Electron–Nuclear Double Resonance Spectroscopy of the Desulfo-Inhibited Molybdenum(V) Center in Bovine Milk Xanthine Oxidase†

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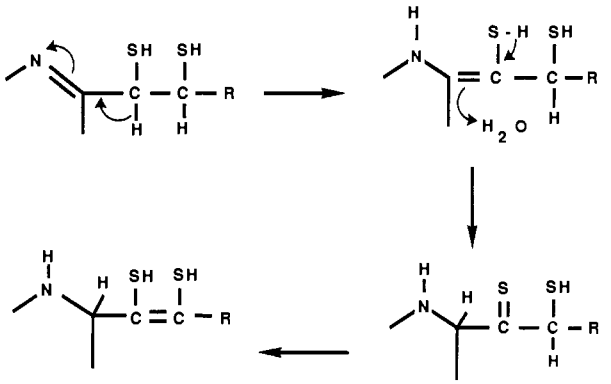
ABSTRACT: The "desulfo-inhibited" Mo(V) center of bovine milk xanthine oxidase has been investigated by electron–nuclear double resonance spectroscopy. Comparison of spectral data obtained from samples prepared with [$^1H_4$]ethylene glycol and with [$^2H_4$]ethylene glycol allowed assignment of proton resonance lines due to the methylene protons of the coordinated ethylene glycol (A_H = 3.6 MHz). Deuterium resonance lines were observed with the deuterated sample (A_D = 0.4 MHz). No spectral evidence was obtained for any weakly coupled nitrogen nuclei to the Mo center under a variety of conditions. Dissolution of the sample in D_2O had little effect on the resonance lines centered about the proton Zeeman frequency, which shows they are not due to exchangeable protons and suggests the Mo center does not have contact with bulk solvent. A deuterium $\Delta m = \pm 2$ "forbidden" transition is observed at high radio-frequency power levels, which suggests either an exchangeable proton on a Mo ligand or a coordinated solvent. Weakly coupled, nonexchangeable proton lines are observed about the free proton frequency, which exhibit properties characteristic of α-protons. A number of arguments are presented to support the proposal that these protons originate from the C(1′) and C(2′) positions on the side chain of the molybdopterin cofactor.

It is now generally accepted that the molybdenum center of xanthine oxidase functions catalytically as the initial electron acceptor in the oxidation of purines. Although the mechanism for this process is yet to be defined, Mo^{6+} is reduced to Mo^{4+} during the initial redox step in catalysis, and the reduced Mo is subsequently oxidized by the Fe/S and FAD centers in the enzyme. Much of our knowledge regarding the ligation of the Mo center has come from EXAFS[1] data (Bordas et al., 1980; Cramer et al., 1981; Cramer & Hille, 1985) and from ESR spectral data on the various Mo(V) spectral species that have been observed [cf. Bray (1980) for a review and Bray and Gutteridge (1982) and George and Bray (1988) for more recent data]. The ligands to the Mo are thought to be two thiol groups originating from the molybdenum cofactor (MoCo) (Johnson & Rajagopalan, 1982), one terminal oxo group, and, in the functional form of the enzyme, a terminal sulfur ligand (Malthouse & Bray, 1980). Removal of this terminal sulfur ligand by cyanide treatment (Massey & Edmondson, 1970) (to form the nonfunctional desulfo Mo center) results in its replacement with a terminal oxo ligand (Bordas et al., 1980) with the oxygen being supplied by solvent. A current proposal for the structure of the coordinated Mo center in molybdenum hydroxylases as suggested by Kramer et al. (1987) is shown in Figure 1. This structure is based on the structural studies of the isolated cofactor after alkylation with iodoacetamide and represents a minimum coordination sphere for the Mo center.

This proposed structure of Mo ligation suggests no direct bonding of the metal to any amino acid side chains of the protein. Evidence supporting this suggestion comes from experiments where the MoCo can be removed from enzymes such as xanthine oxidase and can reconstitute nitrate reductase activity when incubated with the apoenzyme (Kramer et al., 1984; Hawkes & Bray, 1984a). Whether the same coordination structure of MoCo is present in all of the molybdenum hydroxylases is not known at present.

Scheme I: Proposed Redox Tautomerization of Pterins That Could Give Rise to an Unsaturated Side Chain on Resolution and Isolation of the Molybdenum Cofactor from Its Binding Site on the Protein [Taken from Curtius et al. (1985)]



A number of uncertainties remain, however, in our understanding of the nature of Mo ligation to the cofactor. As pointed out by Johnson et al. (1984), the N(5) of the pterin ring could serve as a point of Mo ligation since EXAFS measurements cannot readily distinguish between oxygen and nitrogen. In addition, the 1′,2′-enethiol functionality of the cofactor moiety is assigned on the basis of NMR studies of the isolated metal-free organic derivative and on the basis of known stable model complexes of Mo with 1,2-dithiones (Kusters & de Mayo, 1974). The known redox tautomerization of pterins (Curtius et al., 1985) can occur between the

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[1] Abbreviations: EXAFS, extended X-ray absorption fine structure; ESR, electron spin resonance; ENDOR, electron–nuclear double resonance; ELDOR, electron–electron double resonance; MoCo, molybdenum cofactor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris tris(hydroxymethyl)aminomethane.

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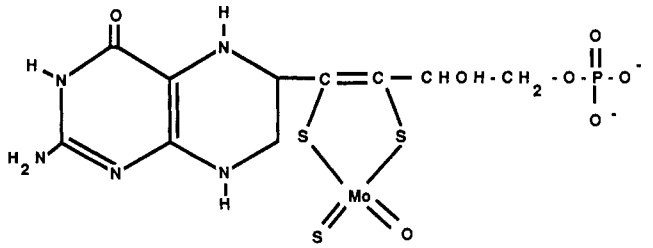


FIGURE 1: Structure of molybdenum cofactor (MoCo) and coordination of Mo in xanthine oxidase as suggested by Kramer et al. (1987).

6-position of the ring and the C-1′ position (see Scheme I) and lead to this type of structure in the isolated cofactor under conditions required for its resolution from the protein and for its purification.

In view of these possible alterations in Mo–cofactor interactions and/or structure that may occur on its release from the enzyme, it seemed worthwhile to apply approaches that have not heretofore been used in structural studies of the Mo environment in the enzyme. ENDOR spectroscopy seemed to be an attractive complement to the extensive ESR data that has been accumulated by Bray and co-workers and has inherent advantages in that higher resolution can be achieved and interpretation of spectral data is less reliant on simulations. This double resonance technique approach has been used increasingly in biological systems (Möbius & Lubitz, 1987) but, to our knowledge, has not been methodically applied to the molybdenum hydroxylases. (Preliminary work on the ENDOR of the Mo and Fe/S centers of xanthine oxidase was presented by Ehrenberg and Bray at the 3rd International Flavin Symposium in 1972 but, to our knowledge, was never published.)

In this study, we report the results of ENDOR spectral studies of the Mo(V) species of xanthine oxidase originally designated "desulfo resting 2" but later renamed "desulfo inhibited" (Bray, 1980). The preparation of this form was described initially by Lowe et al. (1976). The advantages of performing initial ENDOR studies on this Mo(V) species are the following: (1) it can be prepared in high yields (~50% of the total enzyme-bound Mo); (2) since it is stable to oxidation and reduction, the sample can be treated aerobically to reoxidize the Fe/S and flavin centers, thus leaving Mo(V) as the only ESR-detectable species present; (3) this situation allows for ENDOR measurements to be performed over a temperature range down to liquid helium temperatures without complications arising from interference from the intense ESR signals of the two Fe_2/S_2 centers and the known spin-coupling that can occur between these centers and the Mo center (Lowe et al., 1972; Barber et al., 1982; Lowe & Hyde, 1975; Lowe & Bray, 1978). The ability to perform ENDOR measurements at very low temperatures is important since the nuclear spin relaxation properties of certain nuclei (e.g., ^{14}N) may be such that their ENDOR resonances may not be observable at higher temperatures (120 K) usually used for the detection of Mo(V) ESR signals.

Experimental Procedures

Materials and Methods. Xanthine oxidase was isolated form unpasteurized buttermilk as described by Massey et al. (1969). Fresh raw cream was obtained form the University of Georgia dairy and churned to separate the buttermilk fraction from the butter. Desulfoxanthine oxidase was prepared as described by Massey and Edmondson (1970) and the desulfo-inhibited Mo(V) form of the enzyme prepared as described by Lowe et al. (1976). After removal of excess

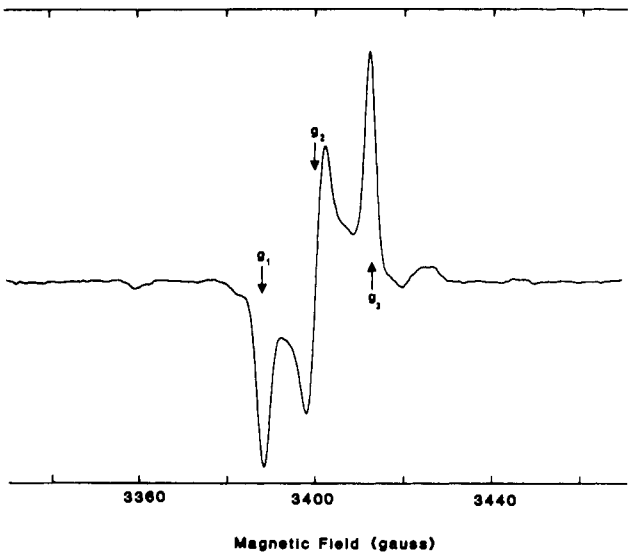


FIGURE 2: ESR spectrum of the desulfo-inhibited Mo(V) species of milk xanthine oxidase. The spectrum was recorded at 77 K. The arrows denote magnetic field positions used a recording the ENDOR spectra.

reagents by either dialysis or ultrafiltration, enzyme samples were concentrated to 0.5–1 mM with a Savant Speed-Vac.

[$^1H_4$]Ethylene glycol was purchased from Sigma, and [$^2H_4$]ethylene glycol (98% isotopic enrichment) was obtained from MSD Isotopes. ^2H_2O (99.8%) was obtained from Aldrich.

X-band ESR spectra were measured with an IBM/Bruker ER 220 spectrometer equipped with Oxford Instruments cryogenic equipment. ENDOR spectra were obtained with the same instrument using a Bruker ER 150 LPE ENDOR cavity and Air Products cryogenic equipment. A 200-W EMI RF amplifier supplied power to the 10-turn Dewar-mounted RF coil in the cavity which terminates in a 50-Ω load giving an unattenuated RF field with a nominal value of ~10 G. Frequency-modulated (FM) ENDOR spectra were obtained under computer control with an Aspect 2000 system using software supplied by the manufacturer. Spectra were obtained at 12.5-Hz field modulation at a microwave frequency of 9.26 GHz. In the 14-MHz frequency range, the modulation depth was set at 50 kHz, and in the 1–10-MHz range the modulation depth was 100 kHz. RF and microwave power levels were adjusted with each sample to give the optimum ENDOR signal.

Data Analysis. ENDOR spectra were further processed by transferring the data files (usually a 1K data set) to an IBM-XT computer with the KERMIT data transfer program. The files were then converted to ASYSTANT files for processing by ASYSTANT software (MacMillan and Co). This program allowed facile spectral manipulations such as first-derivative presentation for the estimation of proton coupling constants from partially overlapping ENDOR lines.

Results

The ESR spectrum of the desulfo-inhibited Mo(V) species is shown in Figure 2. In agreement with previous results (Lowe et al., 1976) no proton coupling is observed when the sample is dissolved in D_2O or when the sample was prepared with [$^2H_4$]ethylene glycol. Double integration of each spectrum corresponding to each sample used in this study showed that ~50% of the Mo in the enzyme was present as Mo(V). As pointed out by Bray (1980), this rhombic signal displays the lowest g tensor anisotropy of any of the known Mo(V)

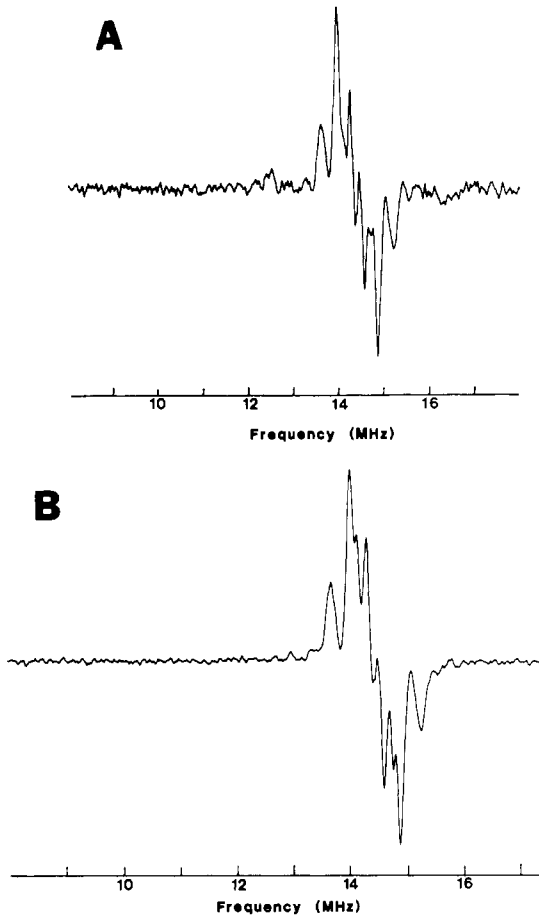


FIGURE 3: ENDOR spectrum of desulfo-inhibited Mo(V) species of xanthine oxidase prepared with (A) [$^1H_4$]ethylene glycol or (B) [$^2H_4$]ethylene glycol. Spectra were obtained under the following conditions: microwave frequency, 9.25 GHz; field modulation, 12.5 kHz; modulation depth, 50 kHz; microwave power, 0.6 mW; RF power, −5 dB below 200 W; scan time, 120 s; time constant, 500 ms; temperature, 15 K. The field setting was at g_2 (see Figure 2). The following coupling constants (A_H) were determined from analysis of the first-derivative presentation of each spectrum: ([$^1H_4$]ethylene glycol) 3.6, 2.1, 1.4, 0.8, 0.5, and 0.2 MHz; ([$^2H_4$]ethylene glycol) 2.0, 1.5, 0.8, 0.6, and 0.2 MHz.

signals observed for this enzyme. The arrows in Figure 2 designate the magnetic field positions used in recording the ENDOR spectra. ESR spectral measurement at 10 K were performed on each sample as a check to be sure that no Fe/S centers were reduced and that Mo(V) was the only ESR-active species present.

The ENDOR spectrum of the [^{1}H]ethylene glycol treated enzyme dissolved in H_2O in the 10–18-MHz region is shown in Figure 3A. A group of highly resolved weakly coupled lines are observed symmetric about the free proton Zeeman frequency (14.4 MHz) and are therefore assigned to be due to protons weakly coupled to the Mo(V) center. The first-order resonance condition for proton signals is

$$\nu_i = |\nu_n \pm A_i/2|$$

where ν_n is the nuclear Zeeman frequency of the free nucleus, A_i is the hyperfine coupling in MHz, and ν_i is the observed signal in the ENDOR spectrum. Analysis of the spectrum in Figure 3A by measurement of ν_+ and ν_- pairs symmetrically placed about the free proton frequency gives four proton resonance lines with the following hyperfine coupling constants: 1.4, 0.8, 0.5, and 0.2 MHz. The accuracy of these measured coupling constants is estimated to be ±0.1 MHz. A pair of lines with weak intensities is also observed with an A_H value of ~2.1 MHz. In addition, a pair of lines with weak intensities

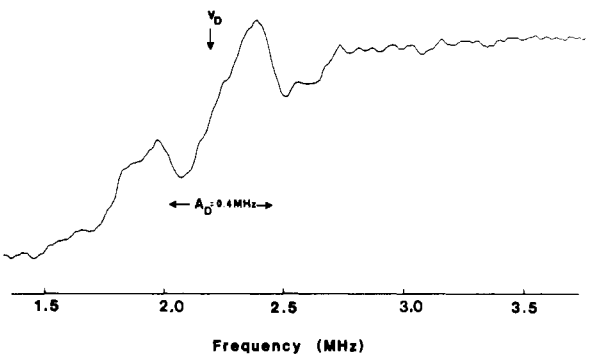


FIGURE 4: Low-frequency ENDOR spectrum of the Mo(V) signal of [$^2H_4$]ethylene glycol inhibited xanthine oxidase. The same spectral conditions as in Figure 3 were employed with the following changes: sweep time, 60 s; modulation depth, 100 kHz; RF power, −3 dB below 200 W. No signal was observed in this region with the sample prepared with [$^1H_4$]ethylene glycol.

is also observed at 12.6 and at 16.2 MHz (A_H = 3.6 MHz) and, as will be shown below, is assigned to the methylene protons of ethylene glycol. Methylene protons are known to exhibit ENDOR lines of weak intensity in powder spectra (Eriksson et al., 1970), and these data would suggest the $-CH_2-$ functionalities remain intact in the ethylene glycol moiety and that it is bound directly to the Mo. This type of binding of ethylene glycol to the Mo center was proposed previously by Bray and Gutteridge (1982), and as will be shown here, the ENDOR data are in support of that proposal.

Proof that the signals with a 3.6-MHz coupling are due to the methylene protons of ethylene glycol is shown in Figure 3B. The ENDOR spectrum of a sample prepared with [$^2H_2$]ethylene glycol no longer exhibits the weak intensity signals with a 3.6-MHz coupling. The ENDOR lines from the other weakly coupled protons are still present and exhibit the same coupling constants, which demonstrates they arise from protons on the cofactor, from amino acid residues on the protein, or from solvent.

Since the ethylene glycol protons exhibited a reasonable coupling (3.6 MHz) to the Mo center, it was of interest to determine if the ^{2}H resonance lines of deuterated ethylene glycol could be observed in the low-frequency region of the ENDOR spectrum. The magnetic moment of a ^{2}H nucleus is 0.15 times that of a proton, and thus, a deuterium coupling of ~0.5 MHz should be observed centered about the free nuclear frequency of ^{2}H (2.2 MHz) since $A_i/2 < \nu_D$. ENDOR lines due to deuterium might be expected to be further split by the quadropolar interaction of the $I = 1$ deuterium nucleus. Depending on the anisotropy of the hyperfine A tensors, this coupling may result in broad, unresolvable ENDOR lines. The spectrum in Figure 4 shows the expected deuterium ENDOR lines centered about ν_D with a separation approximating that expected from the measured proton couplings (Figure 3). Proof that these lines are in fact due to deuterium is their absence in the sample prepared with [$^1H_4$]ethylene glycol. Control experiments showed no signals in this spectral region when the magnetic field was set outside of the ESR absorption envelope. Some splitting of the ν_- and ν_+ components is observable in the spectrum, which could result either from quadrupolar splitting or from anisotropy of the hyperfine coupling. Lack of resolution and the low intensity of the signals precluded any further detailed analysis. No other ENDOR lines were detected which might arise from weakly coupled nitrogen nuclei although a variety of temperature and RF power levels were used. These negative findings for any Mo–nitrogen hyperfine interactions will be discussed in more detail under Discussion.

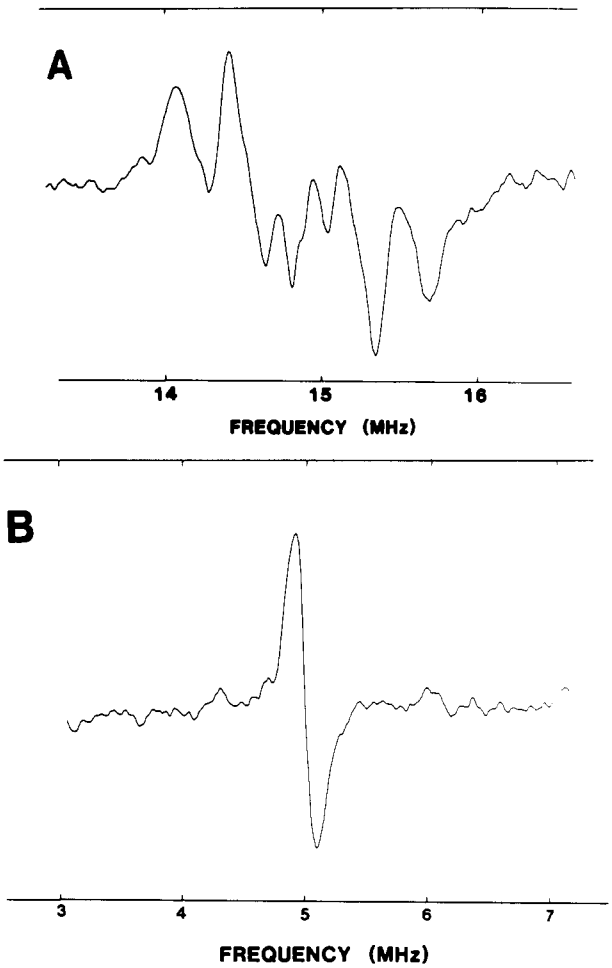


FIGURE 5: (A) ENDOR spectrum of sample prepared with [$^2H_4$]-ethylene glycol in 99.8% D_2O. Twenty scans were averaged under the same spectral conditions as described in Figure 3A. The coupling constants determined for the coupled, nonexchangeable protons are 2.0, 1.5, 0.8, 0.6, and 0.2 MHz. (B) Low-frequency ENDOR spectrum of the same sample with 1-dB attenuation below 200 W of RF power. The same signal at 4.9–5.0 MHz was observed with samples prepared with [$^1H_4$]ethylene glycol in D_2O. No signal in this frequency region was observed with samples dissolved in H_2O.

With the assignment of the ethylene glycol protons, the task remaining was to investigate the origin of the weakly coupled protons apparent about the free proton frequency. Possible origins include solvent protons, cofactor protons, amino acid side chains near the Mo site, or possibly buffer (HEPES) protons. The spectral data in Figure 5A show that dissolving the sample in D_2O has little influence on the ENDOR spectrum about the free proton frequency. Two conclusions can be made from these data: (1) weakly coupled protons are not due to weak interactions of Mo(V) with solvent protons or with exchangeable protons of either the protein or the pterin cofactor, and (2) the small alterations in the matrix ENDOR line show the Mo center to be relatively restricted from contact with bulk solvent. In this respect, it is interesting to note that previous ELDOR experiments on the desulfo "slow" Mo(V) signal of xanthine oxidase (Lowe & Hyde, 1975) also observed the ELDOR line at the free proton frequency was also insensitive to changing the solvent from H_2O to D_2O. These data led Lowe and Hyde to conclude that "few of the protons in the neighborhood of the molybdenum are exchangeable". The ENDOR data reported here are in excellent agreement with the ELDOR data even though different Mo(V) species are under investigation. ^{31}P NMR data on xanthine oxidase (Davis et al., 1984) demonstrated that the resonance assigned to the cofactor phosphate residue was unaffected by the addition of paramagnetic Mn^{2+}, which also shows that this portion of the side chain is shielded from bulk solvent.

When the low-frequency region of the ENDOR spectrum is scanned in an attempt to look for a matrix line at the deuterium Zeeman frequency, an ENDOR signal is observed at 4.9–5.0 MHz but only at high RF power levels (Figure 4B). This signal was not observed with samples of enzyme dissolved in H_2O, and thus, the most likely explanation for its origin is that it is a $\Delta m = \pm 2$ "forbidden" transition of the $I = 1$ deuterium nucleus. In the absence of any hyperfine coupling, this forbidden transition transition should occur at $2\nu_D$ (4.4 MHz).

That this transition occurs at a frequency 0.5–0.6 MHz higher than that expected suggests there is small, but observable hyperfine coupling (0.3 MHz) of the solvent deuterium such that the observed transition occurs at $2(\nu_D + A)$. The corresponding $2(\nu_D - A)$ transition (expected at 3.8 MHz) is not observed (Figure 4B). Differential transition probabilities for the low- and high-frequency ENDOR lines of weakly coupled nuclei (with small gyromagnetic ratios) in transition metal complexes are not uncommon [cf. Schweiger (1982)].

Previous ELDOR studies (Lowe & Hyde, 1975) also observed a double proton spin flip at 28 MHz at high pump power levels for the "slow" defulfo Mo(V) signal. It remains to be determined whether this double proton transition can be observed in ENDOR experiments, and further investigation is required to probe the relation of this unusual transition to the structure to the Mo center.

Previous ENDOR studies of Fe/S centers in ferredoxins (Sands, 1979) have demonstrated that buffer (Tris) protons can exhibit weak coupling to the paramagnetic metal cluster. Since the spectra shown in Figures 3–5 are on samples containing HEPES buffer, the possibility remained that the weakly coupled proton lines observed are from the buffer rather than from the cofactor or from the protein. To test this possibility, ENDOR spectra were measured on a sample prepared with [$^2H_4$]ethylene glycol in D_2O/pyrophosphate buffer. The spectral data in Figure 6 demonstrate that at least three resolved sets of weakly coupled proton resonances are still present and originate from either the cofactor or the protein. Comparison of the spectra in Figure 5A with those in Figures 3 and 4A shows similar couplings although the ENDOR lines are slightly broader for the pyrophosphate-containing samples.

A similar coupling pattern is observed in the ENDOR spectrum when the magnetic field is set on a "turning point" of the ESR spectral envelope, which selects for those molecules with like orientations with the magnetic field (Rist & Hyde, 1970). This observation suggests these sets of proton lines originate from protons which exhibit a reasonable degree of isotropic coupling to the Mo center. One proton line set with a coupling of 0.8 MHz (designated by arrows in Figure 5) does exhibit a greater change in intensity than the other resonance lines with changes in position in the magnetic field. This behavior is characteristic of α-protons, and the structural assignment will be discussed in more detail under Discussion.

ESR evidence for the presence of weakly coupled nonexchangeable protons to the Mo(V) of desulfo-inhibited xanthine oxidase has been published by George (1985). Proton spin flip lines corresponding to $\Delta m = \pm 1$ transitions are observed at high microwave power levels and at low temperatures. Simulation of the ESR spectral data demonstrated the noncollinearity of the g and A tensors. The resolution of these experiments was not sufficient to discriminate the protons of

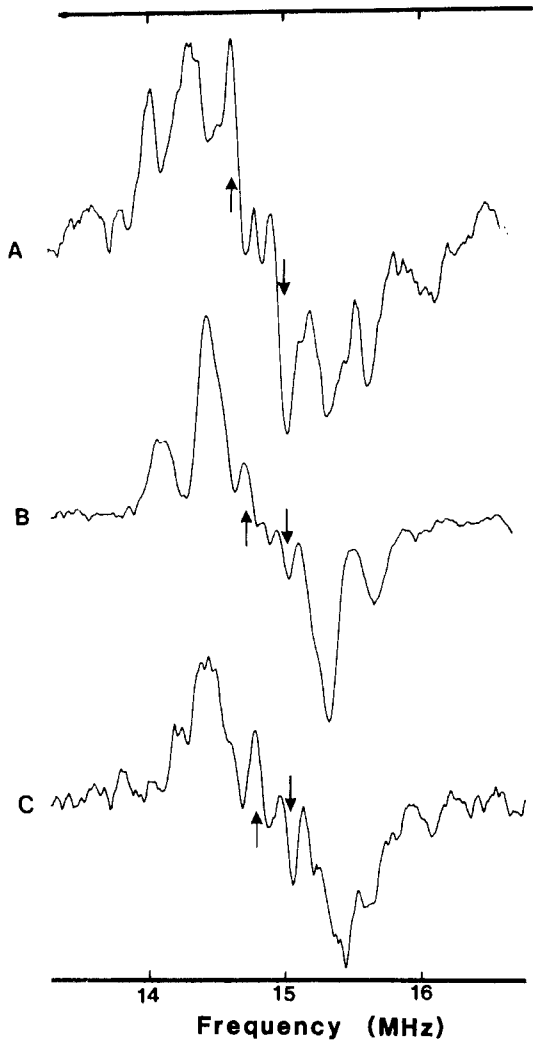


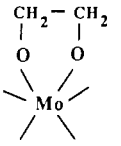
FIGURE 6: ENDOR spectra of [$^2H_4$]ethylene glycol treated desulfoxanthine oxidase in D_2O/pyrophosphate buffer, pH 8.5. The same spectral conditions were employed as in Figure 3A. (A) Field setting at g_1; (B) field setting at g_2; (C) field setting at g_3 (see Figure 2). The arrows denote the ENDOR lines which show the largest dependence of intensity on magnetic field setting.

the coordinated ethylene glycol from those of the cofactor or of the protein.

Discussion

The ENDOR data presented here provide additional insights into the structure of the desulfo-inhibited Mo(V) center and, therefore, are of relevance to our understanding of the structure of the catalytic center in the enzyme. It should again be emphasized that the desulfo-inhibited Mo(V) center was chosen for these initial ENDOR studies in order to optimize the levels of Mo(V) in the enzyme and to preclude any interference from signals arising from coupled nuclei to the Fe/S centers in the enzyme. No evidence was found for any nitrogen coupling to the Mo center, which suggests a ring nitrogen of the pterin cofactor is *not* a site for Mo ligation at least in this enzyme-bound species of Mo(V). This view is further supported by unpublished electron spin-echo modulation spectral data we have measured on xanthine oxidase (in collaboration with Drs. J. Peisach and J. McCracken, Albert Einstein School of Medicine, Bronx, NY) which shows no nitrogen modulation of the spin-echo decay envelope. Since this pulsed EPR technique is quite sensitive for weak nitrogen couplings to paramagnetic metal centers, we feel reasonably confident in excluding this nucleus as a ligand for this Mo(V) species. This conclusion, however, cannot be generalized for the Mo coordination sphere in the resting form of the functional enzyme since the possibility remains that a nitrogen ligand may have been displaced during the manipulations required to form the desulfo-inhibited Mo(V) species.

A second conclusion regarding the structure of the desulfo-inhibited Mo(V) center is that the protons on the ethylene glycol moiety are apparently equivalent, exhibit ENDOR properties characteristic of methylene groups, and exhibit a coupling constant to the Mo center of 3.6 MHz. Due to the weak intensity of the signal and to spectral overlap with other proton signals, we are unable to determine whether this signal is due to $A_\parallel$ or to $A_\perp$. Solution ENDOR studies of a model d^1 system of relevance, chromyl(V) ethylene glycolate anion (Möhl et al., 1982), show an isotropic proton hyperfine coupling of 1.74 MHz with apparent equivalency of the two bis-coordinated ethylene glycol ligands and magnetic equivalency of the methylene protons. This isotropic coupling is in reasonable agreement with the anisotropic coupling constant determined in this study for the ethylene glycol protons (A_H = 3.6 MHz). thus, by analogy, a reasonable structural interpretation of the ENDOR data presented here is that ethylene glycol is bound to the Mo center in the following manner:



This type of cyclic structure was proposed by Bray and Butteridge (1982) for the formaldehyde-inhibited and the desulfo-inhibited Mo(V) signals of xanthine oxidase from ^{17}O solvent exchange experiments. The data presented here are fully supportive of that proposal.

The finding of a forbidden $\Delta m = \pm 2$ deuterium transition when the sample is dissolved in D_2O is of interest. Its origin is probably either from a coordinated solvent molecule or from an exchangeable proton on a ligand to the Mo. The observed hyperfine coupling value (A_D = 0.3 MHz) is consistent with either possibility. Further investigation of this transition may provide some interesting structural insights into the Mo center. We have also observed this transition with the Mo(V) signal obtained on treatment of the functional enzyme with formaldehyde (Pick et al., 1971) when the sample is dissolved in D_2O (D. E. Edmondson, unpublished data). Signal intensity and position exhibited little dependence on the magnetic field, suggesting the interaction to be highly isotropic. Thus, whatever the origin, this behavior appears to be independent of the type of Mo(V) species examined.

The final point of discussion concerns the origin of the weakly coupled protons to the Mo that are nonexchangeable and shown not to be due to buffer protons. If the structure of the molybdenum cofactor as suggested by Kramer et al. (1987) is considered (cf. Figure 1), these protons would have to originate from through-space, dipolar coupling to amino acid residues located in close proximity to the Mo ion within a 5–6-Å distance. While some contribution from dipolar-coupled protons on amino acid side chains undoubtedly exists in the spectra about the free proton frequency, the dominant contribution arises from protons that exhibit ENDOR properties of α-protons. Powder spectra of α-protons exhibit anisotropic hyperfine couplings approximated by $A_{iso}/2$, A_{iso}, and $(3/2)A_{iso}$ (McConnell et al., 1960). The dominant ENDOR lines observed about ν_H show A_H values of 0.5, 0.8, and 1.5 MHz (Figure 3), which suggests they are due to α-protons. The

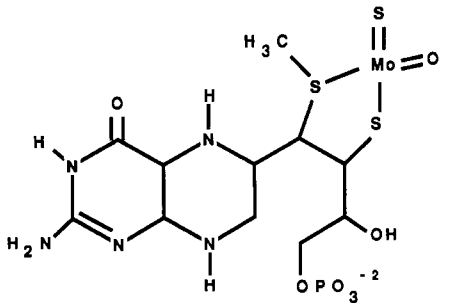


FIGURE 7: Structure of the xanthine oxidase molybdenum cofactor suggested by Fish and Massey (1987). This structure represents a minimum Mo coordination sphere for the Mo center.

intensity differences observed at various field positions (Figure 6), particularly for the 0.8-MHz lines, are also consistent with their assignment as α-protons. Methylene protons such as from a coordinated cysteinyl residue would be expected to exhibit a weaker intensity such as observed with the ethylene glycol protons (Figure 3). Through-space interactions with amino acid protons about the Mo center are unlikely to exhibit these properties. Published ENDOR data on blue copper proteins (Roberts et al., 1984) show relatively broad and unresolved ^{1}H matrix lines, and the resolved ^{1}H ENDOR lines observed for NO derivatives of hemoglobin, its isolated chains, and myoglobin (Höhn et al., 1983) exhibit somewhat different spectral properties than observed here. A reasonable interpretation is to assign these α-protons to the C(1′) and the C(2′) positions on the side chain of the cofactor. This assignment is based on the assumption that the two protons are magnetically equivalent. The low anisotropy observed for the hyperfine coupling is due probably to the low spin density on the 1′- and 2′-carbon atoms (Kwiram, 1968). Final proof for such an assignment awaits isotopic substitution of the cofactor side chain, a task that is not technically feasible at present.

Arguments supporting this assignment of a saturated side chain rather than an unsaturated moiety come from a number of considerations. Dithione metal complexes generally exhibit intense visible absorption spectral bands (Boyde et al., 1986); a property not observed with the Mo center of xanthine oxidase. Preliminary ^{1}H NMR studies of Fish and Massey (1987) suggest the side chain of their preparation of isolated cofactor contains saturated carbons (Figure 7), which also provides independent evidence for the interpretation of the ENDOR data presented here. The differences in side-chain saturation of the cofactor preparations of Kramer et al. (1987) and of Fish and Massey (1987) could result from differences in isolation conditions which, in the former case, could give rise to the redox tautormerization described by Curtius et al. (1985) (cf. Scheme I). The oxidation level of the pterin cofactor has been found to be the tetrahydro form in xanthine oxidase (Rajagopalan et al., 1986). Thus, partial reoxidation of the cofactor to the dihydro form during isolation could result in the difference in structures found on isolation by the above two groups. Davis et al. (1988) have shown that the product rearrangements observed on oxidation of tetrahydrobiopterin are strongly dependent on the type of buffer ion and on the temperature.

The possibility of a cysteinyl ligand to the Mo center has been suggested by Hawkes and Bray (1984b) on the basis of their studies on the isolated Mo cofactor. The data presented here do not rule out that possibility, and the observation of nonexchangeable proton signals of weak intensity with a 2.0–2.1-MHz coupling constant is entirely consistent with their suggestion. Previous publications have suggested one of the thiol groups of the cofactor to be methylated in a thioether linkage (Johnson et al., 1984, 1980). This suggestion was based on the known correlation of sulfite oxidase deficiency and lowered levels of urothione, a possible catabolite of the cofactor, in patients with genetic deficiency in sulfite oxidase. Recent NMR data (Kramer et al., 1987) failed to observe this methyl proton resonance while a methyl resonance was suggested in the NMR data of Fish and Massey (1987). The ENDOR data presented here do not show any definitive evidence for this proposed methyl group although the possibility remains that the spectral resolution achieved is not sufficient for its observation.

Final confirmation of these suggestions awaits more detailed experimentation. Furthermore, although it had been assumed that the cofactor structure is identical for all the molybdenum hydroxylases, there may be subtle, but significant, differences among them. The application of ENDOR spectroscopy promises to be a valuable approach along with other spectroscopic techniques in future studies to probe the nature of Mo ligation in enzyme systems.

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Proton NMR Assignments and Regular Backbone Structure of Bovine Pancreatic Ribonuclease A in Aqueous Solution†

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ABSTRACT: Proton NMR assignments have been made for 121 of the 124 residues of bovine pancreatic ribonuclease A (RNase A). During the first stage of assignment, COSY and relayed COSY data were used to identify 40 amino acid spin systems belonging to alanine, valine, threonine, isoleucine, and serine residues. Approximately 60 other NH-αCH-βCH systems were also identified but not assigned to specific amino acid type. NOESY data then were used to connect sequentially neighboring spin systems; approximately 475 of the possible 700 resonances in RNase A were assigned in this way. Our assignments agree with those for 20 residues assigned previously [Hahn, U., & Rüterjans, H. (1985) *Eur. J. Biochem. 152*, 481-491]. Additional NOESY correlations were used to identify regular backbone structure elements in RNase A, which are very similar to those observed in X-ray crystallographic studies [Wlodawer, A., Borkakoti, N., Moss, D. S., & Howlin, B. (1986) *Acta Crystallogr. B42*, 379-387].

Bovine pancreatic ribonuclease A (RNase A)[1] has played a pivotal role in studies of protein structure (Scheraga & Rupley, 1962; Richards & Wyckoff, 1971), folding (Kim & Baldwin, 1982), and enzyme catalysis (Blackburn & Moore, 1982). We propose extending these studies through the use of NMR spectroscopy, which recently has begun to yield detailed information about the solution structure of small proteins (Wüthrich, 1986).

NMR studies of RNase A date from 1957, with the publication of the first ^{1}H NMR spectrum for a protein (Saunders et al., 1957). Work since then has led to assignments for many of the aromatic protons (Bradbury & Scheraga, 1966; Meadows et al., 1968; Patel et al., 1975; Lenstra et al., 1979; Tanokura, 1983) as well as a number of carbon resonances (Walters & Allerhand, 1980; Howarth & Lian, 1984). Hahn and Rüterjans (1985) made sequence-specific ^{1}H NMR as-

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[1] Abbreviations: COSY, two-dimensional chemical shift correlation spectroscopy; NMR, nuclear magnetic resonance; DQF-COSY, double-quantum-filtered COSY; FID, free induction decay; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional NOE spectroscopy; RELAY, relayed COSY; RNase A, bovine pancreatic ribonuclease A (EC 3.1.27); DSS, 2,2-dimethyl-2-silapentane-5-sulfonate. In discussions of NOESY data, $d_{AB}(i,j)$ designates the distance between proton types A and B located in amino acid residues i and j, respectively, where N, α, β, and γ denote the amide protons, αCH, βCH, and γCH, respectively. The $d_{AB}(i,j)$ notation is also used as an adjective; e.g., $d_{\alpha N}$-type NOE, referring to the NOE associated with the $d_{\alpha N}(i, i+1)$ distance.

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