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Electron spin echo envelope modulation spectroscopy of the molybdenum center of xanthine oxidase

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Abstract

The pulsed EPR technique of electron spin echo envelope modulation (ESEEM) has been utilized to examine both the 'very rapid' and 'desulfo inhibited' Mo(V) signals of xanthine oxidase in order to probe for magnetic interactions with nitrogen, phosphorus, and hydrogen nuclei. No ^{14}N modulation is observed in the 'desulfo inhibited' EPR signal, indicating that histidine is unlikely to be a ligand to molybdenum. Strong ^{14}N modulation is observed in the 'very rapid' EPR signal formed with 2-hydroxy-6-methylpurine substrate bound to molybdenum. We interpret this modulation as arising from nitrogens of the bound purine substrate. This interpretation is consistent with the present evidence indicating that the purine ring present in the species giving rise to the 'very rapid' EPR signal is coordinated to the molybdenum center through the catalytically introduced hydroxyl group. No modulation is observed from non-exchangeable deuterons in experiments performed with deuterated 2-hydroxy-6-methylpurine. Given the signal-to-noise level of the spectra, the lack of modulation indicates that each of the substrate methyl group deuterons is greater than 4.9 Å from the Mo(V). The deuteron removed from the C_8 position in the binding of the substrate is also exchanged to a site or sites greater than 4.9 Å from the Mo(V) in the time-course of sample preparation. Moderately deep deuteron modulation arises from exchangeable sites. A large portion of this modulation can be accounted for by the exchangeable N_7 deuteron of the 2-hydroxy-6-methylpurine substrate, which we estimate to be approximately 3.2 Å from the molybdenum. Additional exchangeable deuterons on the protein or within the buffer must be present within 5 Å of the molybdenum to account for the remaining modulation. No modulation from weakly-coupled ^{31}P nuclei is observed in either the 'desulfo inhibited' or 'very rapid' EPR signal.

Key words: ESEEM; Xanthine oxidase; 'Very rapid' EPR signal; 'Desulfo inhibited' EPR signal; EPR

1. Introduction

Xanthine oxidase catalyzes the hydroxylation of xanthine to uric acid, a reaction that takes place at the molybdenum center of the enzyme (for recent reviews, see [1,2]). The structure of the molybdenum center of this enzyme and other molybdenum hydroxylases has been extensively studied by electron paramagnetic resonance [3–9], X-ray absorption spectroscopy [10–15], and, more recently, resonance Raman spectroscopy [16–17], with the aim of elucidation of the chemical mechanism for the hydroxylation reaction. X-ray absorption spectroscopy has demonstrated that the ligand-coordination sphere of the molybdenum center of xanthine oxidase is dominated by Mo = O and Mo = S groups (at distances of 1.68 Å and 2.15 Å, respectively), with the remainder of the ligand-coordination sphere consisting of thiolate sulfur (at a mean distance of 2.44 Å). EPR [6] and resonance Raman [17] evidence suggest that two of these thiolate ligands are contributed by the dithiolene side chain of a pterin cofactor that is known to be present at the molybdenum center [18]. Of the several EPR signals exhibited by the molybdenum center of xanthine oxidase in the Mo(V) oxidation state, one has been unequivocally shown to be an

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Abbreviations: ESEEM, electron spin echo envelope modulation; ENDOR, electron nuclear double resonance; CW EPR, continous wave electron paramagnetic resonance; ESE, electron spin echo; NQR, nuclear quadrupole resonance; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*-3-propanesulfonic acid.

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authentic catalytic intermediate. This signal, which is termed 'very rapid' on the basis of the rate at which it is generated in the course of the reaction of enzyme with xanthine [19], is observed transiently in the course of the reaction of enzyme not only with xanthine, but also with a variety of other substrates [20]. One of these, 2-hydroxy-6-methylpurine (also called 2-oxo-6-methylpurine), reacts very slowly with xanthine oxidase on a timescale of seconds (by contrast xanthine reacts on a timescale of milliseconds). Furthermore, a maximum of approximately 80% of the active site molybdenum is generated in the 'very rapid' form during the course of the reaction (at approximately 40 s after mixing the enzyme with the substrate at pH 10, 4°C) [8]. Since this reaction is slow, the experiment can be done under aerobic conditions with the significant advantage that reducing equivalents do not accumulate in the other redox-active sites of the enzyme (xanthine oxidase possesses two 2 Fe/2S ferredoxin-type clusters and a flavin adenine dinucleotide moiety in addition to the molybdenum center). It is thus possible to prepare enzyme samples in which the molybdenum center, in the form of the species giving rise to the 'very rapid' EPR signal, is the only paramagnetic center in the enzyme. It is possible to generate another Mo(V) EPR signal, termed 'desulfo inhibited', that is also the only paramagnetic center in the enzyme. This species is generated by first reacting the enzyme with cyanide, which removes the catalytically essential Mo = S sulfur as thiocyanate (it is replaced in the molybdenum coordination sphere with a second oxo group), followed by reduction in the presence of ethylene glycol and partial reoxidation [4,21]. This 'desulfo inhibited' form is generated in the absence of any purine substrate.

We have probed the 'very rapid' and 'desulfo inhibited' EPR signals arising from the paramagnetic molybdenum center of xanthine oxidase with the pulsed EPR technique of electron spin echo envelope modulation (ESEEM). This technique allows us to detect nuclear spin transitions of magnetic nuclei (specifically ^{14}N, ^{31}P, ^{1}H, and ^{2}H) that are weakly-coupled to the electron spin of the Mo(V). No nitrogen interaction is observed in the case of the 'desulfo inhibited' signal where no substrate is present. However, ^{14}N modulation is observed in the ESEEM spectra of the 'very rapid' EPR signal generated in the course of reaction of enzyme with 2-hydroxy-6-methylpurine, presumably arising from the nitrogens of the substrate purine ring. The non-exchangeable deuterons of the 2-hydroxy-6-methylpurine methyl group are too distant to be observed in the ESEEM data obtained with deuterated substrate. Given the signal-to-noise achieved in the ESEEM data, we estimate that deuterons of the methyl group are greater than 4.9 Å away from the Mo(V). Under the present experimental conditions, the deuteron removed from the C_8 position of the substrate has exchanged with solvent and is not detected. Deuteron modulation is demonstrated to arise from exchangeable sites, and a large portion of this modulation can be accounted for by the N_7 deuteron of the 2-hydroxy-6-methylpurine substrate. Additional exchangeable deuterons on protein sites or within the nearby aqueous phase must be present within 5 Å of the molybdenum to account for the remaining modulation. No modulation from weakly-coupled ^{31}P nuclei is observed in either the 'desulfo inhibited' or 'very rapid' EPR signal.

2. Materials and methods

Sample preparation

Xanthine oxidase was purified from unpasteurized cow's milk using the method of Massey et al. [22]. A CM-52 column chromatography step (equilibrated with 0.01 M Mes at a pH of 6.0) was added as a final step to ensure the removal of contaminating lactoperoxidase [23]. The enzyme obtained by this procedure typically had AFR values (ratio of catalytic activity to absorbance) [24] on the order of 170 (i.e., approx. 80% functional). Routine enzyme assays and the determination of the specific activity of xanthine oxidase were performed as described by Massey et al. [22].

The 2-hydroxy-6-methylpurine substrate was obtained from the Alfred Bader Division of the Aldrich Chemical Company. The reagent was recrystallized by being dissolved in dilute base and decolorized over activated charcoal, then acid precipitated, filtered and dried prior to use. Deuteration of 2-hydroxy-6-methylpurine was carried out by dissolving the purine in alkaline D_2O (99.9% enriched) and refluxing for 24 h at 1 atm in a sealed tube, followed by acid precipitation and filtration [8]. The extent of deuteration was determined by NMR (carried out at the Ohio State Chemical Instrumentation Center using a Bruker 500 MHz spectrometer) to be greater than 95%, and further indicated that all proton sites on the 2-hydroxy-6-methylpurine substrate had exchanged with the D_2O solvent buffer during the 24 h exchange reaction.

For the reaction of the deuterated 2-hydroxy-6-methylpurine with enzyme in water, the deuterated purine substrate was diluted in H_2O and used within 1 h of preparation. A separate NMR experiment in which proteated substrate was dissolved in D_2O indicated that the N_7-H and N_3-H protons exchanged with the solvent within 3 min of preparing the sample, while the C_8-H and C_6-methyl protons did not exchange over the course of 37 min. Thus, in the experiments reported here the N_7 and N_3 proton positions on the substrate are expected to be of the isotope found in the solvent, while the methyl protons will be of the isotope present in substrate prior to preparation of the stock solution.

The 'very rapid' EPR signal was prepared by reacting 1.7 equivalents of 2-hydroxy-6-methylpurine with 1.46 mM functional xanthine oxidase (for 40 s on ice in aerobic 0.1 M CAPS buffer, 0.1 N KCl, 0.3 mM EDTA (pH 10.0)). The EPR spectra at both 150 K and 20 K demonstrated that in samples prepared in this manner, the 'very rapid' signal was the only one observed. When samples were prepared in D_2O, the reaction time was extended to 80 s at a temperature of 4°C. The enzyme concentrations varied between 247 and 680 μM. The 'desulfo inhibited' EPR signal was formed by reacting xanthine oxidase (in 0.1 M EPPS, 0.1 M KCl, 0.3 mM EDTA, pH 8.5) with 10 mM cyanide at 25°C for 1.5 h. The enzyme was then reduced with sodium dithionite in the presence of an excess of ethylene glycol, incubated for 30 min, and finally reoxidized by exposure to room temperature air for 1 h [4]. The final enzyme concentration was 350 μM. As in the case of the 'very rapid' EPR signal generated as described above, the Mo(V) signal generated in this manner is again the only paramagnetic center in the enzyme.

ESE spectroscopy

All electron spin echo (ESE) field swept spectra and ESEEM spectra were obtained with a laboratory-built pulsed EPR spectrometer described in detail elsewhere [25]. Samples were placed in 3.8 mm o.d. quartz EPR tubes loaded into a probe assembly [26] immersed in liquid helium at 4.2 K. The ESE experiments were executed with a $\pi/2$ microwave pulse length of 12 ns and a microwave pulse power of approx. 20 W. The ESE field swept ($\pi/2$-τ-π-τ-ESE) spectra for both the 'very rapid' and 'desulfo inhibited' EPR signals were collected at a constant τ of 210 ns. The two-pulse ESEEM ($\pi/2$-τ-π-τ-ESE) data were collected over a τ range (period between the first and second microwave pulses) of 140 ns to 4140 ns in 10 ns τ increments. The three-pulse ESEEM ($\pi/2$-τ-$\pi/2$-T-$\pi/2$-τ-ESE) data were collected at two different magnetic field values of 3458 G and 3465 G, with a constant interpulse time τ of 204 ns to supress proton modulation, covering a T range (period between the second and third microwave pulses) of 76 ns to 8076 ns in 40 ns increments. At 4.2 K, the 'very rapid' and 'desulfo inhibited' EPR signals exhibit relatively long spin-lattice relaxation times (T_1 $\approx$ 100 ms), as seen by utilizing a signal saturation recovery pulse sequence described elsewhere [27]. The repetition delay time between successive microwave pulse sequences was therefore set at 150 ms for all experiments in order to avoid signal saturation. The

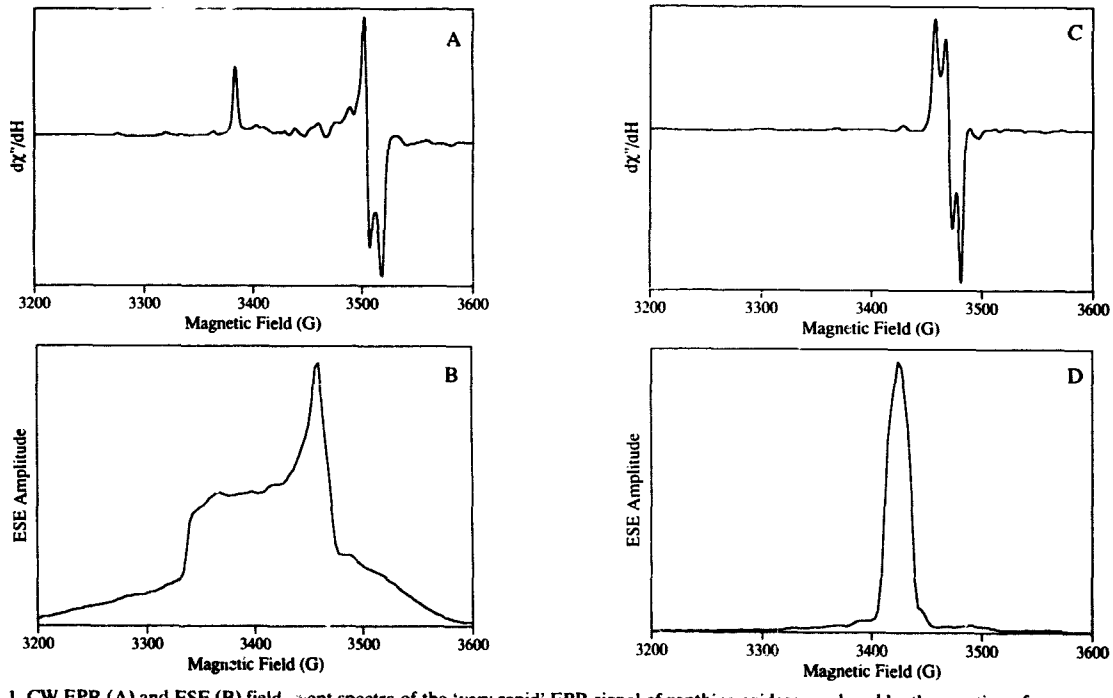

Fig. 1. CW EPR (A) and ESE (B) field swept spectra of the 'very rapid' EPR signal of xanthine oxidase produced by the reaction of enzyme with 2-hydroxy-6-methylpurine substrate in water. The CW EPR (C) and ESE (D) field swept spectra of the 'desulfo inhibited' enzyme of xanthine oxidase in water. The CW EPR spectra were obtained at a temperature of 50 K, a microwave frequency of 9.58 GHz, a field modulation frequency of 100 kHz, and a microwave power of 10 mW. The ESE spectra were obtained at 4.2 K, a microwave frequency of 9.4404 GHZ, with a constant interpulse time τ of 210 ns, and with a repetition time between pulse sequences of 150 ms.

frequency domain of all ESEEM spectra are shown in the form of a cosine Fourier transform. The experimental dead time was reconstructed by utilizing a cosine Fourier backfill method [28].

CW EPR spectroscopy

X-band CW EPR spectra were measured with a Bruker ER 200 D spectrometer equipped with an Oxford Instruments Model ITC4 temperature controller and ESR 900 continuous flow cryostat. The temperature was maintained at 50 K. A Macintosh IIci with a National Instruments NB-MIO-16-L board interfaced directly with the spectrometer was utilized to record the spectra.

Modulation depth measurement

The deuteron modulation depth was calculated according to a modified version of the method established by Mims et al. [29]. A bandpass filter was applied to the three-pulse ESEEM (D_2O/H_2O) ratioed data and the resultant modulation depth d was calculated according to the following formula:

$$d = \frac{\frac{1}{2}[y(peak\ A) + y(peak\ B)] - y(trough)}{\frac{1}{2}[y(peak\ A) + y(peak\ B)]} \quad (1)$$

In this expression, y represents the electron spin echo (ESE) amplitude on the ordinate corresponding to a time $(T+\tau)$ on the abscissa. The modulation depth d represents the (average peak)-to-trough amplitude of the envelope modulation pattern normalized by the average peak amplitude of the echo envelope quotient for a cycle centered upon a trough. The cycle is centered upon the first $y(trough)$ minimum at 630 ns with maximum values of $y(peak\ A)$ at 414 ns and $y(peak\ B)$ at 870 ns on both sides of the trough. The high and low pass filters were applied to remove any interferring modulation components (i.e., ^{14}N and ^{1}H) that remained after the ratioing procedure. Similarly, the modulation depth for the subsequent half-cycle was calculated with the following expression:

$$d = \frac{y(peak) - \frac{1}{2}[y(trough\ A) + y(trough\ B)]}{y(peak)} \quad (2)$$

where the modulation depth d represents the peak-to-(average trough) amplitude of the envelope modulation pattern normalized by the peak amplitude of the echo envelope quotient for a cycle centered upon a peak. [1] The cycle is centered upon the second $y(peak)$ in the spectrum at 870 ns, with minimum values of $y(trough\ A)$ at 630 ns and $y(trough\ B)$ at 1080 ns surrounding the maximum peak.

[1] Equation (12) in the Mims et al. (1990) paper [29] is incorrect. The 1/2 should be replaced by 2.

3. Results

EPR spectroscopy

The CW EPR and ESE field swept spectra of the 'very rapid' EPR signal of xanthine oxidase arising from the addition of 2-hydroxy-6-methylpurine to the enzyme in water are shown in Figs. 1A and 1B. The ESE field swept experiment is the pulsed analog of CW EPR except that the ESE spectrum is displayed as an absorption signal rather than as a derivative signal seen in a field-modulated CW EPR spectrum. In agreement with the literature, the 'very rapid' EPR signal exhibits no resolved couplings to protons and is notably axial with g-values of 2.0229, 1.9518, and 1.9446 [7]. The CW EPR and ESE field swept spectra of the 'desulfo inhibited' signal are shown in Figs. 1C and 1D. The signal is devoid of resolved proton superhyperfine couplings and exhibits g-values of 1.977, 1.973, and 1.967 [4]. It is to be emphasized that there is no evidence in either the 'very rapid' or 'desulfo inhibited' samples for the EPR signals of FADH· or either of the two iron-sulfur centers of xanthine oxidase. The excellent agreement between the CW EPR and ESE field-swept spectra confirms the integrity of the samples used in this ESEEM study.

ESEEM results

The two-pulse ESEEM of the 'desulfo inhibited' Mo(V) signal (lacking substrate) is displayed in Fig. 2. In the frequency domain, the spectrum exhibits a single peak at 14.6 MHz resulting from weakly coupled protons resonating at or near the ^{1}H Larmor frequency. There are no major features that can be attributed to coupled ^{14}N nuclei. This result is consistent with unpublished work cited by both Cammack et al. [30] and Edmondson and D'Ardenne [31] (in collaboration with J. Peisach and J. McCracken), in which ^{14}N modulation is not observed in the ESEEM spectra of the 'desulfo inhibited' EPR signal. Furthermore, Edmondson and D'Ardenne [31] did not see any evidence of ^{14}N coupling to the Mo center via ENDOR (electron nuclear double resonance).

Fig. 3 shows the two-pulse ESEEM and the corresponding Fourier transform of the 'very rapid' EPR signal of xanthine oxidase generated in the course of the reaction with 2-hydroxy-6-methylpurine in H_2O. The spectrum obtained with the bound purine substrate now shows ^{14}N modulation in the low frequency region in addition to the proton peak at 14.7 MHz. The ^{14}N modulation is better resolved with the three-pulse ESEEM results displayed in Fig. 4. Three narrow peaks at 0.63, 0.78, and 1.40 MHz and two broad peaks at 4.06 and 4.52 MHz are observed in the spectrum. Additional peaks are also observed at 1.05, 2.25, 2.58, and 2.94 MHz.

Figs. 5, 6, and 7 display the three-pulse ESEEM

results of experiments designed to examine distances from the Mo(V) to exchangeable and non-exchangeable hydrogen nuclei of the bound 2-hydroxy-6-methylpurine substrate, the surrounding protein, and the aqueous medium. The experiments were performed at a slightly higher g-value than that employed in Fig. 4 because the ^{14}N peak at 2.25 MHz in Fig. 4 interfered with the observation of a deuteron peak at the 2.2 MHz ^{2}H Larmor frequency. This spectral region was found to be free of ^{14}N modulation at the field and frequency conditions used in Figs. 5, 6, and 7.

Fig. 5 displays the three-pulse ESEEM and the corresponding Fourier transform of the 'very rapid' EPR signal of xanthine oxidase generated in the course of the reaction with deuterated 2-hydroxy-6-methylpurine in normal H_2O buffer. Control NMR experiments demonstrate that the methyl hydrogens remain deuterated under these conditions (data not shown). However there is no observable ^{2}H peak at 2.2 MHz, the ^{2}H Larmor frequency, indicating that the methyl group deuterons on the 2-hydroxy-6-methylpurine substrate are too far away from the molybdenum center to be detected. In addition, the deuteron removed from the non-exchangeable C_8 position of the substrate is

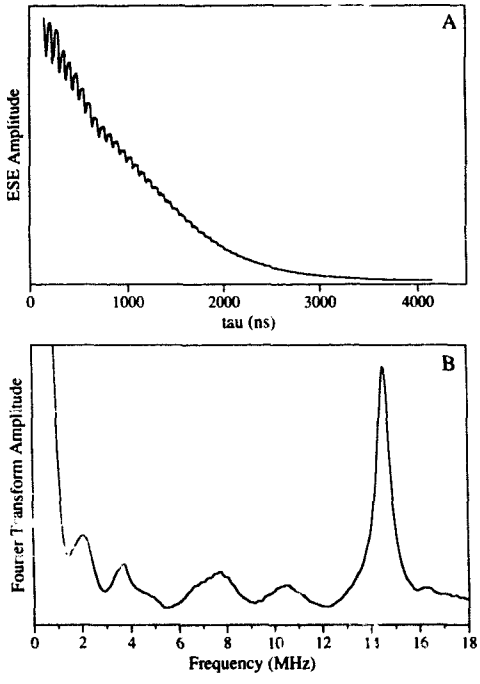

Fig. 2. Two-pulse ESEEM (A) and Fourier transform (B) for the 'desulfo inhibited' enzyme in H_2O. The spectra were obtained at a temperature of 4.2 K, a microwave frequency of 9.4404 GHz, a magnetic field of 3425 G, and with a repetition time between pulse sequences of 150 ms.

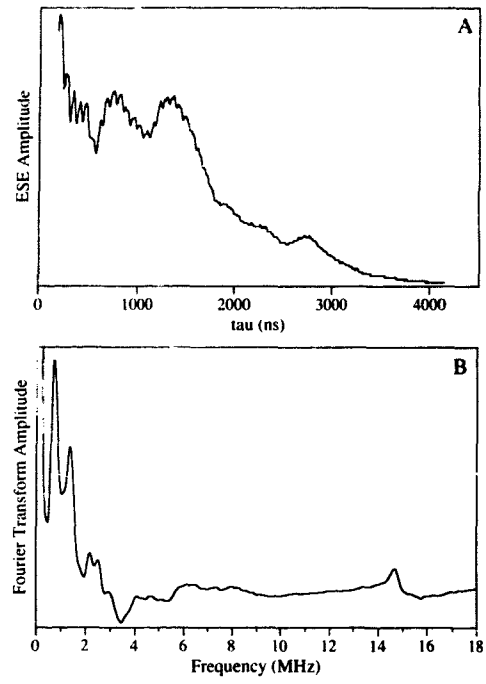

Fig. 3. Two-pulse ESEEM (A) and Fourier transform (B) for the 'very rapid' EPR signal of xanthine oxidase with 2-hydroxy-6-methylpurine in H_2O. The spectra were obtained at a temperature of 4.2 K, a microwave frequency of 9.4404 GHz, a magnetic field of 3458 G, and with a repetition time between pulse sequences of 150 ms.

also exchanged to distant sites, including possibly bulk solvent, in the time-course of sample preparation.

Fig. 6 shows the three pulse ESEEM results when the 'very rapid' EPR signal is generated using proteated 2-hydroxy-6-methylpurine, but in D_2O enriched buffer. A weakly-coupled ^{2}H peak at 2.2 MHz is evident, indicating that solvent exchangeable protons are close to the molybdenum center. These could include the exchangeable N_3 and N_7 protons of the 2-hydroxy-6-methylpurine substrate as well as exchangeable sites on the protein and in the deuterated buffer. The results are very similar when deuterated 2-hydroxy-6-methylpurine is used to produce the 'very rapid' signal in a D_2O enriched buffer (Fig. 7), emphasizing that no detectable ESEEM contribution arises from the non-exchangeable methyl deuterons.

In order to isolate the ESEEM contribution from exchangeable hydrogen sites, we have ratioed the time domain of Fig. 7 (deuterated substrate in D_2O buffer) by the time domain of Fig. 5 (deuterated substrate in H_2O buffer). This ratio procedure acts to eliminate contributions from ^{14}N nuclei (as well as the negligible contribution from the non-exchangeable deuterons) [29]. Fig. 8 (solid line) shows the resulting ratioed

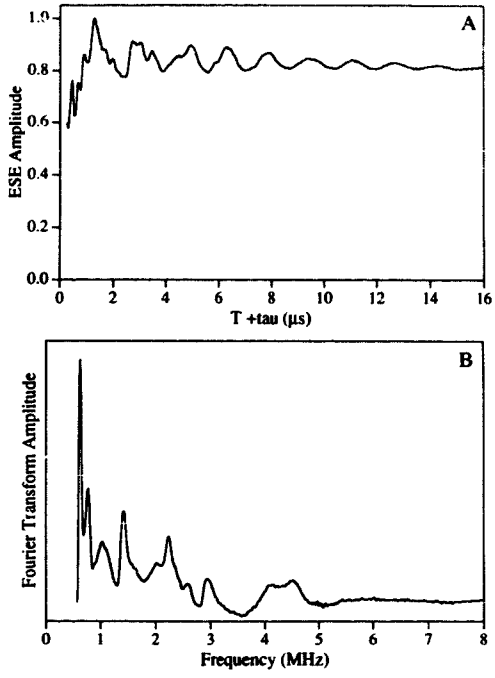

Fig. 4. Three-pulse ESEEM (A) and Fourier transform (B) for the 'very rapid' EPR signal of xanthine oxidase with 2-hydroxy-6-methylpurine in H_2O. The spectra were obtained at a temperature of 4.2 K, a microwave frequency of 9.4751 GHz, a magnetic field of 3458 G, and with a repetition time between pulse sequences of 150 ms. The interpulse time τ between the first and second microwave pulse was set at 204 ns to suppress proton modulation.

time-domain data after filtering through high and low pass filters with cutoff frequencies of 1.5 and 4.0 MHz to remove any residual ^{14}N and ^{1}H components that remained after the ratioing process. The 454 ns deuteron period is evident in the data thus generated. The modulation depth for the cycle centered upon the first trough in the spectrum was measured to be 0.170 using equation (1). Similarly, half a cycle later, that is for the cycle centered upon the second peak in the spectrum, the modulation depth as determined with equation (2) is reduced by 11% to 0.152.

4. Discussion

^{14}N modulation

Electron spin echo envelope modulation is a very sensitive technique for the detection of nitrogen nuclei that are weakly-coupled to a paramagnetic metal center. The ESEEM pattern of the 'desulfo inhibited' Mo(V) EPR signal exhibits no ^{14}N modulation (Fig. 2). It is therefore unlikely that histidine is a ligand to molybdenum, as one of the two imidazole ring nitrogens of the bound histidine would be expected to give rise to detectable modulation. Similarly, it is unlikely that the pterin cofactor of the enzyme is coordinated to the molybdenum via nitrogen.

In contrast, the ESEEM spectra of the 'very rapid' EPR signal of xanthine oxidase with bound 2-hydroxy-6-methylpurine substrate does exhibit ^{14}N modulation (Figs. 3 and 4). The very sharp lines at low frequency are characteristic of ^{14}N nuclei when the hyperfine and applied magnetic fields are similar in magnitude. In this case, for one electron orientation the two magnetic field components tend to cancel one another, and the frequencies approach the zero-field frequencies resulting from the quadrupole moment of the ^{14}N nucleus interacting with the electric field gradient at the site [32]. Such an interaction provides three sharp lines, typically in the 0 to 3 MHz range as is observed in the three-pulse Fourier transform (Fig. 4B). We observe more than three lines, however, indicating that multiple nitrogen nuclei give rise to the modulation. The additional lines may arise not only from transitions due to inequivalent nuclei, but also as combination lines that are due to the the multiplicative nature of the

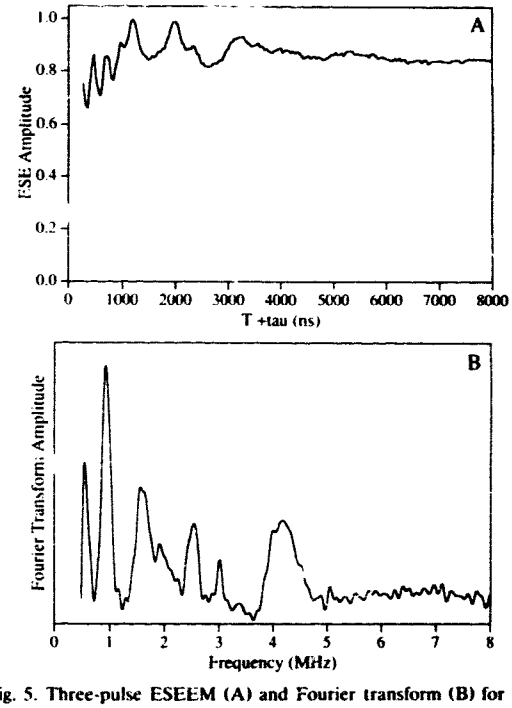

Fig. 5. Three-pulse ESEEM (A) and Fourier transform (B) for the 'very rapid' EPR signal of xanthine oxidase with deuterated 2-hydroxy-6-methylpurine in H_2O. The spectra were obtained at a temperature of 4.2 K, a microwave frequency of 9.7684 GHz, a magnetic field of 3465 G, and with a repetition time between pulse sequences of 150 ms. The interpulse time τ between the first and second microwave pulse was set at 204 ns to suppress proton modulation.

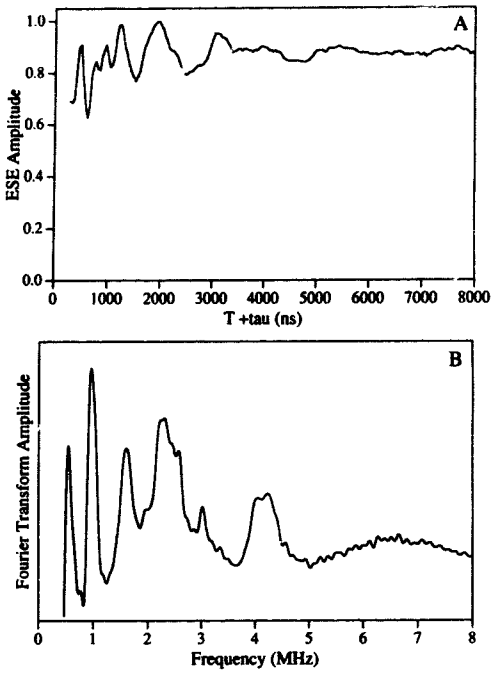

Fig. 6. Three-pulse ESEEM (A) and Fourier transform (B) for the 'very rapid' EPR signal of xanthine oxidase with protonated 2-hydroxy-6-methylpurine in D_2O. The spectra were obtained at 4.2 K, a microwave frequency of 9.7684 GHz, a magnetic field of 3465 G, and with a repetition time between pulse sequences of 150 ms.

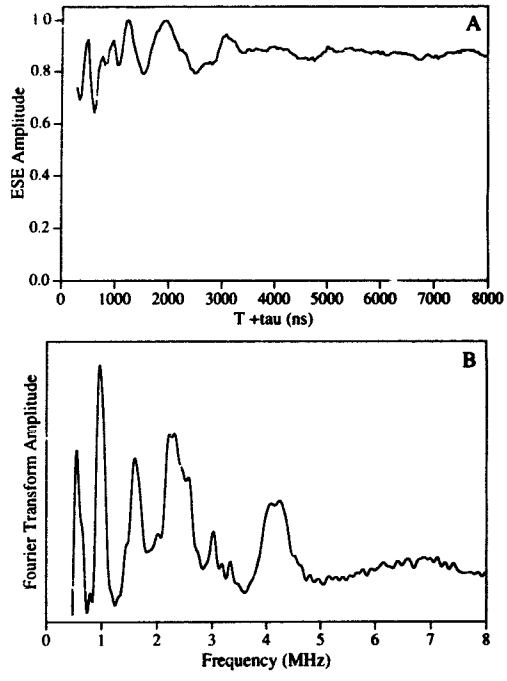

Fig. 7. Three-pulse ESEEM (A) and Fourier transform (B) for the 'very rapid' EPR signal of xanthine oxidase with deuterated 2-hydroxy-6-methylpurine in D_2O. The spectra were obtained at a temperature of 4.2 K, a microwave frequency of 9.7684 GHz, a magnetic field of 3465 G, and with a repetition time between pulse sequences of 150 ms.

envelope modulation from multiple nuclei (shown later in equation (3)). When the hyperfine and applied magnetic fields are of similar magnitude, one typically observes in addition to the zero-field lines a broad line at higher frequency due to the outermost transition for the electron spin orientation where the two field components add. This 'double-quantum' transition is typically in the 4 to 6 MHz range and in Fig. 4 we observe a pair of partially overlapping transitions in this region. Overall, the ESEEM data are qualitatively similar to simulated ESEEM spectra for two nitrogens coupled to an $S = 1/2$ metal center [33].

NMR [34], resonance Raman [16], and EPR [9,35–37] studies have indicated that the 2-hydroxy-6-methylpurine substrate is bound to molybdenum in the species giving rise to the 'very rapid' EPR signal. Specifically, the EPR and resonance Raman evidence indicates that the purine ring of the substrate is ligated to the molybdenum center via the catalytically introduced hydroxyl group. Thus, the ^{14}N ESEEM observed in our experiments appears to arise from two or more of the four ^{14}N nuclei of the substrate, none of which are directly ligated to molybdenum. This is the first measurement of magnetic interactions between the Mo(V) and substrate nitrogens for xanthine oxidase poised in the form giving rise to the 'very rapid' EPR signal. The only nitrogen couplings previously observed for such a complex were seen with the addition of alloxanthine to

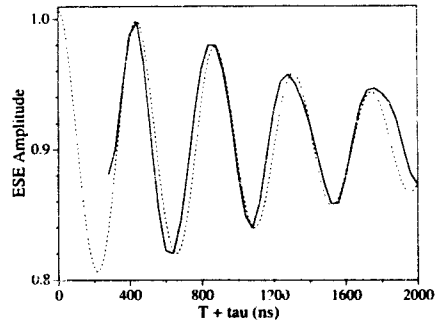

Fig. 8. The time domain ratio of Fig. 7 (D_2O) divided by Fig. 5 (H_2O) to suppress ^{14}N modulation. The ^{2}H modulation period of 454 ns is clearly evident. The spectrum was filtered via a low (1.5 MHz) and high (4.0 MHz) pass filter to eliminate any residual ^{1}H and ^{14}N modulation that remained after ratioing. The dashed line displays the result of a numerical simulation of the ESEEM spectrum, with the modulation arising from two dipolar coupled deuterons, one at a distance of 3.2 Å and another at a distance of 3.9 Å.

enzyme, which appears to result in ligation of nitrogen directly to the Mo(V) [38]. Work in progress to specifically label nitrogens of the 2-hydroxy-6-methylpurine substrate with ^{15}N will enable us to make more detailed assignments of the magnetic interactions with each nitrogen.

We have also considered the alternate possibility that a protein-derived ligand gives rise to the observed ^{14}N ESEEM in the 'very rapid' signal. This seems very unlikely, however, given the clear proximity of substrate nitrogens to Mo(V) in this form of the complex with the substrate bound to molybdenum and the absence of couplings to ^{14}N in the 'desulfo inhibited' species. The possibility that the chemical procedure giving rise to the 'desulfo inhibited' signal displaces an otherwise present nitrogen ligand such as histidine from the molybdenum coordination sphere cannot be absolutely ruled out, but this is inconsistent with the proposed structure of the signal-giving species in which the hydroxyl groups of the ethylene glycol have replaced the two Mo = O groups, leaving the remainder of the molydenum coordination sphere intact [39]. Moreover, since the ^{14}N modulation appears to arise from more than one nitrogen, this would require the displacement of multiple nitrogen ligands by the treatment used to generate the 'desulfo inhibited' species.

^2H modulation

Our ESEEM results on the 'very rapid' EPR signal generated in the course of the reaction with deuterated 2-hydroxy-6-methylpurine in normal H_2O buffer show no modulation from non-exchangeable deuterons (Fig. 5), indicating that the C_6-methyl group deuterons on the 2-hydroxy-6-methylpurine substrate are too distant from the molybdenum center to be detected. In addition, no modulation from the deuteron derived from the C_8 position of the substrate is observed. It is known that the C_8-H proton has been removed from the 2-hydroxy-6-methylpurine substrate at the point in the reaction mechanism that the 'very rapid' species is formed, being replaced by the C_8-O bond of product [8]. This proton, which with xanthine as substrate persists in the active site long enough to be observed as the more strongly coupled of two protons detected in the 'rapid Type 1' Mo(V) EPR signal, is known to exchange relatively rapidly with solvent once abstracted from the purine substrate ($k_{exch} = 25\ s^{-1}$ at pH 8.2, 12°C) [40]. Our ESEEM results provide further indication that the deuteron exchanges rapidly with bulk solvent on the time scale of sample preparation (80 s) once abstracted from the substrate.

The ratioed data of Fig. 8 provide a basis for estimating the distance from the paramagnetic Mo center to solvent-exchangeable protons on the 2-hydroxy-6-methylpurine substrate, solvent-exchangeable sites of the protein, and protons that exist within the aqueous medium. The theory and method for the relationship between the modulation depth parameter and the radial distance measurement in ESEEM spectra has been rigorously investigated [29,41–43]. The modulation depth produced by a weakly dipolar-coupled nucleus is inversely proportional to the sixth power of the radial distance [43]. Thus, a deep ^2H modulation pattern indicates that weakly coupled ^2H nuclei are close to the paramagnet, whereas a shallow modulation pattern indicates that the nuclei are further away. In the two-pulse ESEEM experiment, when multiple magnetic nuclei are coupled to the same electron spin, the modulation function can be represented by the product of single-nucleus modulation functions as indicated by the following [29]:

$$V_{Mod}(I_1, I_2, \ldots, I_N) = V_{Mod}(I_1) \times V_{Mod}(I_2) \times \ldots \times (I_N) \quad (3)$$

In the three-pulse ESEEM experiment, the same expression can be utilized when the magnetic nuclei exhibit weak dipolar couplings (i.e., $\omega_\alpha \approx \omega_\beta \approx \omega$) [29,42]. A complicated ESEEM spectrum consisting of ^{14}N modulation and ^2H modulation prevents the direct observation of the deuteron period (454 ns) in the time domain as seen in Figs. 6 and 7. Nitrogen modulation can be suppressed, however, by dividing the time domain of the deuterated sample with the time domain of the nondeuterated sample. The procedure is evident from an examination of equation (3): division of the modulation pattern of a D_2O sample by the modulation pattern of a H_2O sample permits ^{14}N modulation to be factored out as seen in the following expression:

$$\frac{V_{Mod}(I_1, I_D, \ldots, I_N)}{V_{Mod}(I_1, I_H, \ldots, I_N)} = \frac{V_{Mod}(I_D)}{V_{Mod}(I_H)} \quad (4)$$

where $V_{Mod}(I_1)$ etc. represent ^{14}N modulation components that are suppressed in the ratio. It is to be emphasized, however, that the (D_2O/H_2O) ratioing procedure does not completely eliminate all ^{14}N modulation, since the numerator and denominator represent modulation function averages, and in a non-crystalline sample (i.e., a powder pattern) the average of the product is not rigorously the same as the product of the averages [29,42]. Nevertheless, the ratioing technique reduces the ^{14}N modulation considerably, and allows the deuteron modulation pattern to be observed more clearly, as evident in Fig. 8 (454 ns deuteron period).

The Mims et al. [29] method for calculating a radial distance between a weakly-coupled deuteron and a paramagnetic metal center was used in the following analysis to establish the range of deuteron distances that could give rise to the observed modulation. For a weakly-coupled spin $I = 1$ deuteron, the modulation

depth parameter (k) can be expressed by the following equation:

$$k = 6\left(\frac{g\beta}{H_o r^3}\right)^2 \sin^2(2\theta) \quad (5)$$

where g is the electron g factor, β is the Bohr magneton for an electron, H_o is the magnitude of the applied magnetic field, θ is the angle between the magnetic field vector and the vector connecting the electron and the deuteron, and r is the distance between the electron and the deuteron. The effect of the electric quadrupole moment is neglected in this expression. This modulation depth parameter is 8/3 larger for an $I = 1$ nucleus than for an $I = 1/2$ nucleus. This equation can be modified by averaging over a sphere to account for all of the possible orientations of the electron-nucleus position vector with respect to the magnetic field, resulting in the following expression:

$$\bar{k} = \frac{16}{5}\left(\frac{g\beta}{H_o r^3}\right)^2 \quad (6)$$

where $\bar{k}$ represents the spherical average of the modulation depth parameter. Errors in calculating the modulation depth parameter by the oversimplification of the electron-nucleus orientation within the magnetic field by averaging over a sphere is considered to be minimal [29]. Also, the small deuterium nuclear quadrupole interaction does not significantly affect the modulation depth over the first two cycles of the modulation pattern. The experimental parameters used in obtaining Fig. 8 can be introduced into equation (6) which reduces to the following:

$$2\bar{k} = \frac{186.0}{r^6} \quad (7)$$

where g is equal to 2.014, the magnetic field is at 3465 G, and for convenience r is expressed in Å units. The modulation depth parameter ($\bar{k}$) has been multiplyed by 2 in equation (7) so that it can be directly compared with the experimental modulation depth (d) described in equations (1) and (2) which will be applied to the ratioed ESEEM data of Fig. 8. The factor of 2 is evident by examining the numerical expression for the three-pulse modulation function [29,43]. The deuteron modulation depth for the first cycle centered upon the trough in Fig. 8 calculated according to equation (1) is 0.170. This value for $2\bar{k}$ gives a radial distance measurement of 3.2 Å using equation (7). However, we need to compensate for modulation decay. Modulation depth decays along the ESEEM time axis at a rate that depends upon the magnitude of the hyperfine anisotropy induced by the dipolar interaction ($\alpha 1/r^3$) between the paramagnetic center and the nucleus. Therefore, the modulation pattern produced from a nucleus that is close to a paramagnetic center decays quickly along the ESEEM time axis, whereas the modulation pattern produced from a nucleus that is more distant exhibits slower decay.

We can explore the modulation decay directly with the use of an analytical expression for the modulation depth decay. The expression for a single $I = 1$ nucleus with dipolar coupling is given by the following [29]:

$$\cos\left[0.378\left(\frac{m\pi}{r^3}\right)\frac{g\beta}{H_o}\right] \quad (8)$$

where the integer m depends upon the time $(T + \tau)$ corresponding to the center of a deuteron modulation cycle from which the modulation depth is measured. Specifically, for the cycle centered upon a trough 1.5 deuteron periods from $(T + \tau) = 0$, m is equal to 3. Similarly, two deuteron periods from $(T + \tau) = 0$, m is equal to 4. Thus, m is odd for a cycle centered upon a trough and even for a cycle centered upon a peak. Introducing, the experimental values from Fig. 8 simplifies Eq. (8) to the following expression:

$$\cos\left[2.038\left(\frac{m\pi}{r^3}\right)\right] \quad (9)$$

Multiplying the theoretical modulation depth parameter ($2\bar{k}$) by the dipolar depth decay factor in Eq. (9) results in a corrected modulation depth parameter that can be compared to the experimental modulation depth. This can be used to establish a distance range over which deuterons may contribute to the observed ESEEM spectrum. For example, a single deuteron at 3.0 Å will produce a corrected modulation depth of 0.193 ($m = 3$) which is greater than the experimental modulation depth of 0.170. The outer limit for detection of deuterons is dependent on the signal-to-noise level of the data, We estimate that the minimum modulation depth that can be detected is approx. 0.014, which corresponds to detection of a single deuteron out to a distance of 4.9 Å.

In order to obtain a more precise estimate of distance we have simulated the powder pattern ESEEM spectrum using the density matrix formalism of Mims [44,45]. Details of the numerical calculations are described by Britt et al. [46]. The simulation was optimized to match the modulation depth and the modulation decay over the first four cycles of the experimental data. The dipolar hyperfine interaction utilized in the simulation is identical to that employed in generating the previous analytic expressions for the spherical average modulation depth [29]. However, in the numerical simulations, we have added the effect of the deuteron quadrupole interaction, using values of $e^2qQ = 0.22$ MHz and $\eta = 0.1$ [47]. In order to model the damping of the modulation, a deuteron at relatively short distance (≈ 3.2 Å) is required. One or more additional deuterons at greater distance are required to achieve

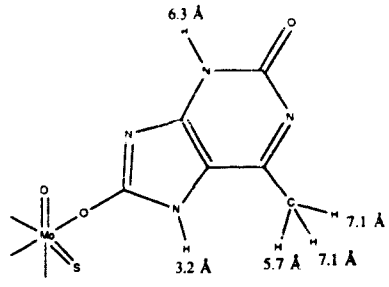

Fig. 9. Proposed model for the geometric arrangement between the Mo center of xanthine oxidase and the 2-hydroxy-6-methylpurine substrate, based upon the experimental deuteron modulation depth obtained from Fig. 8. The model was constructed by utilizing a molecular modeling program (see footnote, this page) in which the structure of the ligand coordination sphere (consisting of the sulfur and oxygen ligands along with the 2-hydroxy-6-methylpurine substrate coordinated via the hydroxyl group) surrounding the Mo center was modeled. The distances between the hydrogens on the 2-hydroxy-6-methylpurine substrate and the molybdenum center are shown next to each individual hydrogen.

the overall modulation depth. For example, a good fit to the data (Fig. 8, dashed line) can be obtained with one deuteron at 3.2 Å and a second deuteron at 3.9 Å.

In the species giving rise to the 'very rapid' EPR signal, several lines of evidence have led to the proposal that the C_8 position of the purine substrate is coordinated to the molybdenum center via the catalytically introduced hydroxyl group, as shown in Fig. 9 [7–9,14–16,36,37,39]. Specifically in Fig. 9, the orientation of the hydroxyl bound substrate has been varied with a molecular modeling program [2] so that the exchangeable N_7 deuteron is 3.2 Å from the Mo(V), thus giving the dominant contribution to the deuteron modulation of Fig. 8. This places the methyl deuterons at a minimum distance of 5.7 Å from the molybdenum, which is consistant with lack of deuteron modulation from non-exchangeable sites. The exchangeable N_3 site is also beyond the range of detection. The N_7 deuteron is thus the only deuteron of the 2-hydroxy-6-methylpurine substrate that gives rise to observable modulation. However, there must be additional exchangeable sites on the protein or in the aqueous media that give rise to the additional modulation. The simulation gives a good fit with one such additional deuteron at 3.9 Å.

^{31}P modulation

^{31}P NMR studies on xanthine oxidase [48] have found evidence for a phosphoserine residue in the active site of the enzyme, and a specific catalytic role for this residue has been proposed [49]. The presence of a phosphoserine residue in the active site of the enzyme has become controversial [50, 51]. We do not observe any modulation in the 'desulfo inhibited' signal (Fig. 2) corresponding to or centered about the ^{31}P Larmor frequency of 5.9 MHz. Likewise, the ESEEM spectra of the 'very rapid' EPR signals generated in the course of reaction of enzyme with 2-hydroxy-6-methylpurine show no evidence of ^{31}P modulation. The ^{14}N modulation in the 'very rapid' signal complicates the spectra, but once again no peaks are observed that are centered upon the ^{31}P Larmor frequency. Utilizing the theoretical modulation depth parameter obtained in equation (6), we can establish a maximum radial distance between a ^{31}P nucleus and the Mo center that could be detected with ESEEM. Given a modulation depth ($2\bar{k}$) that is 3/8 of the value shown in Eq. (6), and a minimal experimental modulation depth of approx. 0.010 based upon the signal-to-noise ratio of the 'desulfo inhibited' signal in Fig. 2, we estimate a maximum distance of 4.3 Å for detection of a single weakly-coupled phosphorous nucleus. We can rule out the presence of a weakly-coupled ^{31}P nucleus at a distance closer than 4.3 Å.

Summary

The ESEEM study reported in this paper provide useful information on the structure and the reaction mechanism of xanthine oxidase. No ^{14}N modulation is observed in the 'desulfo inhibited' EPR signal, indicating that histidine is not a ligand to molybdenum. Strong ^{14}N modulation is observed in the 'very rapid' EPR signal with 2-hydroxy-6-methylpurine substrate bound to molybdenum, which we interpret as arising from nitrogens of the bound purine substrate. This interpretation is consistent with the purine ring being coordinated to the molybdenum through the catalytically introduced hydroxyl group in the species giving rise to the 'very rapid' EPR signal. Modulation due to the C_6-methyl deuterons of 2-hydroxy-6-methylpurine are not observed, indicating that they are each greater than 4.9 Å from the Mo(V), and the deuteron removed from the C_8 position in the course of generating the signal-giving species is also exchanged to a site or sites at least this distance from the Mo(V) on the time scale of sample preparation. We observe moderately deep deuteron modulation from solvent-exchangeable sites near the Mo(V). A large portion of this modulation can be accounted for by the N_7 deuteron of the 2-hydroxy-6-methylpurine substrate, which we estimate to be approx. 3.2 Å from the molybdenum. Additional exchangeable deuterons from protein or solvent within 5 Å of the molybdenum must be present to account for the remaining modulation. We were unable to detect any weakly-coupled ^{31}P modulation in any of the results examined in this paper, indicating that a phospho-

[2] The molecular modeling program PCModel (4th Edn., 1990) obtained through Serena Software was used for the simulation.

serine residue, if present, is no closer than 4.3 Å to the enzyme molybdenum.

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