Coupling of [^{33}S]sulphur to molybdenum(V) in different reduced forms of xanthine oxidase

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(Received 5 June 1981/Accepted 24 July 1981)

Different reduced forms of xanthine oxidase, labelled specifically in the cyanide-labile site with ^{33}S , were prepared and examined by electron paramagnetic resonance. Coupling of this isotope to molybdenum(V) was quantified with the help of computer simulations and found to differ markedly from one reduced form to another. The xanthine Very Rapid signal shows strong, highly anisotropic, coupling with $A(^{33}\text{S})_{\text{av}}$, 1.27 mT. For this signal, axes of the g - and $A(^{33}\text{S})$ -tensors are rotated relative to one another. One axis of the A -tensor is in the plane of g_{xx} and g_{yy} , but rotated by 40° relative to the g_{xx} axis, whereas the direction of weakest coupling to sulphur deviates by 10° from the g_{zz} axis. In contrast with this signal, only rather weaker coupling was observed in different types of Rapid signal [$A(^{33}\text{S})_{\text{av}}$, 0.3–0.4 mT], and in the Inhibited signal coupling was weaker still [$A(^{33}\text{S})_{\text{av}}$, 0.1–0.2 mT]. Clearly, there must be substantial differences in the structures of the molybdenum centre in the different signal-giving species, with the sulphur atom perhaps in an equatorial type of ligand position in the Very Rapid species but in a more axial one in the other species. Structures are discussed in relation to the mechanism of action of the enzyme and the nature of the proton-accepting group that participates in turnover.

The molybdenum centre of xanthine oxidase (Bray, 1980) contains a sulphur atom that is essential for catalytic activity. This atom may be removed by treating the enzyme with cyanide and replaced, with partial restoration of enzymic activity, on treatment with sulphide (Massey & Edmondson, 1970; Bray, 1975). The precise nature of the sulphur atom and its role in the catalytic reaction of the enzyme have been controversial. Olson *et al.* (1974) suggested that it was a persulphide, which acted as a nucleophile, whereas Gutteridge *et al.* (1978*b*) proposed that in the oxidized enzyme it was a terminal sulphur ligand of molybdenum, acting as a proton acceptor in the catalytic reaction. The nature of the sulphur was recently studied by e.x.a.f.s. (X-ray absorption extended fine structure) (Bordas *et al.*, 1980). It has also been studied by e.p.r. of molybdenum(V) in the enzyme, after re-activation of the cyanide-treated enzyme with [^{33}S]sulphide, by observation of the hyperfine splitting of the signals due to this isotope (Malthouse & Bray, 1980). In the latter work, only one of the three molybdenum(V)

signal-giving species from the functional enzyme (the Very Rapid signal obtained on treatment with xanthine) was studied. In the present paper we describe an extension of e.p.r. studies with this isotope. The work was facilitated by investigations (Malthouse *et al.*, 1981) of the use of additional substrates to elicit some of the e.p.r. signals.

Materials and methods

General

Unless otherwise stated, all materials and e.p.r. and other procedures were as described by Malthouse *et al.* (1981). When required, enzyme samples in $^2\text{H}_2\text{O}$ (about 98% final concentration) were prepared as described by Gutteridge *et al.* (1978*a*).

^{33}S -enriched samples of xanthine oxidase for e.p.r.

Xanthine oxidase specifically enriched in the cyanide-labile site with ^{33}S at an enrichment of 59.2% was prepared as described by Malthouse & Bray (1980). The various molybdenum(V) e.p.r. signals were elicited as described below. In all cases control samples of the signal-giving species were obtained with unenriched enzyme in parallel with enriched samples. Care was taken that the concen-

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tration of functional enzyme was about the same in the enriched and unenriched samples. In some cases (e.g. Fig. 4a) for the controls, instead of using ordinary enzyme, we used enzyme that had been re-activated with unenriched sulphide from the desulpho form.

Procedures for obtaining the signals were as follows. The xanthine Very Rapid signal was obtained as described by Gutteridge & Bray (1980a). The xanthine Rapid signal was obtained under similar conditions, but with the reaction time increased to 45 ms. The 2-amino-4-hydroxy-6-formylpteridine Very Rapid signal (Malthouse *et al.*, 1981) was conveniently obtained by first reducing the enzyme, in 20 mM-Bicine [N-bis-(2-hydroxy-ethyl)glycine]/NaOH buffer, pH 8.3, with xanthine (1 mM) for 2 min, then adding the pteridine (5 mM), bubbling the sample gently with air and freezing about 1 min later. The Very Rapid signal from 6-methylpurine was obtained by reducing the enzyme with this substrate (5 mM; from Sigma Chemical Co., Poole, Dorset, U.K.) for 1 min at pH 10.2 [in 20 mM-Ches [(N-cyclohexylamino)ethanesulphonic acid]/NaOH buffer; final pH value, measured with a micro-electrode after the experiment], then bubbling O₂ through the sample for 20 s, before freezing. The Rapid type 1 signal was obtained by reducing the enzyme with 1-methylxanthine (2 mM), with 1 min reaction time, anaerobically at pH 8.2 (20 mM-Bicine/NaOH buffer). The borate Rapid type 2 signal was similarly obtained, by reduction with dithionite (0.4 mM) in the same buffer (40 mM) in the presence of sodium borate (40 mM). Conditions for obtaining the various Inhibited signals were those described by Malthouse *et al.* (1981) (see caption to Fig. 4 of that paper). The Slow signal was obtained by reduction with dithionite in Bicine/NaOH buffer, pH 8.2 (Gutteridge, *et al.*, 1978b).

Computer simulation of e.p.r. spectra

All simulations were conducted on a PDP 11/10 computer. Generally, the assembly code version of the simulation program of Lowe (1978) was used.

For simulation of the ³³S-substituted Very Rapid signal it was found necessary to allow for non-coincidence of the axes of the **g**- and **A**-tensors. A program similar to that of Lowe (1978) was written in BASIC. Spectra were calculated by using the first-order perturbation solution of the $S = \frac{1}{2}$ spin Hamiltonian:

$$\mathcal{H} = \beta \cdot \mathbf{H} \cdot \mathbf{g} \cdot \mathbf{S} + \mathbf{S} \cdot \mathbf{A} \cdot \mathbf{I}$$

All symbols have their usual meanings (cf. Abragam & Bleaney, 1970, chapters 1–3), and **g** and **A** are diagonal in the axis systems *x,y,z* and *p,q,r* (Scheme 1) respectively (**g** and **A** are assumed to be

symmetric). The hyperfine tensor **A** is rotated to the *g* co-ordinate system by using the relation:

$$\mathbf{A}' = \mathbf{T}^{-1} \cdot \mathbf{A} \cdot \mathbf{T}$$

where **T** is the relevant transformation matrix (Golding & Tennant, 1973), with angles of non-coincidence, α and β , as shown in Scheme 1 (note that q is constrained to the *x-y* plane). The angular variation of hyperfine splitting is calculated from the general formula given by Abragam & Bleaney (1970, p. 170), and the spectrum obtained by convoluting the calculated line positions with a Gaussian line-shape function.

For simulations with coincident axes of the **g**- and **A**-tensors, an iterative version (in BASIC) of the program of Lowe (1978) was developed to facilitate fitting of simulated to experimental spectra. Input data are the experimental spectrum and initial estimates of *g*- and *A*-values and line-widths. The program varies any or all of these parameters, in order to minimize *S*, the sum of squares of the differences between experimental and simulated points. Initially, the direction of the line of steepest descent of *S* is found by using the change in *S* produced by small changes in each of the scaled variable parameters. The second derivative in this direction (taken as positive) is similarly estimated. If the second derivative is positive, new starting values of the parameters are determined by the Newton–Raphson method. If it is negative the program searches stepwise for a minimum of *S* along the line of steepest descent. The minimization is complete when the fractional change in the parameter values is less than a predetermined value, otherwise the procedure is repeated. After each iteration a complete minimization of the relative amplitude

Scheme 1. Relationship between the *g* and *A* co-ordinate systems for the Very Rapid signal

Numerical values shown are those used in the simulation (Fig. 2c'); values of α and β are considered accurate to $\pm 4^\circ$; *g*-values are from Tanner *et al.* (1978). Note that *q* is constrained to the *x-y* plane.

and sloping straight base-line is performed. After the final iteration the program tests for a saddle point by varying each simulation parameter in turn; if one is detected, a stepwise search for a lower value of S is performed for each parameter, and the whole procedure is repeated.

Generally, the program has yielded simulations apparently superior to those obtained by many cycles of non-iterative simulations. Time taken for a relatively simple simulation, in which, say, g -values and one set of hyperfine splittings only was varied, was about 24 h.

Determination of ^{33}S splittings

To obtain experimental spectra corresponding to 100% enrichment with ^{33}S , it was necessary to subtract from the spectrum of enriched enzyme appropriate amounts of the corresponding unenriched spectrum. This was done by using a computer and visual display system (Bray *et al.*, 1978). It was important that the two spectra should be aligned accurately. Generally the amount of the ^{32}S spectrum to be subtracted was determined by adjusting the relative amplitudes of the two spectra until they corresponded to equal integrated intensities. Then 40.8% of the ^{32}S spectrum was subtracted from the enriched spectrum to give the pure ^{33}S spectrum. For the Very Rapid signal, because of the wide span of g -values and possible minor contamination with other signals, integration was not considered sufficiently accurate for the above purpose. Therefore the pure ^{33}S spectrum was obtained by subtracting increasing amounts of the ^{32}S spectrum, until the appropriate features just disappeared from the difference spectrum.

^{33}S splittings were determined by comparing ^{32}S and pure ^{33}S spectra, with the help of computer simulation procedures (see above). The procedure was first to simulate the ^{32}S spectrum, then to simulate the ^{33}S spectrum from it by using the same parameters but with added ^{33}S splittings, which were then varied until a good fit was obtained. In the case of the Very Rapid signal it was necessary to vary the angles α and β , also; some 50 simulations were performed before the final fit was obtained. For other signals, the simulations assumed the axes of the g - and A -tensors to coincide, and in most cases the fitting was refined, and the goodness of fit quantified, by using the iterative simulation procedure.

Generally for the simulations g -values, and where appropriate $A(^1\text{H})$ values, were taken from the literature, but line-widths were re-determined with the iterative procedure. Deuterium splittings were approximated by appropriate increases in the line-widths. In a number of cases iterative simulations in which g -values were varied confirmed data in the literature (e.g. for the formaldehyde Inhibited signal

g -value discrepancies were not greater than 0.0004). However, for the 1-methylxanthine Rapid signal, discrepancies were larger, and values of g_1 1.9886, g_2 1.9691 and g_3 1.9646 from the present work are considered more reliable than those obtained by Gutteridge *et al.* (1978a). Errors in $A(^{33}\text{S})$ -values were arbitrarily taken, with a lower limit of 0.02 mT, as the difference between values from iterative simulations in which $A(^{33}\text{S})$ -values, only, were varied and similar simulations in which both g - and $A(^{33}\text{S})$ -values were simultaneously varied. (The former set of A -values are considered the more reliable and are those given in Table 1.)

Results

Control experiments with reconstituted xanthine oxidase

Fig. 1 shows two important controls. Reconstitution of desulpho xanthine oxidase by incubation with sulphide (Massey & Edmondson, 1970; Malt-house & Bray, 1980) has to be done under rather vigorous conditions, with incubation times of up to 2 h at 45°C and pH 9. It was therefore desirable to check that active enzyme so produced gave e.p.r. signals identical with those from the native enzyme. We tested this by observation of the Rapid type 1 signal generated with 1-methylxanthine in $^2\text{H}_2\text{O}$. The null difference spectrum of Fig. 1(c), obtained by subtracting the signal of native enzyme (Fig. 1a) from that obtained with enzyme reconstituted with unenriched sulphide (Fig. 1b), shows that signals to be indistinguishable.

Massey & Edmondson (1970) further showed that, presumably as a result of side reactions in the reconstitution process, substantial amounts of extra radioactive sulphide (about 2 mol of S/mol of Mo) could become bound to the enzyme. The sites at which these extra sulphur atoms are bound is uncertain. Obviously it is important to know that effects of ^{33}S , considered below, depend solely on the isotope incorporated into the cyanide-labile site, rather than on ^{33}S bound elsewhere in the molecule. As a check that such extra sulphur atoms do not interact with molybdenum, we studied e.p.r. signals due to residual desulpho enzyme, in a sample that had been re-activated by treatment with ^{33}S -enriched sulphide. Fig. 1(f) shows a near-null difference spectrum obtained by subtracting the Slow signal generated from untreated enzyme (Fig. 1d) from that obtained with the [^{33}S]sulphide-treated enzyme (Fig. 1e). The findings thus suggest strongly that any extra ^{33}S that may be present in the reconstituted active molecules is unlikely to interfere with studies of the cyanide-labile site. (Observed intensity of the Slow signal in the untreated enzyme sample was substantially higher than that in the treated sample, possibly indicating that further degradation of some

Fig. 1. Controls for ^{33}S -substitution experiments with xanthine oxidase

Spectra (a) and (b) show the Rapid type 1 e.p.r. signal generated from xanthine oxidase with 1-methylxanthine in $^2\text{H}_2\text{O}$. For spectrum (a) normal enzyme was used and for spectrum (b) enzyme that had been reconstituted with ^{32}S sulphide from a cyanide-treated sample. Spectrum (c) is the difference spectrum obtained by subtracting spectrum (a) from spectrum (b). Spectra (d) and (e) show the Slow signal obtained by reducing, with dithionite, desulpho enzyme present as a contaminant of active enzyme. For spectrum (d) a normal enzyme sample was used, whereas for spectrum (e) enzyme incompletely reconstituted with ^{33}S sulphide from a cyanide-treated sample was used. Spectrum (f) is the difference spectrum obtained by subtracting spectrum (d) from spectrum (e). Here and in Figs. 2–6 the arrow indicates $g = 2.0037$.

of the desulpho enzyme to unknown products accompanies the reconstitution process.)

Spectra from ^{33}S -enriched enzyme

Spectra of the various signal-giving species obtained with ^{33}S -enriched enzyme are compared with the corresponding spectra from unenriched samples in Figs. 2–6. In Figs. 2 and 4–6 (a) corresponds to the normal ^{32}S -containing-enzyme spectrum and (a') is the corresponding simulation, (b) is the spectrum from the ^{33}S -enriched enzyme and (c) is the difference spectrum corresponding to 100% ^{33}S . Finally (c') corresponds to the simulation of the spectrum for ^{33}S -containing enzyme. ^{33}S splittings determined from the spectra are summarized in Table 1.

Fig. 2. Effect of ^{33}S substitution on the xanthine Very Rapid e.p.r. signal from xanthine oxidase

In spectrum (a) normal enzyme was used and in spectrum (b) enzyme reconstituted with ^{33}S , and spectrum (c) is the difference spectrum obtained from (a) and (b) and corresponds to 100% enrichment with ^{33}S . Spectra (a') and (c') are computer simulations of spectra (a) and (c) respectively, and spectrum (b') is a simulation of spectrum (b) made by adding theoretical proportions of spectra (a') and (c'). The samples were prepared and the simulations were conducted as described in the text (see the Materials and methods section, Table 1 and Scheme 1).

Only the Very Rapid signal gave clearly resolved ^{33}S splittings (Figs. 2 and 3). Fig. 2(c) shows a rather clearer ^{33}S difference spectrum for the Very Rapid signal generated with xanthine than that presented by Malthouse & Bray (1980). It is abundantly clear that the ^{33}S splittings are quite large and highly anisotropic.

Attempts (not illustrated) to simulate the signal, assuming the axes of the g - and A -tensors to coincide, were only partly successful. Thus, features in the central part of the hyperfine structure about the g_{xx} and g_{yy} region of Fig. 2(c) were badly reproduced in the simulation, and more specifically

the lack of symmetry of the hyperfine structure about the g_{zz} position could not be reproduced. On the other hand, when non-coincidence of the axes was allowed for in the simulations, as described in the Materials and methods section, then good simulations (Figs. 2c' and 2b') could be obtained, both for the ^{33}S difference spectrum (Fig. 2c) and, by adding in the simulation of the ^{32}S spectrum, for the original experimental spectrum (Fig. 2b) also. Parameters of the xanthine Very Rapid signal are given in Scheme 1 and Table 1. $A(^{33}\text{S})_{\text{av.}}$ has the high value of 1.27 mT.

Fig. 3 shows experimental signals for enriched and unenriched enzyme for two other substrates, yielding signals provisionally allocated in earlier work to the Very Rapid class, namely 2-amino-4-hydroxy-6-formylpteridine (Malthouse *et al.*, 1981; see Figs. 3c and 3d) and 6-methylpurine (Pick, 1971; see Figs. 3a and 3b). Coupling to ^{33}S is clearly, for both substrates, producing structure similar to that observed in the xanthine Very Rapid signal (Fig. 2b), confirming allocation of the three signals to the same class. Preliminary simulations of the spectra of Fig. 3 (G. N. George & R. C. Bray,

unpublished work) suggest that both $A(^{33}\text{S})$ values and angles of non-coincidence of the tensors are similar to those for the xanthine signal.

For signals other than Very Rapid, ^{33}S enrichment caused marked broadening but did not give rise to resolved splittings. Results for the Rapid type 1 signal, obtained on reducing the enzyme with 1-methylxanthine, are presented in Fig. 4. Figs. 4(a')–4(c') show spectra obtained in $^2\text{H}_2\text{O}$. A value of $A(^{33}\text{S})_{\text{av.}}$ of 0.35 mT was obtained (Table 1). Spectra generated in $^1\text{H}_2\text{O}$ are shown in Figs. 4(d) and 4(e) and indicate ^{33}S splittings in agreement with those in $^2\text{H}_2\text{O}$.

Fig. 3. Effect of ^{33}S substitution on the Very Rapid e.p.r. signals from 6-methylpurine and 2-amino-4-hydroxy-6-formylpteridine

In spectra (a) and (c) normal enzyme was used, and in spectra (b) and (d) enzyme reconstituted with ^{33}S . Spectra (a) and (b) were obtained with 6-methylpurine, and spectra (c) and (d) with 2-amino-4-hydroxy-6-formylpteridine. For the latter pair of spectra small amounts of the appropriate Inhibited signals have been subtracted.

Fig. 4. Effect of ^{33}S substitution on the Rapid type 1 e.p.r. signal from xanthine oxidase

The signal was generated by reducing the enzyme with 1-methylxanthine. Spectra (a')–(c') correspond to solution in $^2\text{H}_2\text{O}$ and spectra (d) and (e) to solution in $^1\text{H}_2\text{O}$. Spectra (a) and (d) were obtained from normal enzyme and spectra (b) and (e) from enzyme reconstituted with ^{33}S . Spectrum (c) is the difference spectrum obtained by subtracting spectrum (a) from spectrum (b) and corresponds to 100% enrichment with ^{33}S . Spectrum (a') is a simulation of (a) and spectrum (c') is a simulation of (c). Samples were prepared and the simulations conducted as described in the text (see the Materials and methods section and Table 1).

Spectra of the Rapid type 2 signal are presented in Fig. 5. We studied particularly the signal obtained in

Fig. 5. Effect of ^{33}S substitution on the Rapid type 2 e.p.r. signal from xanthine oxidase

Spectra (a')–(c') correspond to the signal obtained by reducing the enzyme with dithionite in the presence of borate and spectra (d) and (e) to that obtained on reduction with xanthine. Spectra (a) and (d) were obtained from normal enzyme and spectra (b) and (e) from enzyme reconstituted with ^{33}S . Spectrum (c) is the difference spectrum obtained by subtracting spectrum (a) from spectrum (b) and corresponds to 100% enrichment with ^{33}S . Spectrum (a') is a simulation of (a) and spectrum (c') is a simulation of (c). Samples were prepared and the simulations conducted as described in the text (see the Materials and methods section and Table 1). Small amounts of an $\text{FADH}^{\bullet}$ signal have been subtracted from spectra (a) and (b) and small amounts of Very Rapid signal from spectra (d) and (e). The Rapid type 1 signal is present along with the type 2 signal in spectra (d) and (e).

the presence of borate, generated in $^1\text{H}_2\text{O}$ (Figs. 5a'–5c'). The splittings for this signal (Table 1) are similar to, or a little smaller than, those for the Rapid type 1 signal. Figs 5(d) and 5(e) show Rapid signals generated with xanthine. These are a mixture of the type 1 and type 2 forms. Though we have not examined these signals in detail, it seems that ^{33}S is

Fig. 6. Effect of ^{33}S substitution on the Inhibited e.p.r. signal from xanthine oxidase

Spectra (a')–(c') correspond to the signal obtained with glycolaldehyde, spectra (d) and (e) to that obtained with ^2H formaldehyde and spectra (f) and (g) to that with 2-amino-4-hydroxy-6-formylpteridine. Spectra (a), (d) and (f) were obtained from normal enzyme and spectra (b), (e) and (g) from enzyme reconstituted with ^{33}S . Spectrum (c) is the difference spectrum obtained by subtracting spectrum (a) from spectrum (b) and corresponds to 100% enrichment with ^{33}S . Spectrum (a') is a simulation of (a) and spectrum (c') is a simulation of spectrum (c). Samples were prepared and simulations conducted as described in the text (see the Materials and methods section and Table 1).

Table 1. ^{33}S hyperfine splittings of the various molybdenum(V) e.p.r. signals from xanthine oxidase

Hyperfine splittings (in mT) were determined from the spectra in Figs. 2–6, as described in the Materials and methods section. Error estimates are indicated only where the iterative simulation procedure was used. g -values, line-widths etc. were taken from the references given. Values of rhombicity parameters $(g_2 - g_3)/(g_1 - g_3)$ are given for comparison with $A(^{33}\text{S})_{\text{av.}}$ values.

Signal	$A(^{33}\text{S})$				$g_2 - g_3$	Reference
	1	2	3	Average	$g_1 - g_3$	
Xanthine Very Rapid*	0.30	2.80	0.70	1.27	0.07	Tanner <i>et al.</i> (1978)
1-Methylxanthine Rapid type 1	0.34 (± 0.04)	0.36 (± 0.05)	0.36 (± 0.03)	0.35 (± 0.04)	0.19	See the Materials and methods section
Borate Rapid type 2	0.37	0.22	0.35	0.31	0.23	Malthouse <i>et al.</i> (1980)†
2-Amino-4-hydroxy-6-formyl-pteridine Inhibited	0.19 (± 0.02)	0.11 (± 0.02)	0.28 (± 0.02)	0.19 (± 0.02)	0.60	Malthouse <i>et al.</i> (1981)
Glycoaldehyde Inhibited	0.14 (± 0.03)	0.09 (± 0.02)	0.22 (± 0.02)	0.15 (± 0.02)	0.60	Malthouse <i>et al.</i> (1981)
Formaldehyde Inhibited	0.06 (± 0.02)	0.03 (± 0.02)	0.20 (± 0.02)	0.10 (± 0.02)	0.65	Tanner <i>et al.</i> (1978)

* Non-coincident g - and A -axes; see Scheme 1.

† Parameters in close agreement with those given in this reference have been confirmed by measurements at 35 GHz and in $^2\text{H}_2\text{O}$ (S. Gutteridge & R. C. Bray, unpublished work).

affecting both of them in much the same way as it affects those Rapid signals that we studied more fully.

Fig. 6 shows ^{33}S effects on the Inhibited signal. These are the smallest effects from the isotope that we observed. However, because of the simple form of the signals, splittings were particularly easy to measure. Figs. 6(a')–6(c') illustrate full analysis of the Inhibited signal obtained with glycoaldehyde. Figs. 6(d) and 6(e) and Figs. 6(f) and 6(g) show comparable effects respectively for the signals from $[\text{H}]$ formaldehyde and from 2-amino-4-hydroxy-6-formylpteridine. As is indicated in Table 1, ^{33}S splittings varied significantly from one Inhibited signal to another, with $A(^{33}\text{S})_{\text{av.}}$ ranging from 0.10 to 0.19 mT. Non-coincidence of the axes of the g - and A -tensors is probably indicated, e.g., by the skewed form of the g_3 peak in Fig. 6(c), but no attempt has been made to simulate this. Anisotropy of the splitting is particularly marked for the formaldehyde Inhibited signal.

Discussion

So many structural features combine in determining the e.p.r. parameters exhibited by a particular compound that the process of attempting to derive structural information from parameters of unknown compounds is a difficult one. However, in agreement with Malthouse & Bray (1980), we conclude from the relatively large magnitude of the ^{33}S splitting in the Very Rapid signal that, for this signal-giving species, the cyanide-labile sulphur atom of active xanthine oxidase is a ligand of molybdenum. A number of further important conclusions follow from the present work.

A novel feature of the work is the simulation of the Very Rapid signal, showing two angles of non-coincidence of the g - and A -tensors. To our knowledge this is the first occasion on which such a simulation has been achieved for a biological molecule. The large anisotropy of the hyperfine splitting argues, as suggested by Malthouse & Bray (1980), for a substantial sulphur p -orbital contribution in the bonding. Addition of the information about angles to the other information already available is a further step towards deduction of the structure of the signal-giving species. Thus it is likely that the line joining the molybdenum and sulphur nuclei will lie along one of the principal axes of the $A(^{33}\text{S})$ -tensor (Pilbrow & Winfield, 1973). However, which axis is involved remains uncertain, owing to lack of knowledge of the bonding type (Keijzers *et al.*, 1975; Nilges *et al.*, 1977). It is hoped that evaluation of ^{95}Mo couplings in the enzyme as well as work with model compounds will help further to clarify matters.

In all the other signal-giving species from the enzyme, the sulphur atom also interacts with molybdenum, though considerably less strongly than for the Very Rapid signal. In the Inhibited signal obtained with $[\text{H}]$ formaldehyde, $A(^{33}\text{S})_{\text{av.}}$ is only 0.10 mT, a value some 13-fold smaller than that for the Very Rapid signal. With such weak coupling the possibility cannot immediately be excluded that the molybdenum–sulphur bond present in the Very-Rapid-signal-giving species has been ruptured in the Inhibited-signal-giving species. However, an alternative explanation, which is to be preferred, is that the decrease in coupling is due simply to a change in the geometry of the molybdenum centre. It is well

known from work with low-molecular-weight complexes that, among compounds having near-axial symmetry, ligand atoms in the axial position show much weaker hyperfine coupling than do those in equatorial positions. This is exemplified by ^{17}O -coupling studies, where a well-authenticated molybdenum complex having an axial oxygen ligand shows $A(^{17}\text{O})_{\text{av}}$ only 0.2 mT (Cramer *et al.*, 1979), whereas in, e.g., the Very Rapid signal from xanthine oxidase the value for this parameter is 1.37 mT (Gutteridge & Bray, 1980a).

Support for progressive changes of geometry is provided by consideration of the $A(^{33}\text{S})_{\text{av}}$ values for all the species listed in Table 1 and by comparison of these with other e.p.r. parameters. The sulphur hyperfine coupling values fall steadily in the series Very Rapid $\gg$ Rapid type 1 $>$ Rapid type 2 $>$ 2-amino-4-hydroxy-6-formylpteridine Inhibited $>$ glycolaldehyde Inhibited $>$ formaldehyde Inhibited. Though there is parallelism between this and progressive changes in the g -tensor from almost axial for Very Rapid to highly rhombic for Inhibited [compare $A(^{33}\text{S})_{\text{av}}$ with the parameter $(g_2 - g_3)/(g_1 - g_3)$ in Table 1], the precise theoretical significance of this is not certain.

We now consider the bearing of our results on the proposal by Gutteridge *et al.* (1978b) that a terminal sulphur ligand of molybdenum in the oxidized enzyme acts as a proton acceptor in the turnover, being converted in the process into an MoSH group. According to this proposal, in the Rapid signal a strongly coupled proton ought to be bonded to the molybdenum via the cyanide-labile sulphur atom. It may be questioned whether such a structure is compatible with the observed relative magnitudes of the sulphur and proton hyperfine couplings to molybdenum. The strongly coupled protons of the various Rapid signals have $A(^1\text{H})_{\text{av}}$ about 1.0–1.5 mT, whereas $A(^{33}\text{S})_{\text{av}}$ is about 0.3–0.4 mT, indicating (Goodman & Raynor, 1970) much stronger coupling of the unpaired electron to the proton than to the sulphur atom. In contrast, it might, at least at first sight, have been expected that, in MoSH, the reverse situation would apply. Furthermore, in the Rapid type 1 species a strongly coupled oxygen atom is available (Gutteridge *et al.*, 1979), and this could, in principle, serve as the binding site of the proton. However, the results could also be explained if the sulphur were located near the nodal plane of the ground-state Mo (4d) orbital, since then non-linearity of the MoSH group could hold the proton out of the nodal plane into a region of higher electron density, so giving rise to the observed relative magnitudes of the hyperfine couplings. This explanation has been mooted (Stiefel *et al.*, 1977) for MoNH compounds (Gardner *et al.*, 1978), in which coupling to protons is similarly stronger than that to nitrogen. Further information

that might resolve the matter should eventually become available from detailed investigations of ^{17}O coupling in the various Rapid signals.

Finally, the structure of the Inhibited-signal-giving species has to be considered. The possibility of a structure of the type $\text{Mo}-\text{S}-\text{C} \lessgtr$, where S is the cyanide-labile sulphur and C is the α -carbon atom of the aldehyde, has been tentatively suggested (Gutteridge & Bray, 1980b). This structure may not be compatible with very weak coupling of sulphur to molybdenum, accompanied by strong coupling (Tanner *et al.*, 1978; S. Gutteridge, G. N. George, R. C. Bray & F. Bergmann, unpublished work) of the carbon atom, and alternative structures for the Inhibited signal-giving species have to be considered. If the aldehyde residue is linked to the metal through oxygen [as is believed to be the case in Very-Rapid-signal-giving species (Gutteridge & Bray, 1980a)], then work with ^{17}O should provide information.

We thank Dr. S. Gutteridge, Dr. J. W. Williams and Dr. A. Hudson for valuable discussion and Miss T. Wilkinson for technical assistance. The work was supported by the Medical Research Council. G. N. G. was supported by a Studentship from the Science Research Council.

References

- Abraham, A. & Bleaney, B. (1970) *Electron Paramagnetic Resonance of Transition Ions*, Clarendon Press, Oxford
- Bordas, J., Bray, R. C., Garner, C. D., Gutteridge, S. & Hasnain, S. S. (1980) *Biochem. J.* **191**, 499–508
- Bray, R. C. (1975) *Enzymes 3rd Ed.* **12**, 299–419
- Bray, R. C. (1980) *Adv. Enzymol. Relat. Areas Mol. Biol.* **51**, 107–165
- Bray, R. C., Barber, M. J. & Lowe, D. J. (1978) *Biochem. J.* **171**, 653–658
- Cramer, S. P., Johnson, J. L., Rajagopalan, K. V. & Sorrell, T. N. (1979) *Biochem. Biophys. Res. Commun.* **91**, 434–439
- Gardner, J. K., Pariyadath, N., Corbin, J. L. & Steifel, E. I. (1978) *Inorg. Chem.* **17**, 897–904
- Golding, R. M. & Tennant, W. C. (1973) *Mol. Phys.* **25**, 1163–1171
- Goodman, B. A. & Raynor, J. B. (1970) *Adv. Inorg. Chem. Radiochem.* **13**, 135–362
- Gutteridge, S. & Bray, R. C. (1980a) *Biochem. J.* **189**, 615–623
- Gutteridge, S. & Bray, R. C. (1980b) in *Molybdenum and Molybdenum-Containing Enzymes* (Coughlan, M. P., ed.), pp. 221–239, Pergamon Press, Oxford
- Gutteridge, S., Tanner, S. J. & Bray, R. C. (1978a) *Biochem. J.* **175**, 869–878
- Gutteridge, S., Tanner, S. J. & Bray, R. C. (1978b) *Biochem. J.* **175**, 887–897
- Gutteridge, S., Malthouse, J. P. G. & Bray, R. C. (1979) *J. Inorg. Biochem.* **11**, 355–360
- Keijzers, C. P., Paulussen, G. F. M. & de Boer, E. (1975) *Mol. Phys.* **29**, 973–1006

- Lowe, D. J. (1978) *Biochem. J.* **171**, 649–651
- Malthouse, J. P. G. & Bray, R. C. (1980) *Biochem. J.* **191**, 265–267
- Malthouse, J. P. G., Gutteridge, S. & Bray, R. C. (1980) *Biochem. J.* **185**, 767–770
- Malthouse, J. P. G., Williams, J. W. & Bray, R. C. (1981) *Biochem. J.* **197**, 421–425
- Massey, V. & Edmondson, D. (1970) *J. Biol. Chem.* **245**, 6595–6598
- Nilges, M. J., Barefield, E. K., Belford, R. L. & Davies, P. H. (1977) *J. Am. Chem. Soc.* **99**, 755–760
- Olson, J. S., Ballou, D. P. Palmer, G. & Massey, V. (1974) *J. Biol. Chem.* **249**, 4363–4382
- Pick, F. M. (1971) Ph.D. Thesis, University of London
- Pilbrow, J. R. & Winfield, M. E. (1973) *Mol. Phys.* **25**, 1073–1092
- Stiefel, E. I., Newton, W. E. & Pariyadath, N. (1977) in *Chemistry and Uses of Molybdenum* (Mitchell, P. C. H. & Seaman, A., eds.), pp. 265–270, Climax Molybdenum Co., London
- Tanner, S. J., Bray, R. C. & Bergmann, F. (1978) *Biochem. Soc. Trans.* **6**, 1328–1330