Reductive Half-Reaction of Xanthine Oxidase: Mechanistic Role of the Species Giving Rise to the "Rapid Type 1" Molybdenum(V) Electron Paramagnetic Resonance Signal†

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Received September 22, 1992; Revised Manuscript Received January 28, 1993

ABSTRACT: The reaction of xanthine oxidase with xanthine, 1-methylxanthine, and 2-hydroxy-6-methylpurine has been reinvestigated with the aim of elucidating the mechanistic role of the species giving rise to the "rapid" Mo(V) electron paramagnetic resonance (EPR) signal. It is found that addition of 2.0 mM 1-methylxanthine or 2-hydroxy-6-methylpurine to partially reduced enzyme generates substantial amounts of the Type 1 form of the "rapid" EPR signal, characterized by superhyperfine coupling to one strongly interacting (a_{av} = 13 G) and one weakly interacting (a_{av} = 3 G) proton. The "rapid" signals observed with both substrates are identical to those observed in the course of the anaerobic reaction of enzyme with a stoichiometric excess of substrate. With 2-hydroxy-6-methylpurine at pH 10, a burst phase in the formation of the species giving rise to the "rapid Type 1" signal is observed that is fast relative to the rate of formation of the species giving rise to the "very rapid" EPR signal. At pH 8.5, partial reduction of enzyme prior to reaction with xanthine, 1-methylxanthine, or 2-hydroxy-6-methylpurine reverses the relative amounts of "rapid" and "very rapid" EPR signal observed at the shortest reaction times. The substantial amounts of "rapid Type 1" signal formed by addition of substrates to partially reduced enzyme or by reaction of oxidized enzyme with a stoichiometric excess of substrate contrasts with previous work, which has shown that under single-turnover conditions none of the substrates investigated generates an appreciable amount of "rapid" EPR signal. It is concluded that the species giving rise to the "rapid Type 1" EPR signal is not a bona fide catalytic intermediate, as has long been thought, but rather arises from the dead-end complex of substrate with enzyme possessing Mo(V) rather than the Mo(VI) required for the initiation of the reductive half-reaction. Using 2-hydroxy-6-methylpurine labeled with ^2H at the C_8 position, additional evidence is found that the C_8–H proton of bound substrate in the species giving rise to the "rapid Type 1" EPR signal is not detectably coupled magnetically.

The molybdenum-containing hydroxylases are unique among monooxygenase systems in two regards: they utilize water rather than dioxygen as the ultimate source of the oxygen atom incorporated into product, and they generate rather than consume reducing equivalents in the course of the catalytic cycle:

$$RH + 2e^- + 2H^+ + O_2 \xrightarrow{\text{heme, flavin, or pterin}} ROH + H_2O$$

$$\text{vs } RH + H_2O \xrightarrow{Mo^{VI}} ROH + 2e^- + 2H^+$$

Monooxygenase systems possessing flavins, pterins, or hemes utilize the thermodynamic favorability of dioxygen reduction to generate highly reactive oxygenating species (flavin or pterin 4a-peroxides, heme ferryl oxides, etc.) as the oxygenating species in their reaction mechanisms. The molybdenum hydroxylases, by contrast, have only the thermodynamic favorability arising from substrate oxidation to drive the reaction in the physiological direction. In the case of molybdenum enzymes such as sulfite oxidase or xanthine dehydrogenase, which donate the reducing equivalents removed from substrate in the hydroxylation reaction to cytochrome *c* and NAD^+,[1] respectively, physiologically useful reducing equivalents accumulate. While the thermodynamic driving force is relatively low for the molybdenum hydroxylases, these enzymes do have a kinetic advantage over the O_2-utilizing systems in that the reaction catalyzed is not spin-forbidden. The molybdenum hydroxylases thus represent an alternate strategy to the activation of dioxygen in enzymology.

Principally because of its ease of isolation from a convenient source, milk xanthine oxidase has become the paradigm for the molybdenum-containing hydroxylases [for reviews see Bray (1988) and Hille (1992)]. In addition to its molybdenum center, xanthine oxidase contains two 2Fe/2S iron–sulfur centers of the spinach ferredoxin variety and flavin adenine dinucleotide (FAD). In the case of xanthine oxidase, xanthine is hydroxylated at the molybdenum center, reducing it from Mo(VI) to the Mo(IV) (the so-called reductive half-reaction; Bray et al., 1964). The enzyme is subsequently reoxidized at the flavin site by dioxygen, generating either peroxide or superoxide, depending on the experimental conditions (the oxidative half-reaction; Komai et al., 1969). Electron transfer from the molybdenum center to the flavin is thus an integral aspect of catalysis and is presumably mediated by the iron–sulfur centers (Hille & Anderson, 1991). It is a general feature of the molybdenum hydroxylases that they possess redox-active sites in addition to the molybdenum and that the molybdenum center participates directly in only one of the two half-reactions (usually the reductive). It is to be emphasized in the case of xanthine oxidase that dioxygen serves only as the oxidizing substrate and does not participate in the reductive half-reaction; specifically, it is *not* the source

† This work was supported by a grant from the National Institutes of Health (AR 38917) to R.H.

[1] Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EPPS, *N*-(2-hydroxyethyl)-piperazine-*N'*-3-propanesulfonic acid; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide.

0006-2960/93/0432-3973$04.00/0

of the oxygen atom incorporated into product, as is the case with the flavin-, pterin-, and heme-containing monooxygenases.

In the course of the overall reductive half-reaction sequence of xanthine oxidase, molybdenum is reduced from the (VI) to the (IV) valence state, with the paramagnetic (V) valence state observed to varying extents, depending upon the experimental conditions. Of the several Mo(V) EPR signals observed in the course of the reaction of enzyme with xanthine, two exhibit kinetics sufficiently fast to be possible reaction intermediates in the reductive half-reaction. These two signals, designated "very rapid" and "rapid" on the basis of the time scale over which they transiently appear in the reaction of xanthine oxidase with excess xanthine (Bray & Vänngård, 1969), have been extensively studied over the past 20 years. The "very rapid" signal is notably anisotropic ($g_{1,2,3}$ = 2.025, 1.955, 1.949) and is unusual among the Mo(V) EPR signals exhibited by xanthine oxidase in that no superhyperfine coupling to protons is evident in the signal (Bray & Vänngård, 1969). In 0.05 M glycine, pH 10, the "very rapid" signal appears on a time scale of 20–25 ms and subsequently decays over the succeeding 200 ms in the reaction of enzyme with excess xanthine (Edmondson et al., 1973). Xanthine oxidase exhibits two distinct forms of "rapid" EPR signal, designated "Type 1" and "Type 2", whose relative intensities depend on the experimental conditions (higher xanthine concentrations and lower pH favor the "Type 1" form; Bray et al., 1978). The two forms of "rapid" signal are readily distinguished on the basis of the number of strongly coupled protons in the signal-giving species. The "rapid Type 1" signal, favored at higher concentrations of xanthine and/or lower pH, exhibits superhyperfine coupling to one strongly (a = 13 G) and one weakly (a = 3 G) coupled proton. The "rapid Type 2" signal is favored by low (<2 mM) concentrations of xanthine and exhibits strong coupling to two approximately equal protons (a = 10 G). The two types of "rapid" signal have similar g-values ($g_{1,2,3}$ = 1.9906, 1.9707, 1.9654 for the "rapid Type 1" signal and $g_{1,2,3}$ = 1.9951, 1.9712, 1.9616 for the "rapid Type 2" signal; Bray et al., 1978) and ^{95}Mo hyperfine coupling (Malthouse et al., 1981; Wilson et al., 1991), and are significantly less anisotropic than the "very rapid" EPR signal. In addition, studies utilizing ^{33}S- and ^{17}O-labeled enzyme have demonstrated that both types of "rapid" signal arise from species possessing one relatively weakly coupled sulfur and at least one strongly coupled oxygen (Malthouse et al., 1981; Bray & Gutteridge, 1982). The "rapid Type 2" signal is thought to arise from substrate incorrectly oriented in the active site (Bray et al., 1978) and will not be considered further here. The "rapid Type 1" signal, on the other hand, appears on the same time scale as the decay of the "very rapid" signal in the reaction of xanthine oxidase with xanthine and has been long been assumed to arise from a species lying downstream from that giving rise to the "very rapid" EPR signal (Bray et al., 1979; Bray & George, 1985; Bray, 1988).

Recent kinetic studies of the reductive half-reaction of xanthine oxidase using 2-hydroxy-6-methylpurine (McWhirter & Hille, 1991) and xanthine (Kim & Hille, 1993) have demonstrated that neither of the "rapid" Mo(V) EPR signals seen when enzyme is reacted with excess substrate is observed under single-turnover conditions. This is surprising if the "rapid Type 1" signal is in fact an intermediate in the reaction, particularly in the reaction with 2-hydroxy-6-methylpurine, where large amounts of the "very rapid" signal are observed. The principal difference in experiments performed with excess vs limiting substrate is that in the former case the enzyme turns over three times: since xanthine oxidase is capable of accepting a total of six reducing equivalents from substrate (two at the molybdenum center, two at the FAD, and one at each of the iron–sulfur centers; Hille & Massey, 1981), three successive substrate molecules (each donating a pair of reducing equivalents) react with enzyme to fully reduce it. In addition to the kinetic complication of triplicating the reductive half-reaction sequence, the second and third substrate molecules to react must necessarily encounter partially reduced (specifically, two-electron and four-electron reduced enzyme, respectively) rather than fully oxidized enzyme. It has previously been suggested that the "rapid" signal arises not from a bona fide catalytic intermediate but rather from the complex of unreacted substrate with partially reduced enzyme possessing a molybdenum center in the Mo(V) valence state (Pick & Bray, 1969; Gutteridge et al., 1978). This view has subsequently been abandoned, however, in favor of a position for the signal-giving species lying downstream in the reductive half-reaction sequence from that giving rise to the "very rapid" EPR signal (Bray et al., 1979; Bray & George, 1985; Bray, 1988).

In order to further assess the catalytic relevance of the "rapid Type 1" Mo(V) EPR signal, the interaction of several substrates for xanthine oxidase with partially reduced enzyme has been examined. The present work describes experiments using xanthine, 1-methylxanthine, and 2-hydroxy-6-methylpurine as substrates. The results of these experiments indicate that the species giving rise to the "rapid Type 1" Mo(V) EPR signal does not lie on the main catalytic sequence of xanthine oxidase but rather represents the Michaelis complex of substrate with partially reduced enzyme containing Mo(V).

MATERIALS AND METHODS

Xanthine oxidase was purified from unpasteurized cow's milk (obtained from the dairy herd of the Ohio State University) by the method of Massey et al. (1969), with the addition of a CM-52 column chromatography step at the end of the procedure, to ensure the removal of contaminating lactoperoxidase (Morrison & Hultquist, 1963), and a Sephacryl S-200 gel-filtration column. Enzyme obtained by this procedure typically had AFR values (the ratio of catalytic activity to absorbance at 450 nm) on the order of 160, i.e., the enzyme was approximately 75% functional (fully functional enzyme having an AFR of 210; Massey et al., 1970), and it had an A_{280}/A_{450} ratio in the range 5.3–5.8. The well-characterized deflavo form of xanthine oxidase (lacking the FAD cofactor of the enzyme), was used in the majority of the experiments described here to avoid complications arising from formation of FADH$^{\bullet}$ (the FADH$^{\bullet}$ EPR signal overlaps with the g_1 feature of the molybdenum (V) signals being investigated). Deflavo xanthine oxidase was prepared by the following modification of the method of Komai et al. (1969). Concentrated xanthine oxidase in 0.1 M Tris, 0.3 mM EDTA, and 1.0 mM salicylate, pH 8.0, was mixed with 1.5 volumes of 3.4 M $CaCl_2$, pH 8.0, and incubated on ice for 180 min. A Sephadex G-25 column was equilibrated at 2 °C with 0.1 M Tris, 0.3 mM EDTA, 0.1 M KCl, and 10 mM dithiothreitol, pH 8.0. Approximately 0.5 mL of 3.4 M $CaCl_2$, pH 8.0, diluted with the equilibrating buffer in a 1.5:1 ratio was loaded onto the column, followed by the enzyme incubate, and the column was eluted with the equilibrating buffer. The enzyme fraction was collected and centrifuged at high speed to remove any precipitate and then passed through a Sephadex G-25 column equilibrated with the desired buffer. It has been previously shown that removal of the FAD has no effect on

the function or EPR signals of the molybdenum center (Komai et al., 1969).

UV/visible absorption spectra and enzyme assays were obtained with a Hewlett-Packard 8452 diode-array spectrophotometer interfaced to a Hewlett-Packard Chemstation computer. Electron paramagnetic resonance spectra were obtained with a Brüker ER 300 spectrometer equipped with an ER 035M NMR gaussmeter and a Hewlett-Packard 5352B microwave frequency counter. Temperature control was achieved using a Brüker ER4111 VT continuous-flow liquid nitrogen cryostat. All spectra were obtained using the following instrument settings and experimental conditions unless otherwise stated: 10-mW microwave power, 400-G field sweep, 3400 field set, 100-kHz field modulation, 2.0-G modulation amplitude, and 150 K. The microwave frequency varied from sample to sample due to differences in sample tube dimensions but was in all cases near 9.460 GHz (see figure legends for specific values). EPR samples containing partially reduced deflavo xanthine oxidase were prepared as follows. Deflavo enzyme at a concentration typically on the order of 50 μM in functional molybdenum was placed in an anaerobic quartz cuvette (0.4-cm light path) possessing a side port and then sealed with a rubber septum and made anaerobic with cycles of evacuating and flushing with argon on an anaerobic train. After 6–10 cycles over the course of an hour, the enzyme was titrated with a concentrated stock solution of sodium dithionite (using a Hamilton syringe equipped with a threaded drive) to give approximately 50% of the full absorbance change at 450 nm expected for complete reduction of the enzyme. Aliquots were removed through the rubber septum with a 500-μL gastight syringe and placed in septum-sealed, argon-flushed EPR tubes, where any further addition of reagents was carried out prior to freezing in liquid nitrogen. This procedure was found to be adequate for the present purposes given the low oxygen reactivity of reduced deflavo xanthine oxidase (Komai et al., 1969). EPR spin quantitation was performed using the semiquinone of recombinant *Desulfovibrio vulgaris* flavodoxin (Krey & Swenson, 1990) of known concentration as an integration standard (ϵ_{580nm} = 4.70×10^3 M^{-1} cm^{-1}; Dubourdieu & LeGall, 1970); EPR tubes were calibrated using a 1.0 mM $CuCl_2$ and 10 mM EDTA solution. Freeze–quench EPR experiments were performed with an apparatus generally resembling that described previously (Bray, 1961a; Bray & George, 1985) but equipped with a computer-interfaced stepper motor to initiate mixing of reagents. In the longer time studies with 2-hydroxy-6-methylpurine, advantage was taken of the extremely slow rate of reaction and the following manual mixing method was employed. Microliter aliquots of a concentrated substrate solution appropriate to give the desired final concentration of substrate were added to 0.5-mL aliquots of enzyme with vigorous stirring, and the reaction mix was transferred by micropipette to EPR tubes for freezing in dry ice/acetone at the appropriate time intervals. Given the extremely long time scale over which the 2-hydroxy-6-methylpurine/xanthine oxidase reaction takes place at 5 °C (approximately 500 s to go to completion), the uncertainty in time using this procedure (approximately 2 s) is negligible for the present purposes. This procedure has the significant advantage that uncertainties in the EPR spin quantitation owing to variations in the packing density from sample to sample in conventional rapid quench work are avoided.

Initial estimates of g-values for the various signals examined in the present study were determined from the slope of the field position of each feature in the spectra of samples prepared in D_2O over a microwave frequency range from 3 to 35 GHz (S-, X-, and Q-band spectra were acquired, although only the X-band data are presented). Initial estimates for the average values for the hyperfine splitting were determined by inspection from the Q-band spectra obtained from samples prepared in H_2O, where there was minimal overlap of spectral features. g- and A-values were refined by simulation of the EPR spectra using the FORTRAN program EPRSIM, kindly provided by Dr. Graham George (Stanford Synchrotron Radiation Laboratory, Menlo Park, CA), running on a VAXstation 3200 minicomputer. In all cases, good fits to the data were obtained after only minor adjustments to the initial estimates. The final parameters obtained by computer simulation are summarized in Table I. A-values were isotropic to within 10% and the averages are reported in Table I.

Table I: EPR Parameters for the EPR Signals Obtained on Binding Substrate to Deflavo Xanthine Oxidase Partially Reduced by Prior Titration with Sodium Dithionite

	g			
	x	y	z	$a(^1H)_{ave}$ (G)
"rapid Type 1" 1-methylxanthine[a]	1.9654	1.9707	1.9906	13; 3.0
"rapid Type 1" 1-methylxanthine[b]	1.9657	1.9703	1.9893	13; 3.0
"rapid Type 2" xanthine[c]	1.9616	1.9712	1.9951	10
"rapid Type 2" xanthine[b]	1.9639	1.9712	1.9967	10
"rapid Type 1" 2-hydroxy-6-methylpurine[b]	1.9665	1.9710	1.9915	12.8; 2.9

[a] Gutteridge et al. (1978). [b] This work. [c] Bray et al. (1978).

2-Hydroxy-6-methylpurine was purchased from the Alfred Bader division of Aldrich Chemical Co. The reagent was dissolved in dilute base and decolorized over activated charcoal, then acid precipitated, filtered, and dried prior to use. Xanthine and 1-methylxanthine were obtained from Sigma and used without further purification. Deuteration of substrates was carried out as described previously (Edmondson et al., 1973; McWhirter & Hille, 1991) by dissolving in alkaline D_2O (99%) and refluxing for 6 h at 1 atm in a sealed tube, followed by acid precipitation and filtration. The extent of deuteration was determined by NMR (carried out at the Campus Chemical Instrumentation Center of the Ohio State University using a Brüker 500-MHz spectrometer), and was found to be greater than 95%. NMR also demonstrated that the methyl protons of 2-hydroxy-6-methylpurine had exchanged with solvent under the conditions of the exchange reaction. Sodium pyrophosphate buffer was from Sigma, and CAPS [3-(cyclohexylamino)-1-propanesulfonic acid) and EPPS [*N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid] were from Aldrich. Sodium dithionite was from Virginia Chemicals and D_2O was from Cambridge Isotope Laboratories. All other reagents were of the highest quality commercially available and were used without further purification.

RESULTS

Mo(V) EPR Spectra of Deflavo Xanthine Oxidase Complexed with Xanthine, 1-Methylxanthine, and 2-Hydroxy-6-methylpurine. The EPR signals observed in H_2O or D_2O (0.1 M EPPS and 0.1 N KCl, pH/D 8.5) at 9 GHz when deflavo xanthine oxidase is partially reduced with sodium dithionite and subsequently treated with xanthine, 1-methylxanthine, 2-hydroxy-6-methylpurine, or 8-bromoxanthine (at a final concentration of 2.0 mM in each case) are shown in Figure 1. The changes in spectral line shape and increase in signal intensity compared to an enzyme sample prepared

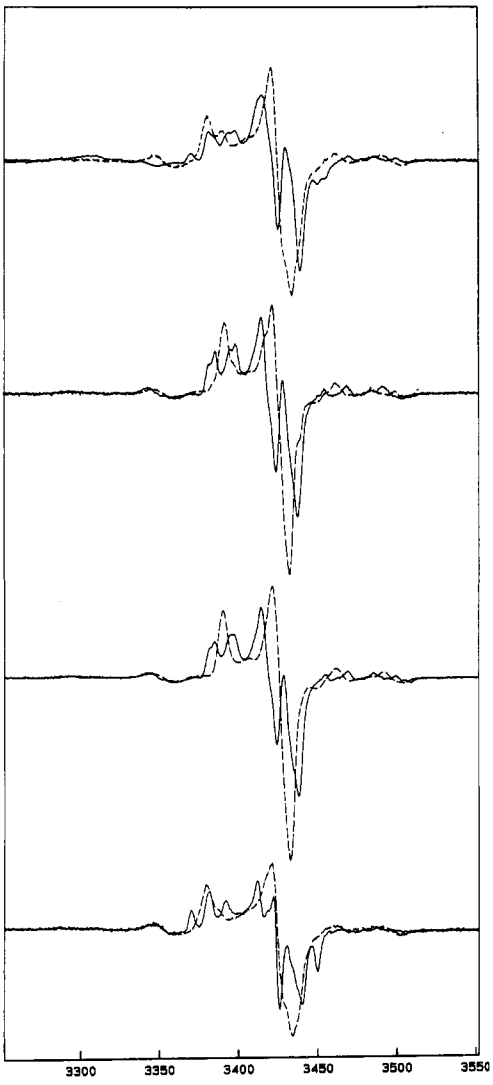


FIGURE 1: EPR spectra of partially reduced deflavo xanthine oxidase with substrates in H_2O (solid lines) and D_2O (dashed lines): from top to bottom, the complexes of partially reduced deflavo xanthine oxidase with xanthine, 1-methylxanthine, 2-hydroxy-6-methylpurine, and 8-bromoxanthine. The enzyme concentrations (in functional molybdenum sites) were 67 μM for the H_2O samples and 48 μM for the D_2O samples. Enzyme was partially reduced by titration with sodium dithionite to give 57% of the full absorbance change for reduction at 450 nm in the case of the H_2O samples and 53% in the case of the D_2O samples. Instrument settings were as follows: 2.0-G field modulation, 100-kHz modulation frequency, 400-G sweep, 150 K; microwave frequencies in gigahertz for the H_2O and D_2O signals, respectively, were 9.4580 and 9.4573 (xanthine), 9.4575 and 9.4582 (1-methylxanthine), 9.4569 and 9.4584 (2-hydroxy-6-methylpurine), and 9.4598 and 9.4587 (8-bromoxanthine). For each substrate or analog, spectra in D_2O and H_2O were normalized to give the same integrated signal intensity so as to facilitate comparison; the relative intensities of the samples in H_2O are accurately represented with respect to one another. Initial estimates for g-values were obtained from the D_2O spectra using the relationship $g = 714.484\nu/H_{res}$, where ν is the microwave frequency (in gigahertz) and H_{res} is the resonant field position of the spectral feature (in gauss); initial estimates for ^1H a-values were likewise obtained by inspection from the H_2O spectra. These parameters were refined by computer simulation of both D_2O and H_2O spectra and are presented in Table I.

in the absence of substrate (data not shown) demonstrate that each of the purines has in fact bound to the enzyme. It can be seen that 1-methylxanthine gives an essentially pure "rapid Type 1" EPR spectrum, with one strongly and one weakly coupled proton; for comparison, 8-bromoxanthine gives a "rapid Type 2" signal with two equally strongly coupled protons evident in the EPR signal. Xanthine gives a mixture of the Type 1 and Type 2 forms of the "rapid" signal (in a ratio of approximately 1:2, respectively, on the basis of the relative amplitudes of the g_1 features for the two types of signal in the D_2O spectrum shown in Figure 1). The results with 1-methylxanthine and xanthine are consistent with the type of the "rapid" signal observed when oxidized enzyme is reacted with excess concentrations of these substrates (Bray, 1988). Like 1-methylxanthine, 2-hydroxy-6-methylpurine also gives a "rapid Type 1" EPR signal, with one strongly coupled and one weakly coupled proton being evident. The g- and a-values for the signals seen with xanthine, 1-methylxanthine, and 2-hydroxy-6-methylpurine, obtained as described in the Materials and Methods section, are summarized in Table I. In all cases, simulations using these parameters gave excellent fits to the data, faithfully reproducing all major features of the spectra. The EPR parameters given in Table I for xanthine and 1-methylxanthine agree well with the literature (Bray et al., 1978; Gutteridge et al., 1978) and serve to emphasize that the EPR signals generated by the addition of substrates to partially reduced deflavo xanthine oxidase are identical in all respects to those observed transiently in the course of the reaction of xanthine oxidase with excess substrate.

Spin quantitation of the "rapid Type 1" EPR signal seen with 2-hydroxy-6-methylpurine indicates that approximately 42% of the functional enzyme molybdenum is in the form of the species giving rise to this EPR signal, somewhat greater than the maximum amount seen transiently in the reaction of xanthine oxidase with excess xanthine (Edmondson et al., 1973). The observation of substantial amounts of the "rapid Type 1" signal in this experiment contrasts, however, with the results of EPR kinetic studies using substoichiometric concentrations of 2-hydroxy-6-methylpurine (McWhirter & Hille, 1991) or xanthine (Kim & Hille, 1993). In these latter experiments, no "rapid" EPR signal is observed in the course of the reaction, even at very long times. The experiments utilizing substoichiometric concentrations of substrate were carried out in such a way that a given enzyme molecule could react with no more than a single molecule of substrate.[2] The absence of the "rapid Type 1" signal in these experiments has been taken as evidence that the signal-giving species is not an authentic catalytic intermediate but is instead simply the complex of substrate with enzyme possessing Mo(V) at the active site. As discussed below, the demonstration that partial reduction of xanthine oxidase with sodium dithionite prior to addition of substrate generates relatively high levels of the "rapid Type 1" signal is consistent with this interpretation.

Reaction of Oxidized and Partially Reduced Deflavo Xanthine Oxidase with Excess 2-Hydroxy-6-methylpurine at pH 10. In order to obtain a more complete picture of the reaction of 2-hydroxy-6-methylpurine with xanthine oxidase, the reaction was examined at long times under conditions of excess substrate, conditions analogous to those used in previous work with xanthine and 1-methylxanthine as substrates (Edmondson et al., 1973). The reaction was carried out at

[2] In these studies, single-turnover conditions were ensured by using a stoichiometric excess (5–10-fold) of enzyme over substrate so that it was statistically unlikely that a given enzyme molecule reacted with more than a single molecule of substrate. Single-turnover conditions are also ensured by the fact that product release is the rate-determining step in the reductive half-reaction of xanthine oxidase (Olson et al., 1974). Thus under conditions of substoichiometric substrate concentrations it is unlikely that there will be any free substrate left in solution by the time a given enzyme molecule completes its first turnover.

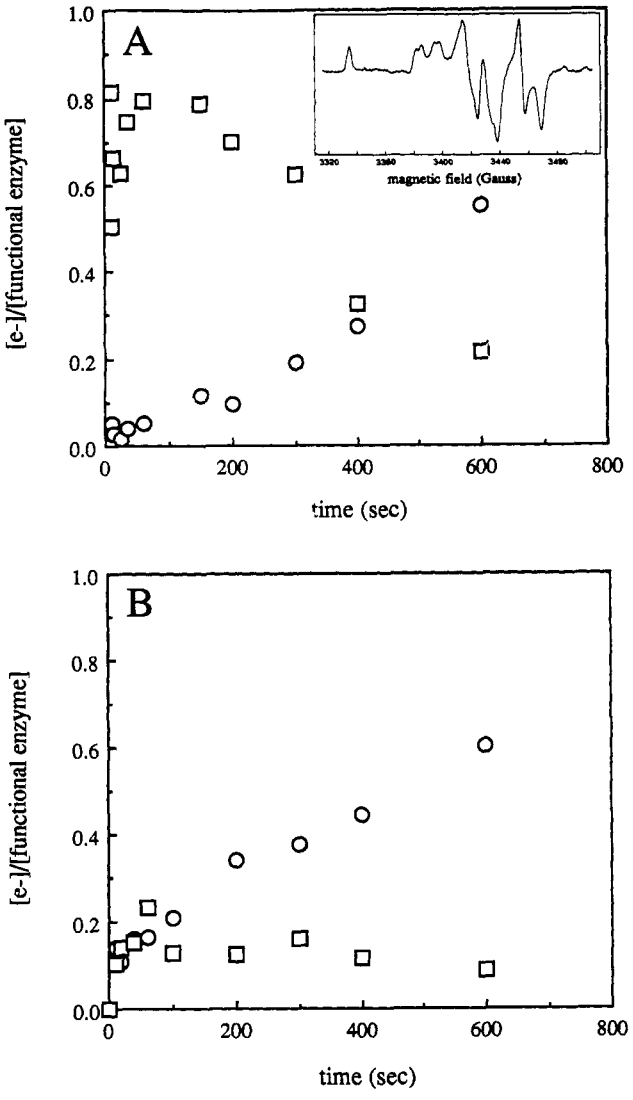


FIGURE 2: Reaction of oxidized and partially reduced xanthine oxidase with 2-hydroxy-6-methylpurine. Panel A, oxidized enzyme; Panel B, enzyme partially reduced (sufficient to give 58% of the total absorbance change at 450 nm expected for full reduction of enzyme) with sodium dithionite. Squares, "very rapid" signal; circles, "rapid Type 1" signal. The reaction conditions for both experiments were 31 μM active deflavo xanthine oxidase and 500 μM 2-hydroxy-6-methylpurine, 0.1 M CAPS and 0.1 N KCl, pH 10, at 2 °C. The reduced amount of "very rapid" signal seen in the reaction with partially reduced enzyme is consistent with the failure of enzyme possessing two or more reducing equivalents to form the species giving the "very rapid" signal, as discussed in the text. Inset, a representative spectrum (that correspoding to 400 s in the reaction of 2-hydroxy-6-methylpurine with oxidized enzyme) from which the data was obtained. The feature to lowest field and the two features to highest field are due to the "very rapid" signal. The instrument conditions were the same as given in Figure 1, except that the field sweep was 200 G rather than 400 G; the microwave frequency was 9.4580 GHz.

pH 10 rather than 8.5 (where enzyme exhibits maximal catalytic velocity) so that the "very rapid" Mo(V) EPR signal could be observed and its kinetics compared vis á vis the "rapid Type 1" signal. As shown in Figure 2A, when 31 μM functional, oxidized deflavo enzyme is reacted with 0.5 mM 2-hydroxy-6-methylpurine in 0.1 M CAPS, pH 10, at 2 °C, the "rapid Type 1" EPR signal begins to accumulate only after an extended lag phase. The "very rapid" signal, on the other hand, accumulates in the first 100 s of the reaction and subsequently decays to a very low level. The kinetics of the "very rapid" signal are consistent with the kinetics of its formation and decay as previously determined under single-turnover conditions (McWhirter & Hille, 1991). It is important to note that the "rapid Type 1" EPR signal generated catalytically in the reaction of enzyme with excess 2-hydroxy-6-methylpurine (Figure 2, inset) is indistinguishable from that shown in Figure 1, formed by addition of this substrate to partially reduced deflavo enzyme.

By contrast to the results seen with oxidized enzyme, when deflavo xanthine oxidase is partially reduced with sodium dithionite (to give 58% of the fractional absorbance change at 450 nm due to enzyme reduction) prior to reaction with 2-hydroxy-6-methylpurine, a significant burst of "rapid" EPR signal formation is observed, followed by a further increase in "rapid" EPR intensity at a rate comparable to that seen with fully oxidized enzyme (Figure 2B, circles). A comparison of the kinetics of "rapid Type 1" signal formation seen with oxidized and partially reduced enzyme as shown in Figure 2 indicates that partial reduction of the enzyme prior to reaction with this slow substrate substantially increases the amount of "rapid Type 1" signal that accumulates in the first 100 s of the reaction. At the shortest time intervals in the reaction with partially reduced enzyme, the integrated intensity of the "rapid Type 1" signal is in fact somewhat greater than that of the "very rapid" signal. Significantly, the burst phase observed in the reaction with partially reduced enzyme takes place too rapidly for the "rapid Type 1" species to have arisen from the breakdown of the species giving rise to the "very rapid" EPR signal (k_{obs} = 0.0085 s^{-1} for decay of the "very rapid" EPR signal at 2 °C; McWhirter & Hille).

Freeze–Quench Kinetic Studies of 2-Hydroxy-6-methylpurine, Xanthine, and 1-Methylxanthine Binding to Partially Reduced Deflavo Xanthine Oxidase at pH 8.5. The above results demonstrate that at pH 10 the early appearance of the "rapid Type 1" EPR signal upon addition of 2-hydroxy-6-methylpurine to deflavo xanthine oxidase is greatly facilitated by partially reducing enzyme prior to reaction with substrate. Similar results are obtained when the reaction of deflavo xanthine oxidase with 2-hydroxy-6-methylpurine is followed at pH 8.5. Figure 3 (top pair of spectra) compares the spectra obtained at 100 s in the reaction of either oxidized or partially reduced deflavo xanthine oxidase with 500 μM 2-hydroxy-6-methylpurine in 0.1 M EPPS and 0.1 N KCl, pH 8.5, at 2 °C (100 s is the point at which the "very rapid" signal accumulates maximally under these experimental conditons; data not shown). As in the case of the experiment at pH 10, partial reduction of the enzyme (to the level of 51% of the absorbance change at 450 nm expected for full reduction of the enzyme) greatly increases the amount of "rapid Type 1" signal observed. The amount of "rapid Type 1" signal observed at 100 s after mixing increases 7-fold upon partial reduction of xanthine oxidase, with the relative intensities of the "rapid Type 1" and "very rapid" EPR signals increasing from a ratio of 0.4:1.0 in the reaction with oxidized enzyme to 12:1.0 in the reaction with partially reduced enzyme.

Results similar to those obtained with 2-hydroxy-6-methylpurine are also observed with xanthine and 1-methylxanthine as substrates. Owing to the much faster reaction time of enzyme with these substrates, rapid quench experiments were performed, mixing partially reduced deflavo xanthine oxidase with substrate and freezing 22 ms afterwards (22 ms is near the point in the reaction at which the "very rapid" EPR signal accumulates maximally under the present experimental conditions with xanthine as substrate; Edmondson et al., 1973). Figure 3 shows the EPR signals observed 22 ms after mixing either oxidized or partially reduced deflavo xanthine oxidase (to give 55% of the absorbance change at 450 nm for full

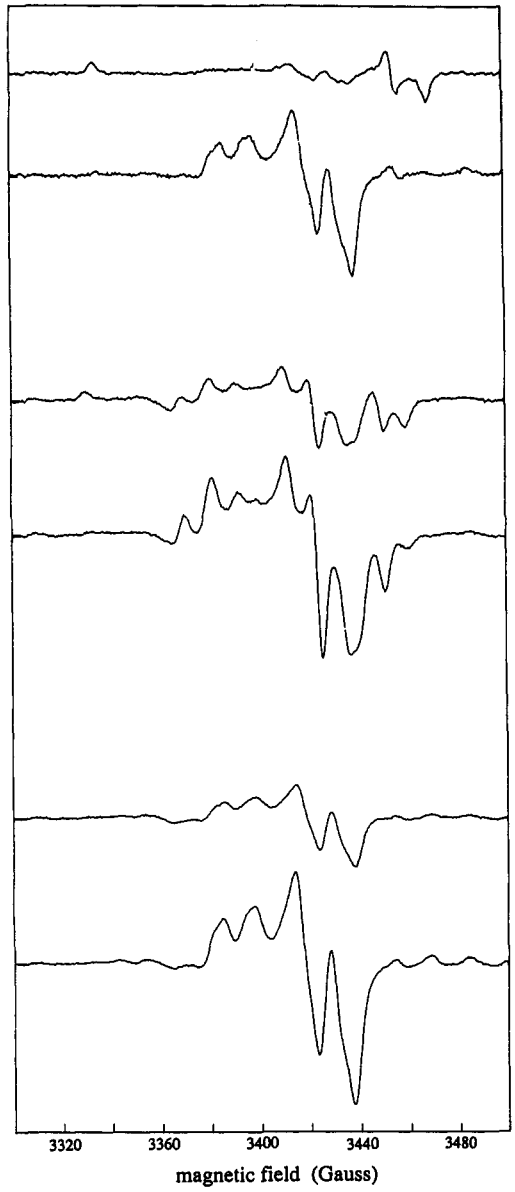


FIGURE 3: Reaction of oxidized and partially reduced deflavo xanthine oxidase with substrate at pH 8.5. Enzyme was reacted with 500 μM 2-hydroxy-6-methylpurine (top) 500 μM xanthine (center), or 500 μM 1-methylxanthine (bottom). For each pair of spectra shown, the upper corresponds to reaction of substrate with oxidized enzyme and the lower with partially reduced enzyme. The reaction conditions were 0.1 M EPPS and 0.1 N KCl, pH 8.5. For the reaction with 2-hydroxy-6-methylpurine, the reaction was carried out at 2 °C and samples were collected 100 s after manual addition of substrate to enzyme. The enzyme concentration was 67 μM, and for the second spectrum the enzyme was reduced with sodium dithionite to give 51% of the spectral change at 450 nm for full reduction of the enzyme. For the reaction with xanthine and 1-methylxanthine, the reaction was carried out at 25 °C and samples were collected 22 ms after mixing in a conventional rapid-quench apparatus. The enzyme concentration was 50 μM, and for the second spectrum the enzyme was reduced with sodium dithionite to give 55% of the spectral change at 450 nm for full reduction of the enzyme. Instrument settings for acquisition of all spectra were identical to those given in the legend to Figure 1.

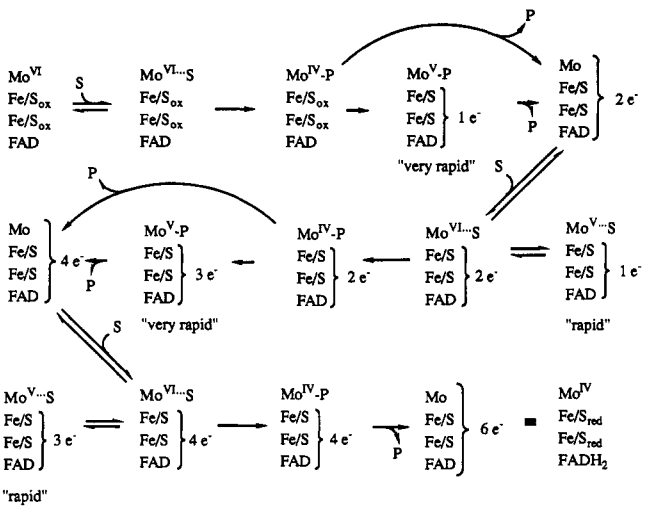
reduction of the enzyme) with 0.5 mM xanthine (center) or 1-methylxanthine (bottom) in 0.1 M EPPS and 0.1 N KCl, pH 8.5. In good agreement with the results shown in Figure 1, xanthine gives "rapid Type 1" and "Type 2" signals in a ratio of approximately 1:2, while 1-methylxanthine gives a "rapid Type 1" signal exclusively. With both substrates, the amount of "rapid" signal increases by a factor of approximately 2.5 upon partial reduction of the enzyme prior to reaction with substrate. In the case of xanthine, the ratio of "rapid" to "very rapid" signal increases from 0.6:1 to <10:1 with enzyme reduction. In agreement with earlier work (Edmondson et al., 1973), no "very rapid" signal is observed with 1-methylxanthine as substrate at pH 8.5. Thus with both xanthine and 1-methylxanthine, partial reduction of enzyme prior to reaction with substrate substantially increases the amount of "rapid Type 1" signal relative to the "very rapid" signal early in the course of the reaction. In the case of xanthine, as with 2-hydroxy-6-methylpurine, partial reduction of enzyme prior to reaction with substrate reverses the order of appearance of the two EPR signals: the "very rapid" signal accumulates to a greater extent at 22 ms after mixing with oxidized enzyme, and the "rapid Type 1" signal accumulates to a greater extent in the reaction with partially reduced enzyme.

When 2-hydroxy-6-methylpurine is added to partially reduced deflavo xanthine oxidase in D_2O (0.1 M EPPS and 0.1 N KCl, pD 8.5), the observed "rapid Type 1" EPR signal exhibits no ^1H coupling (Figure 1), indicating that both strongly and weakly coupled proton sites in the signal-giving species are solvent-exchangeable. Furthermore, indistinguishable spectra are observed when C_8-^1H- and C_8-^2H-labeled 2-hydroxy-6-methylpurine are used in the D_2O experiment (data not shown). Previous studies using a stoichiometric excess of xanthine have shown that the strongly coupled proton seen in the "rapid Type 1" signal is derived from the C_8 position of substrate but has been transferred to a site on the enzyme in the course of becoming strongly coupled (Gutteridge et al., 1978). The independence of the "rapid Type 1" signal seen with 2-hydroxy-6-methylpurine on the isotopic label at C_8 of substrate is consistent with the earlier work with xanthine and illustrates more clearly that it is only after being transferred to the molybdenum center that the proton is strongly coupled; while it remains on substrate the C_8-H is only weakly, if at all, coupled to the molybdenum.

DISCUSSION

The experiments described in the present work demonstrate that the species giving rise to the "rapid Type 1" Mo(V) EPR signal of xanthine oxidase can be generated prior to substantial accumulation of the species giving rise to the "very rapid" EPR signal simply by partial reduction of enzyme prior to reaction with substrate. With all three substrates examined, substantial amounts of the "rapid Type 1" signal can be generated too rapidly to be accounted for by catalytic formation of an intermediate lying downstream from the species giving rise to the "very rapid" EPR signal. The facile formation of the "rapid" species by addition of xanthine, 1-methylxanthine, or 2-hydroxy-6-methylpurine to partially reduced enzyme (as demonstrated here) or by reaction of a stoichiometric excess of substrate with oxidized enzyme (Palmer et al., 1964; Edmondson et al., 1973; Olson et al., 1974; Bray et al., 1978) contrasts with the failure to observe the "rapid" signal under single-turnover conditions with these substrates (McWhirter & Hille, 1991; Kim & Hille, 1993). If the species giving rise to the "rapid Type 1" signal were an intermediate lying downstream from that giving rise to the "very rapid" signal, it should have been observed in these single-turnover experiments. Taken together, these observations indicate that the "rapid Type 1" signal is formed only under those circumstances when substrate encounters partially reduced enzyme in forming the Michaelis complex. Partial reduction of enzyme can be accomplished either by reaction with at least one prior substrate molecule or, alternatively, by partial reduction of enzyme with

Scheme I



dithionite prior to reaction with substrate. Under single-turnover conditions, with enzyme in stoichiometric excess over substrate, all substrate molecules will react with fully oxidized enzyme and the species giving rise to the "rapid Type 1" signal is thus not expected to form. The species giving rise to the "rapid Type 1" EPR signal may thus be thought of as a Mo(V) analog of the Mo(VI)–substrate Michaelis complex. Mechanistically, this species is a dead-end complex rather than a bona fide catalytic intermediate, and it is unable to proceed in the catalytic sequence until the molybdenum center is reoxidized to Mo(VI) (by electron transfer from the molybdenum to one of the other redox-active centers of the enzyme). Elaborating upon the kinetic mechanism proposed for the reaction of xanthine oxidase with limiting 2-hydroxy-6-methylpurine (McWhirter & Hille, 1991), Scheme I for the reaction of xanthine oxidase with excess substrate is obtained. In this scheme, the species giving rise to the "rapid Type 1" and "very rapid" EPR signals are indicated.[3] It is to be emphasized that the "rapid" species is placed upstream rather than downstream from the "very rapid" species *in the oxidation of a given substrate molecule*. Such an assignment for the "rapid" species contrasts with conclusions of recent work in which the "rapid" species is placed downstream from the species giving the "very rapid" EPR signal in a given catalytic sequence (Bray et al., 1979; Bray & George, 1985; Bray, 1988) but is in agreement with earlier studies in which it was concluded that the "rapid" species was a Mo(V)···substrate complex (Pick & Bray, 1969; Gutteridge et al., 1978). The "very rapid" species is shown in Scheme I as an obligatory reaction intermediate, as has been demonstrated to be the case with 2-hydroxy-6-methylpurine as substrate at pH 10 (McWhirter & Hille, 1991), but is partially circumvented in the cases of xanthine and 1-methylxanthine by direct dissociation of product from the Mo(IV)–P complex (Scheme I, dashed arrows). Such a bifurcation in the reaction mechanism is now generally accepted as the explanation for the varying yields of "very rapid" EPR signal with experimental conditions and substrate type (Bray, 1988).

In the reaction of oxidized enzyme with excess substrate, only the latter two of the three catalytic sequences undertaken by each enzyme molecule are able to produce the "rapid" species. Also, the final catalytic sequence cannot form the "very rapid" species, since the flavin and iron–sulfur centers are fully reduced at this stage of the reaction and unable to accept a reducing equivalent from Mo(IV)–P. In the case of deflavo enzyme, only the first catalytic sequence can form the "very rapid" species. This accounts for the significantly lower amount of "very rapid" signal observed when deflavo enzyme is partially reduced prior to reaction with substrate in the experiment shown in Figures 2 and 3, since that portion of the enzyme population possessing two or more reducing equivalents will not be able to form the "very rapid" species. Scheme I adequately explains why partial reduction of enzyme prior to reaction with substrate gives rise to a burst in the appearance of the "rapid" EPR signal: prior reduction of the enzyme permits formation of the species giving rise to the "rapid Type 1" signal simply by binding of substrate to that portion of the enzyme population already possessing Mo(V). Olson et al. (1974) have demonstrated that a scheme similar to that given here accurately describes the kinetics of the appearance and disappearance of the "rapid" EPR signal in the course of the reaction of xanthine oxidase with excess xanthine. [These workers assumed, however, that the "rapid" signal arose principally from partially reduced enzyme lacking substrate bound at the active site, a conclusion that is not consistent with subsequent work clearly demonstrating that the form of the "rapid" signal observed is substrate-dependent (Bray et al., 1978; Bray, 1988).] The uncomplexed forms of partially reduced xanthine oxidase in Scheme I will possess a certain amount of Mo(V), but this must be small in light of the obvious effect of different substrates on the magnitude and form of the "rapid" signal (Figure 1). Scheme I appropriately emphasizes that the species giving rise to the "rapid" EPR signal is not an intermediate per se but rather a dead-end complex, unable to proceed with the catalytic sequence until the molybdenum center becomes fully oxidized by transfer of a reducing equivalent to one of the other redox-active centers of the enzyme. The scheme also provides a clear explanation as to why the "very rapid" and "rapid Type 1" do not constitute a conjugate acid/base pair (Tsopanakis et al., 1978): on the basis of a variety of evidence the former species has been shown to contain product coordinated to molybdenum via its newly introduced hydroxyl group (i.e., as Mo–OR; Bray & Gutteridge, 1982; Bray & George, 1985; Hille & Sprecher, 1987; Oertling & Hille, 1990), while the latter simply has substrate bound to the enzyme (but not necessarily coordinated to the molybdenum itself).

From the above, it follows that formation of the C_8–O bond of product has taken place at the point in the reaction mechanism that the "rapid Type 1" species is formed *for a given substrate molecule*. The species giving rise to the "rapid Type 1" signal is not formed until product release at the end of a given catalytic sequence and a second (or third) molecule of substrate is bound. This conclusion raises questions regarding the nature of the strongly coupled oxygen atom observed when the "rapid Type 1" species is generated in ^{17}O-labeled water. This oxygen exhibits an a_{av} of 14 G with nearly isotropic splitting of each of the principle g-values (Bray & Gutteridge, 1982). The strongly coupled oxygen was originally assigned to a Mo=O group that is generally recognized to be

[3] In Scheme I, only those distributions of reducing equivalents among the several redox-active sites which give rise to Mo(V) are shown. The distribution of reducing equivalents among the several redox-active centers of partially reduced xanthine oxidase has been shown to be determined by the relative reduction potentials of the centers (Olson et al., 1974; Hille et al., 1981). It has also been demonstrated that the equilibrium distribution of reducing equivalents within partially reduced enzyme is rapidly attained relative to the rate of overall catalysis (Hille & Massey, 1985; Hille & Anderson, 1990). The distribution of reducing equivalents in the several partially reduced intermediates in Scheme I is thus expected to at least approximate that predicted at equilibrium on the basis of the relative reduction potentials. For those intermediates complexed with either substrate or product, this distribution is perturbed by the known effect of substrate/product binding on the reduction potentials of the molybdenum center (Barber & Siegel, 1982; Hille & Stewart, 1984).

present in the signal-giving species, but recent work with oxomolybdenum model compounds $[LMoOX]^-$ (X = S or O, L = *N,N'*-dimethyl-*N,N'*-bis(2-mercaptophenyl)ethylenediamine; Dowerah et al., 1987; Wilson et al., 1991) has demonstrated that the Mo=O oxygen is in fact only weakly coupled in the EPR spectra of these Mo(V) complexes. In particular, the ^{17}O splitting in $[LMoOS]^-$ and $[LMoO(SH)]^-$ is relatively small (a_{av} = 4.3 and 2.2 G, respectively; Dowerah et al., 1987, Wilson et al., 1991) compared to that observed in the case of the "rapid Type 1" signal of the enzyme. Consequently, these workers have suggested that the strongly coupled oxygen present in the enzyme species giving the "rapid Type 1" signal is the bridging oxygen of a Mo–OR group, –OR representing product coordinated to molybdenum via the newly introduced hydroxyl group. The present work indicates that this formulation for the "rapid Type 1" species is unlikely, given that the C_8–O bond has not been formed in the signal-giving species. It is possible that the strongly coupled oxygen arises from hydroxide present in the molybdenum coordination sphere, and there is some evidence (albeit weak) from X-ray absorption studies for an additional oxygen (or nitrogen) coordinated to the molybdenum (Hille et al., 1989; Turner et al., 1989).[4] Work is presently in progress to further elucidate the chemical nature of the strongly coupled oxygen atom in the species giving rise to the "rapid Type 1" EPR signal and its role in the reaction mechanism.

The ability to generate relatively large amounts of kinetically stable "rapid Type 1" EPR signal in the absence of contaminating $FADH^•$ signal has also permitted a reexamination of the magnetic interaction between Mo(V) and the C_8-H of substrate. The failure to observe any detectable difference between the "rapid" signal generated by addition of the C_8-^1H and C_8-^2H forms of 2-hydroxy-6-methylpurine in D_2O supports previous work with xanthine as substrate (Gutteridge et al., 1978) and given the very low rate of reaction of enzyme with this substrate provides the clearest indication to date that the C_8-H is only very weakly coupled at best in the Mo(V)–S complex. It is only after substrate has been oxidized and the C_8 proton transferred from the purine ring that the proton becomes strongly coupled. This is in agreement with the earlier demonstration that both magnetically coupled protons seen in the "rapid Type 1" EPR signal reside on the enzyme rather than substrate (Gutteridge et al., 1978). The failure to detect ^1H coupling in the "rapid Type 1" signal generated in the experiment with ^1H-labeled substrate is also further evidence that catalysis has not been initiated in samples prepared as described in the present study, since this proton is known to occupy the strongly coupled site of the species giving rise to the "rapid Type 1" signal once removed from substrate (Gutteridge et al., 1978).

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[4] Hydroxide (or water) has previously been invoked as a ligand in the molybdenum coordination sphere and is displaced by substrate in forming the Michaelis complex. The evidence that substrate directly coordinates to the molybdenum (via N_7) is extremely weak, however, and is not consistent with the absence of ^{14}N hyperfine in the "rapid Type 1" EPR signal at all microwave frequencies examined (Wilson et al., 1991). The presence of a substrate-displaceable hydroxide would in any case be inconsistent with -OH being present in the substrate-complexed form of the enzyme giving rise to the "rapid Type 1" EPR signal.