the present data indicate structure b to be the correct structure. (c) Desulfo Inhibited species, in which an ethylene glycol residue is bound.

without success, for ^{14}N ENDOR over a range of different temperatures (10–40 K; data not shown).

Discussion

Structural Information on the Inhibited and Desulfo Inhibited Signal Giving Species. That the ENDOR spectra (Figure 6) for the Inhibited species, obtained by using normal and deuterium-substituted formaldehyde, are essentially identical with one another permits us to draw an important conclusion about the structure of the signal-giving species. Alternative structures are shown in Chart Ia,b. The basic structure (Bray, 1988) is deduced from the observation by EPR of hyperfine coupling, to ^{13}C of the inactivating formaldehyde or methanol molecule (Tanner et al., 1978), to ^{17}O originating from water (Bray & Gutteridge, 1982), and to ^{33}S originating from the sulfido group (Malthouse et al., 1981). Also, recent EXAFS studies have led to the conclusion (Turner et al., 1989) that, contrary to earlier suggestions, the oxo and not the sulfido ligand of molybdenum in the oxidized enzyme survives in the Inhibited signal giving species.

The ENDOR data now permit us to distinguish between the two structures. In Chart Ia the formaldehyde molecule is shown incorporated into the signal-giving species without having been oxidized. In contrast, in Chart Ib it has been oxidized to the formic acid level. Thus, with the formaldehyde unoxidized, there are two protons on the aldehydic carbon, as shown in Chart Ia. With the structure as drawn, these two protons should be equivalent and therefore equally coupled to the molybdenum; i.e., two equivalent protons should be detected in the EPR spectrum of the formaldehyde Inhibited species rather than the single proton that is actually seen (Pick et al., 1971; Tanner et al., 1978). Enzyme active sites are generally highly distorted, and it is therefore not difficult to imagine the structure of Chart Ia being distorted to the extent that only one of the protons is EPR detectable. Thus a second, weakly coupled proton could escape detection in the EPR spectrum. However, it seems inconceivable that the second formaldehyde proton, if present, would remain undetected by ENDOR. The virtual identity of spectra a and b of Figure 6 leads us to conclude that such a second, weakly coupled, substrate proton is not present in the signal-giving species. Therefore, the structure is not that of Chart Ia but presumably that of Chart Ib, with the aldehyde molecule oxidized to the level of the carboxylic acid.

In contrast to the above result, Morpeth and Bray (1984) concluded that the aldehyde residue of the Inhibited species remained unoxidized. They based this on studies of reactivation of the enzyme following inhibitory treatment with aldehydes. They found reactivation to occur spontaneously but relatively slowly on removing excess aldehyde by gel filtration. When, following gel filtration, the reactivation process was allowed to take place anaerobically, then the enzyme became partly reduced. They concluded that this was due to reduction by the bound substrate molecule following its dissociation from the enzyme. We now have to assume that in the earlier work reduction was brought about not by the substrate molecule bound in the active site but by aldehyde molecules incompletely separated, despite the precautions taken, during the gel filtration process.

Our ENDOR data also provide information on the structure of the Desulfo Inhibited species. The accepted structure [cf. Bray (1988)] for this, based on EPR data, is shown in Chart Ic. Studies of this species, generated in ^{17}O-enriched water, indicated (Bray & Gutteridge, 1982) weak coupling to two oxygen ligands. Weak coupling to ^{13}C was also detected (George, 1983) when enriched ethylene glycol was used to generate the signal. The Desulfo Inhibited species is, uniquely among molybdenum(V) xanthine oxidase species, resistant (Lowe et al., 1976) both to oxidation and to reduction of the metal ion. Some analogy, and indeed some support for the structure of Chart Ic, may be drawn from comparison with a series of molybdenum(V) complexes of the ligand hydrotris(3,5-dimethyl-1-pyrazolyl)borate, studied by Cleland et al. (1987). These workers found that of 19 complexes, having different ligands in the two available coordination sites, that with ethylene glycol bound as in Chart Ic had a much lower redox potential for reduction to the molybdenum(IV) state than had any of the other complexes. This is consistent with the resistance to the reduction noted for the enzyme species.

Detailed interpretation of our ENDOR data on the Desulfo Inhibited species requires further work. However, results seem basically consistent with the structure of Chart Ic. One conclusion immediately derivable from the data is that the four protons from the glycol are not all exactly equivalent in the Desulfo Inhibited signal giving species. That two of these protons are nonequivalent follows from the presence of the two arrowed peaks in Figure 7. That all four may be nonequivalent

is suggested by the further weak features that are present to still higher frequencies than those arrowed, in the bottom trace of Figure 8 (for which the field was set at g_1).

Further Information from ENDOR Data. As we were completing our work, the publication of Edmondson and D'Ardenne (1989) came to our attention. This describes proton ENDOR studies of the xanthine oxidase Desulfo Inhibited species, using both normal and deuterium-substituted glycol. Our spectra are generally similar to those of these workers but are recorded with much better signal-to-noise ratios. They concluded their data were consistent with the structure of Chart Ic, in agreement with earlier work from this laboratory (Bray & Gutteridge, 1982; Bray, 1988), a point that we have now confirmed. Only a single feature from the substrate protons was distinguished by Edmondson and D'Ardenne (1989), which led them to conclude that the four protons are equivalent. However, the better quality of the spectra in our work permitted us, as noted above, to show that this conclusion is in error.

Edmondson and D'Ardenne (1989) noted the absence of ^{14}N ENDOR, and this has been confirmed in the present work, on the basis of more extensive data. This finding presumably indicates the absence of a nitrogen ligand of molybdenum in the enzyme. However, the presence of such a ligand cannot definitely be excluded since ^{14}N ENDOR lines are often difficult to detect (Schweiger, 1982). (In this context our failure to detect ^{14}N ENDOR from a formamide molecule, loosely bound in the Rapid signal giving species, makes it clear that such a molecule is unlikely to be coordinated through nitrogen to molybdenum and suggests it may not be coordinated to the metal.)

On two further points, our data or interpretations, or both, are in serious disagreement with those of Edmondson and D'Ardenne (1989). First, we were not able (cf. Figure 9c) to detect the resonance at 4.9–5.0 MHz observed by Edmondson and D'Ardenne (1989) for a sample of the Desulfo Inhibited species in ^2H_2O. The magnitude of this discrepancy is emphasized by our observation (Figure 9c) of ^{31}P ENDOR at the nearby expected frequency of 5.9 MHz, whereas in the spectra of these workers such signals were not detectable above the noise level. The origin of the signal at 4.9–5.0 MHz in the work of Edmondson and D'Ardenne (1989) must remain uncertain. It was interpreted as the $\Delta M_I = \pm 2$ overtone transition (Tycko & Opella, 1987) at a deuterium nucleus, analogous to $\Delta M_s = \pm 2$ transitions in triplet-state EPR. Such transitions are normally seen only either when there is strong quadrupole coupling at the $I = 1$ nucleus, for which there is no evidence in the present case, or [cf. Bodenhausen (1981)] when the radio-frequency field is sufficiently strong to cause admixture of the nuclear states. The equipment used by Edmondson and D'Ardenne (1989) seems to have been similar to ours, and it is not clear therefore that the much greater rf power needed to account for the discrepancy could have been delivered to their samples and not to ours. In any case we note that, for a $\Delta M_I = \pm 2$ overtone transition, the expected resonance frequency is $2\nu_D \pm A$ and not as stated by these workers.

A second major point of disagreement between our work and that of Edmondson and D'Ardenne (1989) concerns interpretation of the central region of the ENDOR spectrum, due to weakly coupled nonexchangeable protons in the Desulfo Inhibited species [and by analogy in the other Mo(V) species from the enzyme also, though these were not studied by these workers]. Our conclusion, as already noted, is that there are probably at least nine sets of coupled protons. It follows that the nonexchangeable ones are covalently bound or not accessible to the solvent and either on the pterin molybdenum cofactor or on amino acid residues in the protein. We do not consider at the present time that further conclusions can be drawn about these protons, nor can we assign them in any way. In contrast, Edmondson and D'Ardenne (1989) interpreted spectra, basically similar to ours, in an entirely different manner, assigning all the main features in the central region from about 13.7 to 15.3 MHz to a single pair of equivalent protons. According to our interpretation, four to five protons or sets of protons contribute in this region. These workers went on to conclude that the presumed pair were "α-protons", attributed to protons on the C(1′) and C(2′) positions of the side chain of the pterin cofactor, meaning that this side chain is saturated, contrary to the proposals of Kramer et al. (1987).

Recent work on analysis of ENDOR line shapes is of particular importance in clarifying this matter. For organic radicals, Babcock and co-workers (O'Malley & Babcock, 1986; Sandusky et al., 1989; Bender et al., 1989) deduced information about all the principal values of the hyperfine tensor from measurements at a single point in the EPR spectrum. This approach appears satisfactory for the systems studied but is of very limited applicability ouside the organic free-radical field, since it relies on the **g** tensor being isotropic or no more than very slightly anisotropic. For systems with more substantial **g** value anisotropy, more recent workers [e.g., True et al. (1988), Hoffman and Gurbiel (1989), Hurst et al. (1985), and Baker and Raynor (1988)] used procedures of wide applicability that rely on the more stringent conditions available in peak assignment if one observes the variation in position, intensity, and number of features of a given ENDOR peak as the exciting field is varied across the whole EPR spectrum, rather than, as was previously the case, relying on analysis only at fields corresponding to turning points in the EPR spectrum. As is clear particularly from the work of Hoffman et al. (1985), for a rhombic **g** tensor, a single weakly coupled proton (or a set of equivalent protons) having a rhombic **A** tensor would, at field settings corresponding to g_1 and g_3, give only one pair of peaks in the ENDOR spectrum near the free proton frequency, while up to six pairs of peaks might be observed at other field settings.

From spectra a and c of Figure 4, recorded for the Desulfo Inhibited species at g_1 and g_3, respectively, it is abundantly clear, following this reasoning, that in the region of 13.7–15.3 MHz there are considerably more than a single pair of peaks present and hence that more than one set of equivalent protons is contributing to the spectrum. The same conclusion can also be made from the data of Edmondson and D'Ardenne (1989). Though these workers do not cite references, their contrary attribution of these spectral features to a single pair of protons seems to depend on reasoning analogous to that of Babcock and co-workers, which however becomes quite invalid when applied to a spectrum with **g** value anisotropy of the present magnitude. Edmondson and D'Ardenne (1989) also seem, though again no references are cited, to make unjustified extrapolations in their assignments from work [cf. Kevan and Kispert (1976)] on organic radicals. Thus, we can see no justification from the data presented for the conclusion, based on line shapes, that protons contributing in the central region are α-protons or for the accompanying statement that methylene protons "would be expected to exhibit a weaker intensity". Thus there is no evidence for the structural conclusion of Edmondson and D'Ardenne (1989) that the side chain of the pterin cofactor is saturated.

In metalloenzymes whose detailed structures are not known, the single most useful piece of information that can in principle

be derived from ENDOR spectra is probably the distance between the central metal ion and the coupled proton or other nucleus. As is indicated by references already cited, procedures for extraction of hyperfine coupling parameters via spectral simulations are not as yet highly developed. Extracting the dipolar component of the hyperfine coupling is necessary for evaluating distances. The anisotropic component of the coupling can in principle be evaluated by the procedure already mentioned, the recording of a series of ENDOR spectra at different magnetic field positions, spanning the width of the EPR spectrum. As the field position is varied so the hyperfine splitting, measured between the outer ENDOR features corresponding to a particular coupled proton or other nucleus, changes its value. From measurements of the magnitude of the splittings and of the extent of their variation with magnetic field, the anisotropic component may be determined. This in turn, except in cases of very large **g** value anisotropy, is dominated by the dipolar contribution, and the metal–proton distance is then given simply by a constant divided by the cube root of the maximum anisotropic coupling. Such procedures were recently used in studies of a molybdenum complex by Hughes et al. (1990) and also, for example, by Mustafi and Makinen (1988).

For the Desulfo Inhibited species, detailed interpretations of this type, of data such as those in Figure 8, via comparison with corresponding spectra (not shown) obtained with deuterium-substituted glycol, are complicated by the inequivalence of the protons derived from the glycol, and we have not yet completed our evaluation. However, preliminary distance estimates seem consistent with the structure of Chart Ic but with some distortion.

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