Numbers and Exchangeability with Water of Oxygen-17 Atoms Coupled to Molybdenum(V) in Different Reduced Forms of Xanthine Oxidase†

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ABSTRACT: The effect of using [^{17}O]water (24–50% enriched) as solvent on the Mo(V) electron paramagnetic resonance spectra of different reduced forms of xanthine oxidase has been investigated. All the Mo(V) signals are affected. Procedures are described, based on the use of difference spectral techniques, that facilitate interpretation of such spectra. The number of coupled oxygen atoms may be determined by estimation of the fraction of the spectrum that remains unchanged by the isotope at a known enrichment. For a species having two coupled oxygen atoms, the use of two different isotope enrichments permits elimination from the difference spectra of the contribution of the two singly substituted species. From the application of these methods, it is concluded that not only the strength of the hyperfine coupling of oxygen ligands of molybdenum but also their number and their exchangeability with the solvent vary from one reduced form of the enzyme to another. The inhibited species from active xanthine oxidase has been studied in the most detail. It has two weakly coupled oxygen atoms [$A(^{17}O)_{av}$ = 0.1–0.2 mT] that do not exchange with the solvent. A cyclic structure is proposed for this species in which two oxygen ligands of molybdenum are bonded to the carbon of the formaldehyde or other alcohol or aldehyde molecule that reacted in producing the signal. Structures of the other signal-giving species from active xanthine oxidase (Very Rapid and Rapid types 1 and 2) are discussed, as is corresponding information on species from the desulfo enzyme and from sulfite oxidase.

The structures and mechanisms of action of molybdenum centers in enzymes have been extensively studied in recent years (Bray, 1980). The molybdenum atoms are known to have oxygen as well as sulfur ligands, and there is every reason to expect that some of the former at least play a definite role in the catalytic reactions. Experimental investigation of oxygen ligands has depended either on X-ray spectroscopy or alternatively on EPR[1] investigations of the coupling of molybdenum in the Mo(V) oxidation state to the stable oxygen isotope ^{17}O. With the former method,[2] precise quantification of the number of oxygen ligand atoms is not always possible (Tullius et al., 1979; Bordas et al., 1980). Thus, Bordas et al. (1980) were uncertain whether active xanthine oxidase in the oxidized state contains one or two terminal oxygen ligands in addition to its terminal sulfur atom. EPR studies employing ^{17}O, particularly more recent ones (Gutteridge & Bray, 1980a; Gutteridge et al., 1980), have increased the understanding of the enzymes considerably; however, quantification of the number of oxygen ligands has not been stressed in this or in earlier work (Gutteridge et al., 1979; Cramer et al., 1979a) with the isotope. Nevertheless, the number of oxygen ligand atoms in different reduced forms of the enzymes is of considerable interest. Thus, for example, it is important to know whether loss of terminal sulfur from active xanthine oxidase on conversion to the desulfo form (Gutteridge et al., 1978a) is accompanied, as was originally proposed, by its replacement by terminal oxygen or whether no such replacement takes place (EXAFS has not so far clearly distinguished these possibilities[2]).

Studies of exchangeability of oxygen ligands with oxygen from solvent water molecules also forms a part of the studies with ^{17}O on enzymes. Gutteridge & Bray (1980a), in an observation important in relation to the catalytic mechanism of xanthine oxidase, found that an oxygen ligand in the oxidized enzyme exchanged relatively slowly, whereas in the reduced state exchange of this oxygen was extremely rapid. Similarly, ^{17}O from water exchanged into the high-pH and the low-pH forms of sulfite oxidase but not into the inhibitory phosphate complex of this enzyme (Gutteridge et al., 1980).

The present work was undertaken with a view to obtaining more information on the numbers of oxygen ligands in the different reduced forms of xanthine oxidase and on their exchangeability with solvent water. In the course of the work we have also evaluated some of the hyperfine coupling parameters for the oxygen ligands. These should ultimately be useful in more theoretical approaches to the determination of the structures of the signal-giving species. Some of our conclusions have been summarized briefly in the proceedings of a symposium (Bray, 1982).

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[1] Abbreviations: EPR, electron paramagnetic resonance; EXAFS, X-ray absorption edge extended fine structure; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; Ches, 2-(cyclohexylamino)ethanesulfonic acid; DPPH, diphenylpicrylhydrazyl; FADH, reduced flavin adenine dinucleotide.

[2] Since this paper was submitted, recent work by Cramer et al. (1981) has come to our attention. These workers employed EXAFS to deduce information on the ligands of molybdenum in oxidized and in dithionite-reduced active and desulfo xanthine dehydrogenase. With regard to oxygen ligands, they concluded that oxidized [Mo(VI)] active and desulfo enzyme contained, respectively, one and two terminal oxygen ligands with the corresponding reduced [Mo(IV)] forms both containing one terminal oxygen. Their conclusion relating to the oxidized enzyme is thus in agreement with the original proposal (Gutteridge et al., 1978a) that cyanolysis of a terminal sulfur ligand in the active enzyme leads to replacement by a terminal oxygen, a proposal that is further supported by the present work. Though EPR and EXAFS thus provide complementary information on ligation of molybdenum in these enzymes, only the former has so far provided information relating directly to coordination of the metal in the Mo(V) state. Furthermore, existence of multiple species of molybdenum, which are readily distinguished at the Mo(V) level by EPR and which may be studied individually [e.g., Rapid types 1 and 2 species or the phosphate and low-pH species of sulfite oxidase (Bray, 1980)], must in contrast complicate and tend to confuse the interpretation of EXAFS.

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Materials and Methods

Enzyme Samples. Xanthine oxidase was prepared by the salicylate denaturation method (Hart et al., 1970; Bray, 1982). When the desulfo enzyme was required, this was prepared by treatment with cyanide according to Malthouse & Bray (1980). When necessary, concentrated samples of the enzyme in dilute (e.g., 10 mM) NaBicine buffer, pH 8.2, were freeze-dried. In some cases we freeze-dried the enzyme in bead form (Bordas et al., 1980), but as this was rather slow (see below), conventional procedures proved more satisfactory.

Enrichment with ^{17}O. Water, 54% enriched in ^{17}O, was obtained from Monsanto Research Corp., Mound Facility, Miamisburg, OH 45342. Samples of freeze-dried enzyme were dissolved in the enriched water. Reducing and other reagents to be added were in some cases prepared as concentrated solutions in ordinary water; when this was done, correction was made for the dilution of the isotope. Since freeze-dried xanthine oxidase was found (by weight loss after drying at 110 °C) to have a water content of 5%, correction was also made for this dilution of the isotope.

Generation of EPR Signal-Giving Species. We generally worked with freeze-dried xanthine oxidase and for each signal-giving species prepared side by side samples in ordinary water and in ^{17}O-enriched water. The use of concentrated samples (up to about 1 mM functional active centers) was advantageous where the more complex difference techniques were to be applied. Since we found that EPR samples prepared from freeze-dried enzyme sometimes had unexpectedly low pH values, only pH values measured after the samples were thawed, when the EPR spectra had been recorded, are meaningful. Unless otherwise states, all experiments were carried out in the Bicine buffer in which the enzyme had been freeze-dried. Generally the freeze-dried enzyme was dissolved and allowed to stand in the enriched water for about 1 h before additions were made, so as to ensure (Gutteridge & Bray, 1980a) complete equilibration with the isotope. Signals were generated as follows.

The purine Rapid type 1 signal (Bray & Vänngård, 1969) was obtained by reduction with the substrate (10 mM) anaerobically for 1 min at about 20 °C. For the borate Rapid type 2 signal (Malthouse et al., 1980; S. Gutteridge, unpublished results) some problems were encountered because of the low pH values, which tended to lead to contamination with a type 1 species. Finally, small amounts of solid borax were mixed with the freeze-dried enzyme before dissolving, and the signal was generated by reduction with small amounts of dithionite solution, added anaerobically with a microsyringe, samples being frozen after about 1 min. The final boric acid concentration was about 100 mM and the final pH value 7.0–7.5.

The Slow signal was obtained by reduction of the desulfo enzyme for 20 min with dithionite at a final concentration of about 4 mM. For the nitrate Slow signal (Gutteridge et al., 1978a), $NaNO_3$ was present at a concentration of 250 mM. The desulfo inhibited signal was obtained (Lowe et al., 1976) by first developing the slow signal as above, then adding ethylene glycol, anaerobically, to a final concentration of 9 M, and freezing after a further 3 h.

The Inhibited signal was obtained with deuterium-substituted formaldehyde prepared from paraformaldehyde (Malthouse et al., 1981b; Pick et al., 1971). For the samples employing ^{17}O, the hydrolysis was carried out in enriched water, in addition to the freeze-dried enzyme being dissolved in this medium. Enzyme freeze-dried in 50 mM NaChes buffer, pH 9.0, was employed. Generation of the signal was achieved by treatment with the aldehyde at a concentration of 200 mM for 30 min at 20–25 °C in open EPR tubes. When gel filtration was carried out, this was done with Sephadex G-25 in columns 0.5 cm × 2.5 cm, and subsequent concentration used Minicon B-15 concentrators (from Amicon Ltd.) Final pH values were 7.4–7.6.

EPR Spectra. All spectra were recorded with a dual sample cavity containing manganese and DPPH standards (Swartz et al., 1972), on a Varian E9 spectrometer linked to a computer system (Bray et al., 1978). Spectra, as reproduced in the figures after photographic reduction, were plotted directly by the computer system without the use of smoothing procedures. Running conditions for most of the samples (including those giving the Inhibited and Rapid type 2 spectra) were as follows: microwave frequency 9.3 GHz, microwave power 10 mW, modulation amplitude 0.16 mT, temperature about 120 K. For a few samples the power was increased to 20 mW and the modulation to 0.25 mT.

Before obtaining difference spectra with the computer system, alignment was always adjusted with the help of spectra from the reference channel. Alignment could with care be made to ±0.01 mT, though instrument performance (linearity of the scans) may sometimes have been a limiting factor (see Results). Integrations of the spectra were performed on the computer. Our software (Bray et al., 1978) allowed us to check carefully on the effect of carrying out the integrations between slightly different field values. Such tests indicated that, for spectra of good quality, the error for the ratio of integrals of two spectra was unlikely to exceed ±10%.

For some samples we used the computer to subtract an FADH· signal from the observed signal to obtain the pure Mo(V) signal. Amounts subtracted were largest for the borate Rapid signal, but smaller amounts were also subtracted from the purine Rapid and from the Slow signals. We also observed another relatively weak radical signal ($g = 2.011$, peak to peak width 0.17 mT) in samples exposed to extended freeze-drying. This signal was presumably due to some form of degradation, perhaps relating to sulfur, in a small proportion of the enzyme molecules. However, since the signal was present both before and after reducing the samples, it could, like the FADH· signal, also be subtracted out, and such corrections were applied to some of the Rapid and Slow signals. No corrections were applied to the Inhibited signal.

Quantification of the Number of Coupled ^{17}O Atoms. For each reduced form of the enzyme, if only a single exchangeable coupled oxygen atom is present and water of enrichment less than 100% is employed, then only two EPR signal-giving species will contribute, with the ^{16}O and ^{17}O spectra superimposed. If the fractional enrichment with ^{17}O is a, then the relative intensities of the two spectra will be $1 - a$ and a. If the spectrum of a control sample in ordinary water is examined along with the spectrum of an enriched sample, it is a simple matter to adjust the amplitudes of the spectra, with the computer system, so that they have equal integrated intensities. The spectrum corresponding to 100% enrichment with ^{17}O may then be obtained by subtracting, on the computer, a fraction $(1 - a)$ of the spectrum of the unenriched sample from that of the enriched one.

On the other hand, if there is more than a single exchangeable oxygen, the situation is more complicated. In general, if there are n coupled atoms, 2^n species will contribute to the spectrum. For example, if $n = 2$, then four species will be present, which we will designate, respectively, as Enz $^{17}O^{17}O$, Enz $^{17}O^{16}O$, Enz $^{16}O^{17}O$, and Enz $^{16}O^{16}O$. Fractional concentrations of these four species will be a^2, $a(1 - a)$, $a(1$

$-a$), and $(1-a)^2$, respectively. Obviously, the greater the value of n, the more difficult it is to interpret the spectra. However, it is frequently possible to identify in the resultant spectrum the contribution of the species in which no ^{17}O atoms have exchanged (Enz $^{16}O^{16}O$ for $n = 2$). In general, the fraction of the spectrum that is unchanged will be $(1-a)^n$. The method we employed to determine n is based on this principle. Control and experimental spectra were adjusted to equal integrated intensities; then difference spectra were produced by subtracting this proportion of the control spectrum from the experimental, for a series of different assumed values of n. These difference spectra were then examined for features likely to be due either to the unenriched spectrum or to the inverted form of this. If "unenriched" features were present, it was concluded that the value of n was too great. Conversely, if "inverse" features were present, it was concluded that n was too small.

Simulation of Spectra for Two Coupled Oxygen Atoms. If it has been established that $n = 2$ and if spectra at two separate values of the enrichment, a, are available, it is then in principle possible to eliminate contributions both of Enz $^{16}O^{16}O$ and of the two "mixed" species (Enz $^{17}O^{16}O$ and Enz $^{16}O^{17}O$, which will be present at equal concentrations) and so to obtain the pure spectrum of the species Enz $^{17}O^{17}O$. The procedure is as follows. If one assumes spectra at enrichments a_1 and a_2 have been obtained (where $a_1 > a_2$), then, from each spectrum, the contribution of the unenriched species is first eliminated, as described above. The two corrected spectra so obtained are then adjusted to equal integrated intensities. In each of these spectra we have the fraction of Enz $^{17}O^{17}O$ as $a/(2-a)$ and the total fraction of the mixed species as $2(1-a)/(2-a)$. It can then readily be shown that the fraction of the spectrum obtained at low enrichment to be subtracted from that obtained at high enrichment in order to produce the spectrum of pure Enz $^{17}O^{17}O$ is given by

$$(1-a_1)(2-a_2)/[(1-a_2)(2-a_1)]$$

Conversely, to obtain pure mixed Enz $^{16}O^{17}O$ plus Enz $^{17}O^{16}O$, it is necessary to perform the subtraction the other way round, subtracting

$$a_2(2-a_1)/[a_1(2-a_2)]$$

It must of course be emphasized that the two final spectra obtained by these "double-difference" techniques represented, under the conditions used, only a small fraction of the initial signal intensity. Hence, for results to be meaningful it was essential to start from spectra of the highest quality, since any noise or other irregularities in the originals would be greatly magnified in the final difference spectra.

Simulation of the two difference spectra obtained as described above (and which are of course not independent of one another) was then carried out (Lowe, 1978) by conventional trial and error procedures, by starting from the known parameters of the unsplit spectrum and adding ^{17}O hyperfine coupling, with assumed values of the coupling constants, until the best fits were obtained.

Results

Form of Spectra in ^{17}O-Enriched Water. All the molybdenum(V) spectra of xanthine oxidase were found to be modified by the use of ^{17}O-enriched water (see Figures 1–3). The nature of the modification ranged from the appearance of well-separated and readily interpreted six-line hyperfine splitting patterns (for the Very Rapid signal, Gutteridge & Bray, 1980a) through complex splitting patterns in the case of the Rapid and Slow signals to broadenings only of the spectra in the case of Inhibited and Desulfo-Inhibited signals. In general we have not interpreted the spectra fully. Particularly for spectra involving strongly coupled exchangeable protons, it may be difficult to arrive at full interpretations without making use of doubly labeled water ($^2H_2{}^{17}O$), which is however not commercially available.

Number of Exchangeable Oxygen Ligand Atoms Coupled to Molybdenum. In the case of simple spectra, particularly where only one coupled oxygen atom is involved, direct simulation of the spectra as used by Gutteridge & Bray (1980a) is the best way of determining n, the number of coupled oxygen ligand atoms. For more complex spectra, a procedure is described under Materials and Methods for determining n, on the basis of the identification of the amount of unmodified spectrum remaining in the spectra of enriched samples. Application of this procedure to two signals, Rapid type 1 (purine) and Rapid type 2 (borate), is illustrated in Figure 1. The unenriched spectra are given in (a) and again in inverted form in (c), while the spectra of enriched samples are presented in (b). (d–i) show calculated difference spectra after eliminating the theoretical amount of the unenriched spectrum, calculated for increasing assumed values of n. (n has been increased in steps of 0.5, though of course only integral values have real meaning.) To determine the true value of n, it is necessary to judge the value of the parameter at which neither "positive" nor "inverted" features from the unenriched spectrum are present in the difference spectrum. Clearly, both for the type 1 and the type 2 spectra, negative features are present at low values of n (Figure 1d) and positive values at high values of n (Figure 1i). Guidelines on Figure 1 draw attention to prominent features of the unenriched spectrum. However, it is probably wise not to concentrate on any one feature in isolation, since it is possible that the ^{17}O spectrum may contribute at the same point, making interpretation complex. Though obviously the criteria for determining n are somewhat subjective, we conclude that our data indicate a value of $n = 1$ for the Rapid type 1 spectrum (since Figure $1e_1$ shows neither positive nor negative features) and a value of $n = 2$ for the Rapid type 2 spectrum (see Figure $1g_2$).

We now consider factors that would influence the reliability of such estimates of n. First, it is obviously highly desirable that only a single signal-giving species should be investigated. The Rapid purine and borate spectra have both been shown, by studies in ^1H_2O and in ^2H_2O at 9 and 35 GHz, to approximate to a single species.

We tested the question as to how small oxygen splittings would have to be to escape detection by our method, by carrying out spectral simulations (not illustrated). From these we were able to conclude that broadening from an isotropically coupled oxygen with $A(^{17}O) = 0.05$ mT could readily be detected, whereas with $A(^{17}O) = 0.02$ mT broadening could not be detected. We also used simulated spectra to test the accuracy with which n could be determined. For this purpose, in some cases we deliberately misaligned the spectra before carrying out subtractions of the type illustrated in Figure 1. From this work we concluded that (for $A \geq 0.2$ mT) for alignment and integration errors within the limits specified under Materials and Methods, n could be determined to an accuracy of ±20%. This would be fully adequate to distinguish $n = 1$ from $n = 2$ but scarcely adequate for $n = 2$ from $n = 3$. This error estimate was confirmed by repeating some of the experiments; e.g., the determination of n for the borate rapid signal (Figure 1) was carried out several times, and in each case a value of $n = 2$ was indicated to the nearest whole number.

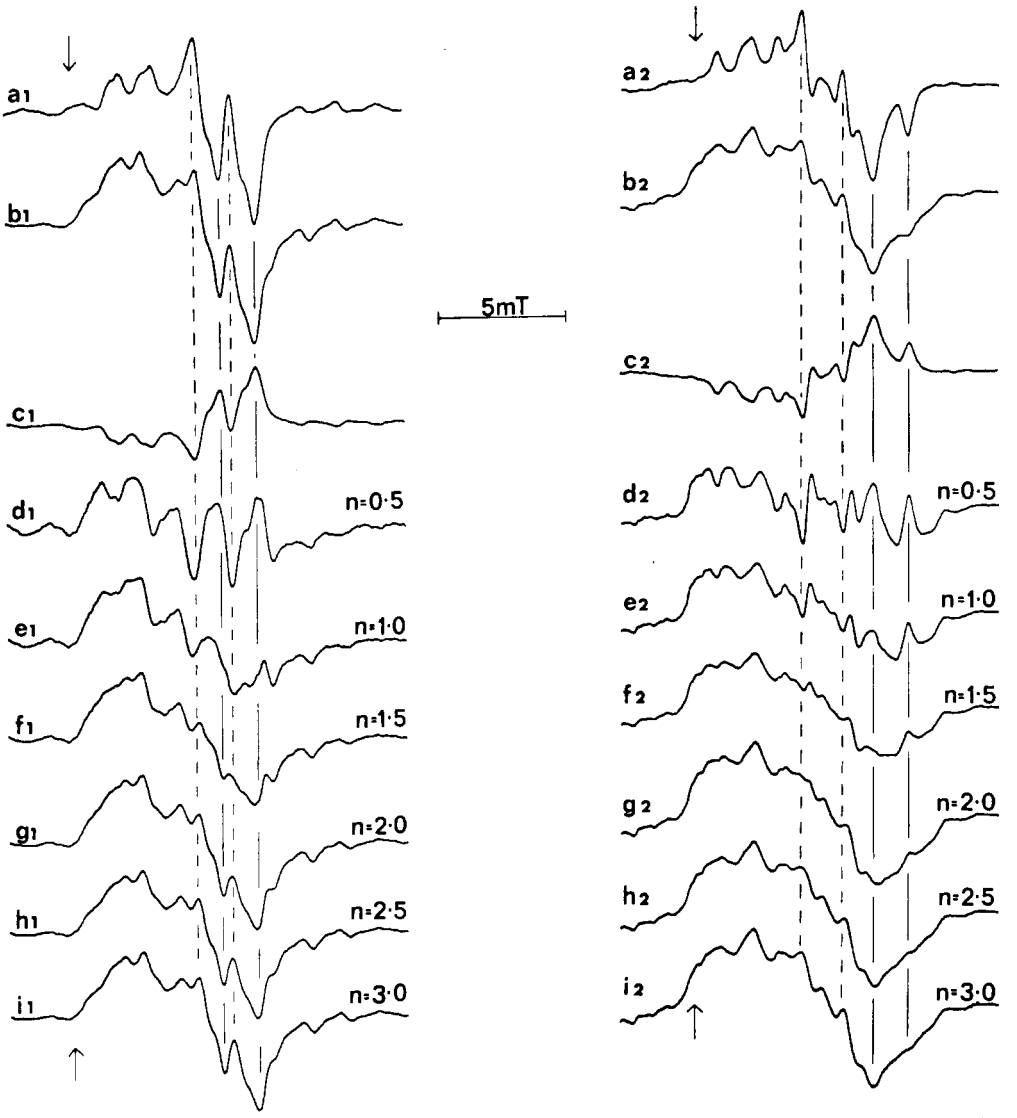


FIGURE 1: Determination of the numbers of coupled oxygen ligands for Rapid type 1 and type 2 species from active xanthine oxidase. Signals were generated as described under Materials and Methods. (a_1–i_1) are the Rapid type 1 signals obtained with purine and (a_2–i_2) the Rapid type 2 signals obtained with dithionite in the presence of borate. In both cases (a) corresponds to the spectrum in ordinary water, and (b) corresponds to that developed in [^{17}O]water [51% enrichment for (b_1) and 49% for (b_2)] while (c) is the spectrum of (a) shown in inverted form. (d–i) are difference spectra obtained by subtracting decreasing amounts of (a) from (b). As discussed in the text, when the difference spectrum contains no contribution either from (a) or from (c), then the contribution of the unenriched spectrum has been eliminated, and the corresponding value of n (calculated as described under Materials and Methods) will give the number of coupled oxygen ligands in the signal-giving species. Arrows here and in the other Figures corresponds to g = 2.0037. Amplifications of the traces shown (relative to the initial spectra adjusted to equal integrated intensities) are in (a_1–i_1) 0.58, 1.00, 0.29, 1.50, 1.50, 1.30, 1.20, 1.00, and 1.00 and in (a_2–i_2) 0.69, 1.00, 0.41, 1.50, 1.50, 1.30, 1.50, 1.30, and 1.30.

Figure 2 shows data comparable to that in Figure 1 on three signals from desulfo xanthine oxidase, namely, the Slow signal obtained in Bicine buffer, the Slow signal generated in the presence of nitrate, and the Desulfo-Inhibited signal. Only the difference spectra for optimal subtraction are illustrated, corresponding, respectively, for the three signals, to n = 2, n = 3, and n = 2. All of our data on xanthine oxidase, together with data on sulfite oxidase, are summarized in Table I.

^{17}O Coupling in the Inhibited Signal. We selected the Inhibited signal for detailed investigation since it represents a species derived from active xanthine oxidase without exchangeable protons and that, in the form obtained by employing deuterium-substituted formaldehyde, exhibits a simple rhombic spectrum. Studies (not illustrated) analogous to those in Figures 1 and 2 showed that development of the signal in the presence of ^{17}O-enriched formaldehyde and water produced broadening rather than resolved splitting of the signal and indicated a value of n = 2.

It is known (Pick et al., 1971) that the Inhibited signal, unlike the Very Rapid, Rapid, or Slow signal, is reasonably stable in the presence of air except at elevated temperatures. We therefore investigated exchange of the coupled oxygen atoms by gel filtration. Comparison of Figure 3c with 3b and 3a indicates that gel filtration did not in any way diminish the ^{17}O effects. The entire procedure from thawing the sample to refreezing took about 40 min at 23 °C. Therefore, the enzyme-bound oxygen atoms must be quite stable to exchange with the solvent.

Although the spectra of Figure 3b and 3c are indeed closely similar to one another, nevertheless the amplified difference spectrum of Figure 3d brings to light another effect of gel filtration on the spectrum. Figure 3d, as is shown by comparison with Figure 3e, clearly shows the rapid signal. This indicates that small amounts of Rapid are present, along with the inhibited, before, but presumably not after, the gel-filtration precedure. This finding is not unexpected, since the

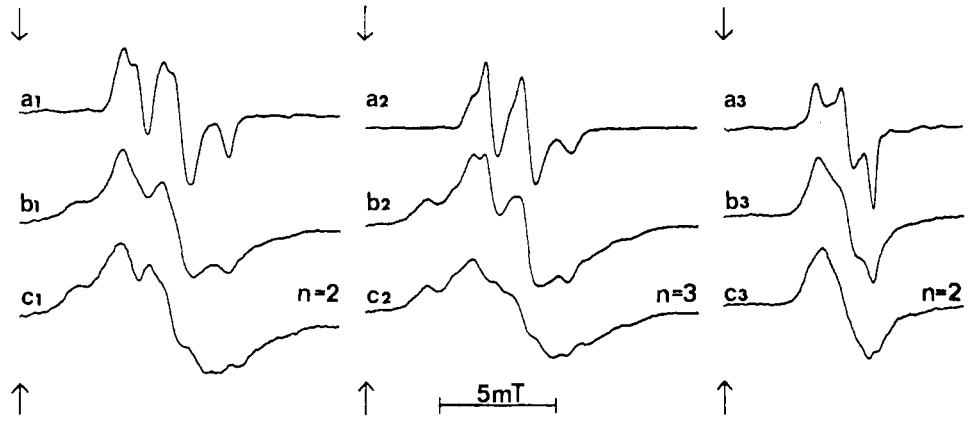


FIGURE 2: Number of coupled oxygen ligands for species from desulfo xanthine oxidase. Signals were generated as described under Materials and Methods. (a_1–c_1) are the Slow signal (in Bicine buffer), (a_2–c_2) are the Slow (nitrate) signal, and (a_3–c_3) are the Desulfo-Inhibited signal. In all cases (a) corresponds to the spectrum in ordinary water and (b) to that in [^{17}O]water (51% enrichment); (c) is the difference spectrum for optimum elimination (cf. Figure 1) of features of the unenriched from the enriched spectrum. Values of n used for (c_1), (c_2), and (c_3) are indicated.

Table I: Number of Oxygen Ligands and Hyperfine Couplings for ^{17}O in Various Mo(V)-Containing Enzyme Species[a]

enzyme	species	no. of ligands	$A(^{17}O)_{av}$ (mT)
active xanthine oxidase	Very Rapid[b]	1	1.37
	Rapid type 1 (purine)	1[c]	1.4[d,e]
	Rapid type 2 (borate)	2[c]	1[d]
	Inhibited (^{2}HC^{2}HO)	2[c]	0.2
desulfo xanthine oxidase	Slow (Bicine)	2[c]	1[d]
	Slow (NO_3^-)	3	1[d]
	Desulfo Inhibited	2	0.3[d]
sulfite oxidase	high pH	1	1.3[d,f]
	low pH	1	0.6[d,f]
	phosphate[g]	1 (?)	0.9

[a] The number of oxygen ligands was determined as described in the text and illustrated in Figure 1, by using ^{17}O at approximately 50% enrichment. In some cases measurements were also made with lower oxygen enrichments. [b] Data of Gutteridge & Bray (1980a). [c] Measurements made both with 50% and with 24–30% enrichment of ^{17}O. [d] Preliminary estimate, for the more strongly coupled oxygen where more than one is present, obtained by inspection of the spectra or from simulations that were only partly satisfactory. [e] Data of Gutteridge et al. (1979). [f] Data from a thesis from this laboratory (Lamy Freund, 1981). Spectra have also been presented by Cramer et al. (1979a). In the present work we estimated the number of ligands from both sets of data. [Computer processing of the spectra of Cramer et al. (1979a) was carried out after photographic enlargement and digitization.] [g] Data of Gutteridge et al. (1980).

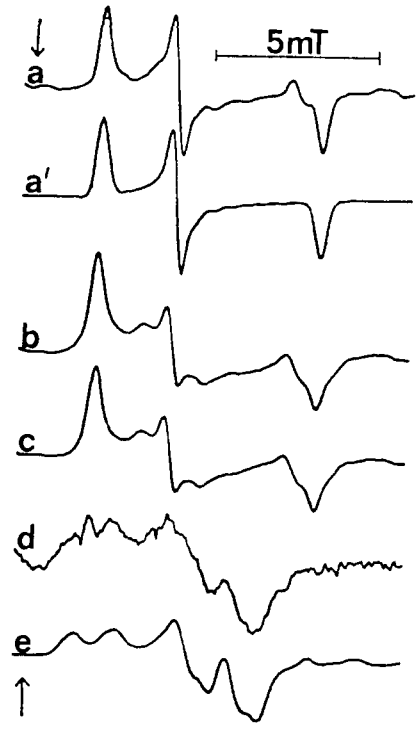


FIGURE 3: Effect of ^{17}O substitution and of subsequent gel filtration on the Inhibited signal obtained with ^{2}HC^{2}HO. (a) shows the Inhibited signal developed as described under Materials and Methods in ordinary water, and (a′) is the corresponding computer simulation (see Table II). (b) shows the signal developed in [^{17}O]water (50% enrichment). (c) shows a sample prepared identically with that in (b) but subsequently freed from excess ^{17}O and formaldehyde by gel filtration in ordinary water, followed by reconcentration. (d) is a difference spectrum obtained by subtracting 1.06 times (c) from (b) and increasing the vertical scale 10-fold. (e) shows for comparison the Rapid signal obtained by reducing xanthine oxidase anaerobically in ordinary water for 1 min with 2 mM glycolaldehyde at pH 8.2. [Note that the sample in (a), like that in (c), was gel filtered.]

conditions used for the formaldehyde treatment are scarcely sufficient [cf. Pick et al. (1971)] to achieve 100% inactivation. Thus, residual unmodified enzyme would be reduced by formaldehyde, yielding the Rapid signal, but would become reoxidized after gel filtration, leading to elimination of the signal. Because of these findings, for our further work with inhibited we used only gel-filtered samples, in order to ensure that we were dealing with a pure species.

We then used the procedure described under Materials and Methods, based on the use of ^{17}O at two different enrichments, to evaluate coupling constants for the two coupled oxygen atoms of the Inhibited signal. Figure 4 illustrates our results. (a) and (b) are respectively $n = 2$ difference spectra, obtained as described in the previous subsection, for enrichments of 24 and 50%. These spectra are not identical with one another (in agreement with $n > 1$). Thus, for example, the central feature in the g_2 region is relatively smaller in (b) than it is in (a). From these two spectra we went on, by using the procedure described, to obtain further difference spectra (Figure 4c and 4d) corresponding respectively to Enz ^{17}O^{17}O and Enz ^{17}O^{16}O + Enz ^{16}O^{17}O. Computer simulation of these spectra, based on the parameters of Table II, is presented in Figure 4c′ and 4d′. Though the signal-to-noise ratio, particularly in Figure 4c, is poor, the main features in both difference spectra are adequately simulated with the single set of parameters given in Table II. (Note that the features in the center of the g_2 features in Figure 4c, not reproduced in the

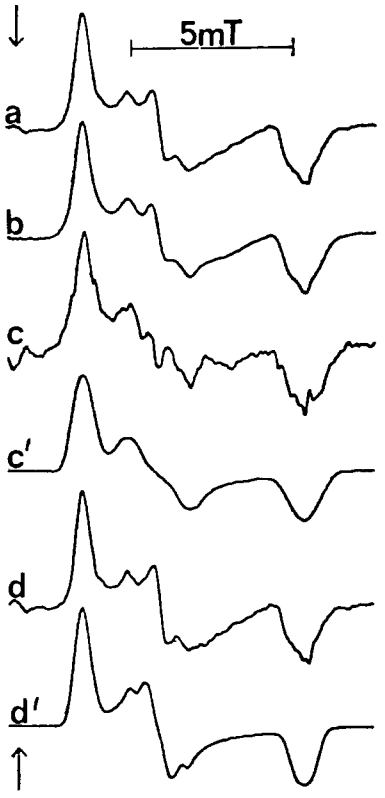


FIGURE 4: Use of difference spectra and computer simulations to evaluate hyperfine couplings for two oxygen ligands in the Inhibited signal. Experimental spectra for the Inhibited signal, with $^2HC^2HO$ in [^{17}O]water at two different enrichments, and also in ordinary water, were obtained by using gel filtration as described under Materials and Methods and as illustrated in Figure 3. Difference spectra for $n = 2$, obtained as illustrated in Figure 1, are shown in (a) for ^{17}O enrichment of 24% and in (b) for enrichment of 50%. If one subtracts (a) from (b) and (b) from (a) in theoretically calculated amounts (see Materials and Methods), spectra corresponding to Enz $^{17}O^{17}O$ and to Enz $^{17}O^{16}O$ + Enz $^{16}O^{17}O$ may be obtained and are shown in (c) and (d), respectively. (c′) and (d′) are simulations of (c) and (d), respectively, obtained by using the parameters listed in Table II. Amplitudes in the four experimental spectra have been adjusted so as to correspond to equal integrated intensities. (c) corresponds to 4.35[(b) − 0.77(a)] and (d) to 1.69[(a) − 0.41(b)]. The two simulated spectra have also been adjusted to equal integrated intensities.

Table II: Oxygen Coupling in the Inhibited Signal[a]

	1	2	3	av
g	1.9911	1.9772	1.9513	1.9732
$A(^{17}O)^b$	0.06	0.31	0.13	0.17
	0.12	0.12	0.17	0.14

[a] The parameters given are those used in the simulations of Figures 3 and 4. g values are those of Tanner et al. (1978); line widths (as half-widths in millitesla) of 0.188, 0.139, and 0.176 allow for unresolved coupling to deuterium. Couplings to oxygen are in millitesla. [b] Note that the combination of hyperfine couplings given is an arbitrary one. Thus, though we can say, for instance, that there are two oxygens giving splittings of 0.06 and 0.12 mT, respectively, in the A_1 direction, we cannot say which of these oxygens is giving the larger splitting and which the smaller in the A_2 and A_3 directions.

simulation of Figure 4c′, may represent instrumental artifacts arising from slight nonlinearity of the field scan. A similar artifact seems to be present in the g_1 region of Figure 3d.) The entire difference spectral procedures of Figure 4 have been repeated, for other Inhibited signal-giving samples of the enzyme similarly prepared, with similar results. We therefore conclude that the value of $n = 2$ is fully confirmed. Values of the hyperfine coupling constants (Table II) are considered to be fairly accurate (perhaps ±0.02 mT), but as is indicated in the footnotes to the Table, the combination of coupling constants given is quite arbitrary.

Discussion

Number of Oxygen Ligands and Hyperfine Coupling Constants in Different Mo(V) Species. Our results provide much new information on oxygen atoms derived originally from water that are coupled to the molybdenum(V) atom in different reduced forms of xanthine oxidase.

The only data in the literature on ^{17}O coupling to Mo(V) in low molecular weight compounds appears to be that on the thiolate anion, $[MoO(SPh)_4]^-$, which has a square pyramidal structure (Bradbury et al., 1978) with oxygen in an axial position. For this ion $A(^{17}O)_{av}$ is 0.24 mT [Hanson et al., 1981; see also Cramer et al. (1979a). This value, for a compound with an axial structure, is sufficiently large as to make it rather unlikely that any exchangeable oxygen ligand in molybdenum-containing enzymes could escape detection by the techniques we used. Further, in comparison with the model compound, the coupling constants for the enzyme species (Table I) are generally quite large, ranging from $A(^{17}O)_{av} = 1.4$ mT in Very Rapid to 0.2 mT in Inhibited, making it highly likely that in all cases we are dealing with oxygen ligands of molybdenum rather than with oxygen atoms remote from the metal but still coupled to it.

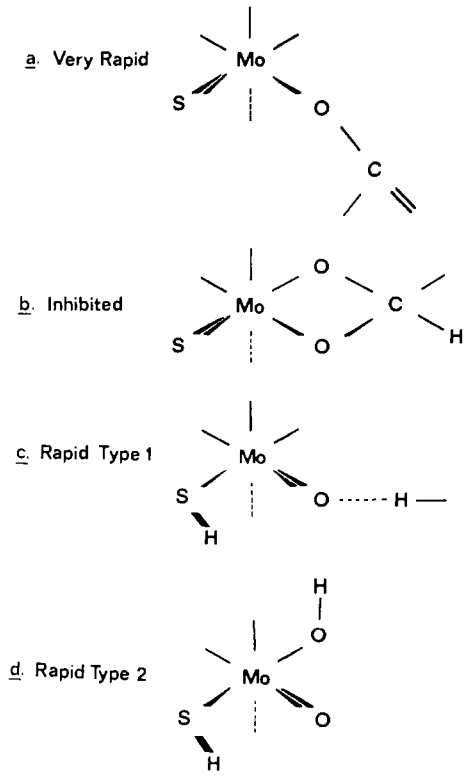
Our data, taken together with that of Gutteridge & Bray (1980a), make it abundantly clear that the strength of the hyperfine coupling of the oxygen ligands, their exchangeability, and their number vary from one reduced form of the enzyme to another. The number of oxygen ligands is only one in the Very Rapid and Rapid type 1 species, but two or even three in all the other species we examined. This makes still more specific earlier conclusions [e.g., Gutteridge & Bray (1980b), Bray (1980), and Malthouse et al. (1981a)], which were based on other EPR parameters, that there are substantial structural differences amongst the different species.

Structures of the Signal-Giving Species. Translating EPR parameters into the structures is not easily achieved, particularly in the absence of any substantial body of information on relevant model compounds. However, to the present data on the oxygen ligands we can add, for species derived from active xanthine oxidase, information (Malthouse et al., 1981a) on coupling of a sulfur ligand and more limited information (Tanner et al., 1978) on coupling of carbon atoms from substrate molecules. Finally, a general knowledge of the relevant chemistry of molybdenum compounds (Stiefel, 1977) is helpful, as of course is EXAFS data (Bordas et al., 1980) on the oxidized enzyme.

Suggested structures of molybdenum(V) species from active xanthine oxidase are shown in Chart I. Chart Ia shows the structure for Very Rapid and is based on a single strongly and isotropically coupled oxygen atom (Gutteridge & Bray, 1980a), a strongly and anisotropically coupled sulfur ligand (Malthouse et al., 1981a), and a weakly coupled C-8 carbon from the xanthine (Tanner et al., 1978). The exchangeability of the oxygen with water molecules is at present not determinable, since the species is a transient with a lifetime of tens of milliseconds at 20–25 °C.

In the Inhibited species, the two weakly coupled oxygen ligands do not exchange with water molecules, making it unlikely that they are terminal ligands. For example, terminal oxygen (Tullius et al., 1979; Bordas et al., 1980) in the oxidized enzyme exchanges with the solvent water with a half-time of

Chart I: Suggested Structures of Mo(V) Species from Active Xanthine Oxidase[a]



[a] Only atoms whose presence is known from hyperfine coupling studies are shown.

about 15 min (Gutteridge & Bray, 1980a), and that in molybdate exchanges even faster (von Felton et al., 1978). The cyclic structure presented in Chart Ib can account for the stability of the oxygens and also for the strong isotropic coupling from a carbon atom of the formaldehyde or methanol that gives rise to the signal. Cyclic complexes with structures somewhat analogous to the one in Chart Ib are known both for vanadium (Wasson, 1971; Day & McClung, 1976) and for molybdenum (Stiefel et al., 1977), having a phosphorus or an arsenic atom bonded to the metal via two sulfur atoms. Strong transannular coupling of phosphorus or arsenic to the metal is observed by EPR analogous to the coupling (Tanner et al., 1978) of the carbon atom to molybdenum in Inhibited. The sulfur atom of Inhibited we take to be a terminal one since there are no exchangeable protons; its coupling to the metal is relatively weak (Malthouse et al., 1981a). For aldehydes other than formaldehyde, the nonexchangeable weakly coupled proton in Chart Ib is replaced by the R group of the aldehyde, RCHO (Malthouse et al., 1981b).

In the Rapid type 1 signal there is a strongly coupled oxygen and a relatively weakly coupled sulfur (Malthouse et al., 1981a), together with one strongly and one rather weakly coupled proton (Gutteridge et al., 1978b). Chart Ic takes this into account, with the strongly coupled proton shown on the sulfur rather than on the oxygen [cf. Bray (1980)]. Reasons for preferring this are as follows. First, arguments similar to those presented in relation to Inhibited may apply, namely, that a ligand of the metal, though relatively weakly coupled because it lies in a region of low electron density, may bear a further substituent that occupies a region of high electron density and so is strongly coupled. Secondly, the mechanism of substrate oxidation is postulated (Gutteridge et al., 1978b; Bray et al., 1979; Gutteridge & Bray, 1980a) to involve attack by a nucleophile on the C-8 position of xanthine, with transfer of two electrons to molybdenum and uptake of the C-8 proton by an accepting group. The nucleophile is identified, in line with the structures of the Very Rapid species (Chart Ia), as the terminal oxygen ligand of the oxidized enzyme, leaving the terminal sulfur as the proton acceptor. The weakly coupled proton of the signal is shown, more speculatively, in Chart Ic as being hydrogen bonded to oxygen from some group in the protein. Recent information supporting these assignments comes from EPR work (G. N. George and R. C. Bray, unpublished data) on mercurial derivatives of the enzyme in which this metal is strongly coupled to molybdenum, presumably through sulfur.

The Rapid type 2 species has been assumed (Bray et al., 1978) to represent a labile inhibitory species rather than a functional catalytic one. It is like the Rapid type 1 signal except that both protons are strongly coupled to the metal and, more definitively, that it has two rather than one coupled oxygen ligand. Our proposed structure is shown in Chart Id, with one hydrogen on oxygen and one on sulfur. [The possibility that both protons are on oxygens (or even on a single oxygen) has also to be considered.] Since the final step of the catalytic mechanism (Gutteridge & Bray, 1980a) is believed to involve hydroxyl ion attack on the molybdenum of the Very Rapid species (or of some analogous species) to displace the uric acid anion, we may speculate that Rapid type 2 represents a species in which premature or "wrong" hydroxyl attack has taken place. For both Rapid type 1 and Rapid type 2 species there will in general be a bound anion, substrate, or product molecule, which is presumably coordinated to molybdenum. These are not shown in Chart I, since no hyperfine coupling from any of their constituent atoms has so far been observed. Other ligands of the metal are probably sulfur (Tullius et al., 1979; Bordas et al., 1980).

The structures of signal-giving species from the desulfo form of xanthine oxidase, as well as from sulfite oxidase, will be considered briefly. That the Slow signal (especially in the presence of nitrate) contains more oxygen ligands than do the Rapid species is consistent with and supports strongly the proposal (Gutteridge et al., 1978a) that cyanolysis of the active enzyme involves replacement of a terminal sulfur ligand by a terminal oxygen. The Desulfo-Inhibited species presumably has a cyclic structure somewhat analogous to that of Inhibited. [Gel filtration experiments (not illustrated), analagous to those we carried out on Inhibited, indicated its oxygens to be nonexchanging.] For sulfite oxidase the data (Table I) indicate one oxygen ligand in both the high-pH and the low-pH species; presumably in the latter form the oxygen is protonated. The presence of a single oxygen ligand in these species contrasts with the situation in the oxidized enzyme for which EXAFS data (Cramer et al., 1979b) indicate two terminal oxygen ligands. Finally, for the phosphate signal from sulfite oxidase (Gutteridge et al., 1980), which represents an inhibitory species with nonexchanging oxygens, one would expect a cyclic structure analogous to Inhibited. Whether further work at improved signal-to-noise ratios will bring the number of coupled oxygen atoms involved in the signal (Table I; Gutteridge et al., 1980) into line remains to be seen.

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