Magnetic Coupling of the Molybdenum and Iron–Sulphur Centres in Xanthine Oxidase and Xanthine Dehydrogenases

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Magnetic interaction between molybdenum and one of the iron–sulphur centres in milk xanthine oxidase [Lowe, Lynden-Bell & Bray (1972) *Biochem. J.* 130, 239–249] was studied further, with particular reference to the newly discovered Mo(V) e.p.r. (electron-paramagnetic-resonance) signal, Resting II [Lowe, Barber, Pawlik & Bray (1976) *Biochem. J.* 155, 81–85]. E.p.r. measurements at 35 GHz near to 4.2 K showed that the interaction has the same sign at all molybdenum orientations and is ferromagnetic. The predicted splitting of the e.p.r. signal from the reduced iron–sulphur centre, Fe/S I, was observed, providing positive identification of this as the other interacting species. Chemical modification of the molybdenum environment in xanthine oxidase can change the size of the interaction severalfold, but interaction always remains approximately isotropic. The interaction in turkey liver xanthine dehydrogenase is indistinguishable from that in the oxidase. However, a bacterial xanthine dehydrogenase with different iron–sulphur centres shows rather larger interaction. Guanidinium chloride disturbs the iron–sulphur centres of the oxidase, and when this occurs there is a parallel and relatively small change in the interaction. Removal of flavin from the molecule, or raising the pH to 12.0, changes the interaction slightly without affecting the chromophores themselves. It is concluded that the Fe/S I centre and the Mo are at least 1.0 nm and probably nearer 2.5 nm apart, and that the conformation of the protein between them is relatively stable up to pH 12.

Many oxidative enzymes contain within their molecules several different kinds of functional groupings. Understanding the detailed mechanisms (cf. Moore & Williams, 1976; Van Heuvelen, 1976) whereby electrons are transferred during the enzymic reaction from one redox-active grouping to another within a multicomponent oxidative enzyme will depend on knowing the relative positions of the various centres within the protein molecule. Unfortunately, there are at present no X-ray-crystallographic data available on the structure of any enzyme of this type. The study of magnetic interactions between the various groupings in such a molecule might in principle, however, be capable of giving geometrical information.

In a number of redox-active proteins, relatively weak magnetic couplings between centres have been observed, e.g. in the eight-iron ferredoxins (Matthews *et al.*, 1974), in vitamin B₁₂-dependent enzymes (Schepler *et al.*, 1975) and in respiratory-chain systems (Ruzicka *et al.*, 1975; Ingledew *et al.*, 1976).

Abbreviations used: e.p.r., electron paramagnetic resonance; ELDOR, electron electron double resonance; S, spin quantum number; ΔM_s , change in magnetic quantum number.

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The first such coupling to be observed was that between molybdenum and iron–sulphur in the enzyme xanthine oxidase (Lowe *et al.*, 1972). This enzyme (mol.wt. 280 000) has two subunits, each of which contains one molecule of FAD and two types of iron–sulphur centres (designated Fe/S I and Fe/S II), in addition to molybdenum [see Bray (1975) for a review]. Although the coupling is small, the very sharp lines of the molybdenum e.p.r. spectra (<0.5 mT) make it a particularly favourable system to study in detail. Thus Mo(V) signals from xanthine oxidase have been observed to be split by one of the reduced iron–sulphur centres (presumed to be Fe/S I), but only at low temperatures (about 40 K or below), when the iron–sulphur electron is relaxing slowly (Lowe *et al.*, 1972). No deviations from an isotropic splitting were detected by these authors; the corresponding splitting of the iron–sulphur e.p.r. signal was not observed. However, ELDOR studies (Lowe & Hyde, 1975) showed that the molybdenum indeed consisted of two species interconverting at about the iron–sulphur spin-lattice relaxation rate. No half-field e.p.r. lines, corresponding to transitions for which $\Delta M_s = 2$, have been observed even in the most concentrated xanthine oxidase samples (Lowe, 1974). On the other hand, the interaction also manifests itself, when iron–sulphur is reduced, by an in-

crease in the microwave power (about 10-fold) required to saturate the Mo(V) e.p.r. spectra (D. J. Lowe, unpublished work). However, quantitative interpretation of saturation data is difficult.

The observation of Lowe *et al.* (1972), that the splitting of the molybdenum signal, and hence the magnetic interaction, was essentially isotropic, led to a paradox. The dipolar (anisotropic) component of the coupling was apparently very small, so that the coupling was assumed to be mainly due to an exchange-type interaction occurring over an extremely long distance, perhaps as much as 4 nm. This suggested that there might be a special pathway through the protein, conceivably of suitably orientated aromatic amino acid residues, which facilitated both electron transfer during the enzyme-turnover reactions and magnetic interaction between the centres (Lowe, 1974; Bray, 1974). However, the theory of such magnetic interaction is not well worked out and it was not possible to exclude the alternative that other mechanisms make the observable dipolar component small.

To obtain more information on these problems, we have now carried out further studies, concentrating particularly on the newly discovered Mo(V) e.p.r. signal from xanthine oxidase, designated Resting II by Lowe *et al.* (1976). This signal has a number of advantages over other signals from molybdenum in this enzyme. It is particularly sharp and is uncomplicated by interactions with protons. It is stable to oxidation and reduction; thus iron-sulphur in the enzyme may be oxidized or reduced without affecting the molybdenum chromophore (Bray *et al.*, 1975). Finally, the splitting of the signal Resting II due to interaction with iron-sulphur is a particularly large one.

In addition to studying magnetic interaction in relation to the Resting II signal of xanthine oxidase, we also carried out chemical-modification studies on the enzyme, as well as a comparison with two xanthine dehydrogenases. We sought species in which selective chemical modification had taken place to the molybdenum e.p.r. chromophore, or to the iron-sulphur chromophore, or to the hypothetical pathway through the protein through which their coupling might occur (see Scheme 1). In different modifications, dipolar and exchange interactions might be altered in different ways, thereby possibly clarifying the nature and mechanism of the coupling and yielding information about the relative positions of the chromophores.

Materials and Methods

Enzyme samples

Xanthine oxidase was prepared by the salicylate denaturation method of Hart *et al.* (1970), xanthine dehydrogenase from turkey liver as described by

Scheme 1. Hypothetical pathways for magnetic interaction between the molybdenum and iron-sulphur centres in xanthine oxidase or xanthine dehydrogenase

The enzyme half-molecule is represented by the oval shape, and the circles placed in arbitrary positions within this represent the molybdenum, flavin and Fe/S groups, together with the atoms in their immediate vicinities. The full arrow represents a possible pathway directly through the protein and the dashed arrow an alternative pathway via the flavin. The length of the pathway Mo to Fe/S is considered in the Discussion section. Although this scheme is considered in relation to magnetic interactions, it is formally similar to the electron-transfer scheme first put forward by Komai *et al.* (1969).

Cleere & Coughlan (1975) (see also Barber *et al.*, 1976) and xanthine dehydrogenase from *Veillonella alcalescens* as described by Dalton *et al.* (1976). Unless otherwise stated, all experiments were performed in 0.05 M-sodium pyrophosphate buffer adjusted to pH 8.2 with HCl; changes of buffer were avoided, since there is a slight variation of linewidths and *g* values with different buffers. Enzyme concentrations, from A_{450} measurements ($\epsilon_{\text{mM}} = 72 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$; Bray, 1975), expressed as total enzyme (2 mol of FAD/mol), ranged from 30 to 900 μM . The Rapid Mo(V) e.p.r. signal from functional enzyme was obtained by brief reduction with purine or substituted purine substrates. The Slow signal from the non-functional desulpho species (prepared as described by Massey & Edmondson, 1970) was obtained by extended reduction with dithionite (see Bray *et al.*, 1975). Xanthine oxidase showing the stable Mo(V) signal, Resting II, was prepared from the desulpho enzyme by treatment with ethylene glycol as described by Lowe *et al.* (1976), and when necessary centres Fe/S I and Fe/S II in these samples were reduced with dithionite (Bray *et al.*, 1975). Guanidinium chloride solution was added anaerobically after the reduction was complete. Reaction times with guanidinium chloride were 1 min, before freezing the samples. For the high-pH (12.0) sample showing the Resting II signal, the modified enzyme (33 μM) was treated with dithionite (1 mM) for about 1 min, then an equal volume of Na_2CO_3 (250 mM;

adjusted to pH 12.6 with NaOH) was added anaerobically and the sample was frozen 1 min later. A final pH of 12.0 was measured on a duplicate sample mixed in the absence of enzyme; buffering by the enzyme would not be expected to lower the pH significantly. Xanthine oxidase lacking the flavin moiety was prepared by the method of Komai *et al.* (1969), and had $A_{450}/A_{550} = 1.87$, indicating complete removal of the FAD. Xanthine oxidase enriched in ^{95}Mo was prepared as described by Bray & Meriwether (1966).

E.p.r. and computer techniques

E.p.r. techniques were as described by Lowe *et al.* (1972) and Smith *et al.* (1973). For 35 GHz spectra near to 4.2 K the cavity was protected by a thin rubber sheath (made from a child's balloon) and immersed in a specially constructed simple glass Dewar (capacity about 1 litre) containing liquid helium, which fitted inside and extended above the modulation coils. Operation for about $\frac{1}{2}$ h on a single filling of liquid helium was possible. A small temperature gradient between the sample and the liquid would be expected. Double integrations were corrected for g -value-dependence of transition probability as described by Aasa & Vännegård (1975). All spectra were automatically digitized during running and stored for later manipulation (double integration, addition, etc.) by using a PDP 11/10 computer (Digital Equipment Co., Galway, Ireland) with a visual display system. Isotropically split (low-temperature) spectra were simulated by displacing the parent (high-temperature) spectrum equally to high and low field, then adding the two shifted spectra together. Values given in the text for calculated relative amounts of two spectra added together to give a third spectrum were obtained from a least-squares computer-fitting procedure. The program corrected for differences in integrated intensities and carried out adjustment of baseline shifts and overall gain, as well as changing the relative amounts of the two species, until an optimal fit was obtained. Errors given are the relative changes required to give a 50% increase in the sum-of-squares error estimate.

Results

Splitting of the Resting II Mo(V) e.p.r. signal by the Fe/S I and the converse

Fig. 1(a) shows the 35 GHz Resting II Mo(V) e.p.r. spectrum at a temperature over 100 K, where no interaction with the iron-sulphur centres is observed; the spectrum is rhombic in type (Lowe *et al.*, 1976). The low-temperature change in the signal is shown in Fig. 1(b), and the splitting at each principal g value is clearly visible. Table 1 shows the values of these splittings, and for the first time a proper measure-

Fig. 1. 35 GHz e.p.r. spectra of the Resting II Mo(V) signal from xanthine oxidase at various temperatures. The sample was prepared as described by Lowe *et al.* (1976) and the Fe/S centres were reduced with dithionite. The microwave attenuations were 30 dB, 30 dB and 40 dB for (a), (b) and (c) respectively, with a modulating magnetic field of 0.5 mT at 100 kHz.

Table 1. Resting II Mo(V) e.p.r. signal of xanthine oxidase: g values and values of the splitting observed at each g value, at low temperatures

The sample was prepared as described by Lowe *et al.* (1976), and the Fe/S centres were reduced with dithionite. The g values differ slightly from those given by Lowe *et al.* (1976) and were obtained by using a dual sample cavity (S. Tanner & R. C. Bray, unpublished work).

	g_1	g_2	g_3
g value	1.978	1.971	1.965
Splitting (mT)	2.2	2.4	2.6

ment of the anisotropy of a split xanthine oxidase signal can be made; this is the difference between the maximum and minimum values given in Table 1 and is 0.4 mT. (The assumption that all the splittings have the same sign, their magnitudes differing because of variations in the relatively small anisotropic contributions, is justified in the next paragraph.)

Fig. 1(c) shows the spectrum of the same sample in liquid helium at a temperature a little above 4.2 K; the lower-field line of each doublet is increased in amplitude by 10–15% relative to the higher-field line. This enhancement was observed at different microwave powers and modulating-field amplitudes and so is unlikely to be due to saturation or passage effects. The two levels of the reduced Fe/S I centre (g approx. 2; $S = \frac{1}{2}$), split by Zeeman interaction, are predicted to have a population difference, because of the Boltzmann distribution, of about 10–15% at a temperature of about 10 K, for a field of 1.3 T. Thus, the observed enhancement of one of the two interconverting species in the molybdenum spectrum is expected. Since the lower-field component is increased for each g value, the sign of the interaction must be the same at all orientations of the Mo(V) species, and furthermore the interaction is ferromagnetic.

In principle, the interaction should have the same effect on the Fe/S I spectrum as on the Mo(V) spectrum. That this has not been observed previously is not unexpected (Lowe *et al.*, 1972), since for most Mo(V) signals (see Table 2, below) the interaction is much less than the linewidth of the Fe/S I signal, and furthermore the amount of Mo in the quinquivalent state has generally been less than 20%. Use of the Resting II signal, however, enables the splitting of the Fe/S I signal to be observed, since the size of the interaction is substantially greater than in other xanthine oxidase signals and since 50% of the Mo can be quinquivalent. Figs. 2(a) and 2(b) show, respectively, the normal Fe/S I signal, little influenced by Mo(V), and that given by xanthine oxidase with 50% of its Mo as Resting II. The broadening of the g_1 feature particularly is clearly apparent in Fig. 2(b) (see linewidth markers). Fig. 2(c) is the same as Fig. 2(b) but after subtraction of an appropriately scaled ^{95}Mo Resting II spectrum, so as to correct for the ^{95}Mo and ^{97}Mo hyperfine lines which overlie the broadened Fe/S I signal. Fig. 2(c') shows a simulation produced from Fig. 2(a) by assuming an isotropic splitting of 2.5 mT in 50% of the molecules. This simulation agrees well with the experimental spectrum; thus the change in the Fe/S I signal is in accordance with expectations, both qualitatively and quantitatively.

Selective modification of the molybdenum centre

A number of spectrally distinct Mo(V) e.p.r. species of milk xanthine oxidase and the xanthine dehydrogenases from turkey liver and from the

Fig. 2. 9 GHz e.p.r. spectra of the Fe/S I centre of xanthine oxidase showing line broadening from interaction with signal Resting II Mo(V)

(a) shows the Fe/S I spectrum of a sample of xanthine oxidase giving the Slow Mo(V) signal (13% of the Mo in the paramagnetic form), and (b) shows that of a sample giving the Resting II Mo(V) signal (50% of the Mo in the paramagnetic form). (c) shows (b) after subtraction of a split ^{95}Mo Resting II spectrum, to correct for interference by Mo hyperfine lines. (c') is a simulation of (c) produced by adding a 2.5 mT isotropic splitting to 50% of (a). Because of the greater splitting from signal Resting II (Table 2) and the high proportion of Mo(V), the Fe/S I signal in (b) is distinctly broader than that in (a) (see particularly the linewidth markers on the g_1 features). Here and in other Figures showing the Fe/S I signal, the central portions of the spectra have been omitted and replaced by broken lines so as to avoid confusion by the intense Mo(V) spectra. The spectra were recorded at 42 K by using 0.5 mT modulation at 100 kHz. The microwave power was 10 mW and the frequency was 9.160 GHz.

anaerobic bacterium *Veillonella alcalescens* were obtained as described in the Materials and Methods section. All of these Mo(V) signals exhibit an interaction with reduced centre Fe/S I when observed at low temperatures, and adequate simulations can always be produced by assuming an approximately isotropic splitting. These different chemical species have different values of the splitting (Table 2), ranging from 0.7 to 2.4 mT, with anisotropies of from 0.15 to 0.4 mT. Fig. 3 shows typical spectra (at 9 GHz) and simulations, in this case for the Rapid signal from milk xanthine oxidase and from *V. alcalescens* xanthine dehydrogenase.

Selective modification of the iron-sulphur centres

The Fe/S I centre in turkey liver xanthine dehydrogenase is very similar (Barber *et al.*, 1976) to the corresponding chromophore in the milk enzyme.

Table 2. *Splittings and anisotropies of Mo(V) e.p.r. spectra from xanthine oxidase and xanthine dehydrogenase and the effects of chemical modifications*

The treatment (other than reduction where this was necessary) of the enzymes to give the observed molybdenum signals was as described in the Materials and Methods section. The values of the additional isotropic splittings of the Mo(V) e.p.r. spectra caused at 40 K by interaction with the unpaired electron on the Fe/S I centre are given in mT. These numbers were obtained by comparing visually the overall goodness of fit obtained between experimental spectra at 35 GHz and simulations produced by the procedure illustrated in Fig. 3, and selecting the splitting giving the best overall fit. Estimates of the maximum anisotropic components of the splittings correspond to the limit of deviations obtained by using the above method when considering different features of the powder spectra separately. Measurements at 9 GHz agreed with those at 35 GHz unless otherwise indicated. [Some of the data have already been published; see Lowe *et al.* (1972), Lowe (1974) and Bray (1974).] ND, Not determined.

Enzyme and treatment	Mo(V) signal	Fe/S I signal	Splitting (mT)	Anisotropy (mT)
Milk	Rapid	Normal	0.7**	0.25*
Turkey	Rapid	Normal	0.7	0.15
<i>V. alcalescens</i>	Rapid	(Normal†)	1.6**	0.2
Milk	Slow	Normal	1.1‡¶**	0.15‡
Turkey	Slow	Normal	1.1	0.15
<i>V. alcalescens</i>	Slow	(Normal†)	1.5**	0.25
Milk	Resting II	Normal	2.4§	0.4§
Milk (in 3.4 M-guanidinium chloride)	Resting II	Modified	2.0	ND
Milk (at pH 12)	Resting II	Normal	2.4	ND
Milk (deflavo enzyme)	Resting II	Normal	2.4	ND

* The corresponding value measured at 9 GHz was 0.15 mT.

† Only one Fe/S signal is seen with the enzyme from *V. alcalescens*. It is quite different from the Fe/S I signal of the milk and turkey enzymes.

‡ Identical values were obtained in $^2\text{H}_2\text{O}$ solution.

§ Data from Table 1.

|| Measured at 9 GHz only.

¶ cf. Lowe *et al.* (1972).

**cf. Lowe (1974) and Bray (1974).

Fig. 3. High- and low-temperature forms of the Rapid Mo(V) signal from (a) xanthine oxidase and (b) *V. alcalescens* xanthine dehydrogenase, with simulations of the low-temperature forms assuming isotropic splitting

The samples (at pH 7.8) were reduced with an excess of purine and frozen after 1 min. Recording conditions were 0.25 mT modulation and 5 mW power at high temperature, or 20 μW at low temperature. Splittings assumed in the simulations were 0.7 mT for the oxidase and 1.6 mT for the dehydrogenase.

It may not be surprising therefore that Mo(V) signals from the turkey enzyme show splittings (Table 2) identical with the corresponding ones from the milk enzyme. On the other hand the bacterial enzyme has

quite a different Fe/S centre (Dalton *et al.*, 1976) and gives larger splittings (Table 2).

In a search for further ways of modifying the interaction in the milk enzyme, we used the protein

denaturant guanidinium chloride at various concentrations. Figs. 4(a) and 4(b) show the 9GHz high-temperature Resting II e.p.r. signal from xanthine oxidase in the absence and presence of guanidinium chloride (3.4M); there is no detectable effect on the high-temperature spectrum. Figs. 5(a), 5(b) and 5(c) show the effect of zero, 3.0M- and 3.4M-guanidinium chloride on the 40K Mo(V) Resting II spectra, showing that although the molybdenum chromophore is unaffected, the guanidinium chloride has significantly changed the interaction. The spectrum in 3.0M-guanidinium chloride is intermediate in form between those in zero and in 3.4M-guanidinium chloride as confirmed by the simulation of Fig. 5(b) shown in Fig. 5(b'). The latter is a least-squares fit (see the Materials and Methods section for details) corresponding to a mixture of $28 \pm 6\%$ of the species in Fig. 5(a) plus $72 \pm 6\%$ of the species in Fig. 5(c). We conclude, on the assumption that all the molecules are modified in 3.4M-guanidinium chloride, that 72% of them must have undergone the same modification in 3.0M-guanidinium chloride.

The modification produced by guanidinium chloride must involve part of the enzyme molecule other than the molybdenum centre. That the Fe/S I centre is involved is shown by Fig. 6. Figs. 6(a), 6(b) and 6(c) show the spectra of centre Fe/S I in the samples of Figs. 5(a), 5(b) and 5(c). Clearly guanidinium chloride has severely modified centre Fe/S I

Fig. 4. Lack of effect of guanidinium chloride on the high-temperature spectrum of the Resting II Mo(V) signal from xanthine oxidase

In (a) guanidinium chloride was absent, but in (b) it was present at a concentration of 3.4M. The spectra were recorded at 120K at 5mW microwave power by using 0.2mT field modulation at 100kHz. The microwave frequency was 9.297GHz.

Fig. 5. Effect of guanidinium chloride on the low-temperature spectra of signal Resting II Mo(V)

The samples of (a) and (c) are those of Figs. 4(a) and 4(b). (b) is a similar sample prepared in the presence of 3.0M-guanidinium chloride. (b') is a simulation of (b) produced by adding 28% of (a) to 72% of (c) (percentage by integrated intensities; see the Materials and Methods section for details). The spectra were recorded at 42K and 0.1mW microwave power by using 0.2mT field modulation at 100kHz. The microwave frequency was 9.160GHz. Signals were recorded at different gain settings; relative corrected integrated intensities in (a), (b) and (c) were 100, 80 and 50 respectively.

(Figs. 6b and 6c). A least-squares fit (Fig. 6b') of the g_2 and g_3 regions shows that the spectrum in 3.0M-guanidinium chloride (Fig. 6b) can be made up from $21 \pm 5\%$ of the species in Fig. 6(a) plus $79 \pm 5\%$ of the species in Fig. 6(c). These findings are fully consistent with the hypothesis that the interaction is changed only when the Fe/S I centre is modified.

The change in interaction caused by guanidinium chloride is not very great, corresponding to a decrease of about 0.4mT (as measured by overall width of the spectrum of Fig. 5c) in the low-temperature splitting of the Mo signal in the sample in 3.4M-guanidinium chloride (Table 2).

The spectra of Fig. 6 were recorded at 40K, at which temperature signal Fe/S II is not normally observable. That this centre was also affected by the guanidinium chloride was shown, however, by additional studies at lower temperatures, in which we were unable to detect its signal in the presence of the denaturing agent.

Anaerobic treatment of active xanthine oxidase by 3.4M-guanidinium chloride for 5min, followed by anaerobic removal of the denaturant by gel filtration,

Fig. 6. Effect of guanidinium chloride on the spectrum of centre Fe/S I in xanthine oxidase

The samples of (a), (b) and (c) were those of Figs. 5(a), 5(b) and 5(c). (b') is a simulation of (b) produced by adding 21% (integrated intensity) of (a) to 79% of (c) (see the Materials and Methods section for details). Spectra were recorded at 42 K and 10 mW microwave power by using 0.5 mT field modulation at 100 kHz. The microwave frequency was 9.160 GHz. The signals were recorded at different gain settings; relative corrected integrated intensities in (a), (b) and (c) were 100, 90 and 60 respectively.

(cf. Pick & Bray, 1969) had no effect on the activity of the enzyme, showing that no major irreversible change in the structure of the protein is caused by the denaturing agent under the conditions used.

Attempts to modify the hypothetical pathway through the protein

All the effect of guanidinium chloride on the interaction, as described above, could be accounted for by its effect on the Fe/S I centre. We therefore sought other methods of modifying selectively the hypothetical interaction pathway (Scheme 1) through the remainder of the xanthine oxidase molecule.

We first studied the effect of removing FAD from the enzyme. Fig. 7(a) shows the high-temperature Resting II Mo(V) e.p.r. spectrum from the deflavo form of milk xanthine oxidase; this spectrum is indistinguishable from the spectrum of the normal form of the enzyme (Fig. 4a). Fig. 7(b) shows the low-temperature spectrum of deflavo enzyme. Comparison of this with Fig. 5(a) shows that the interaction is slightly but significantly altered by loss of FAD.

Fig. 8 shows e.p.r. spectra from a normal FAD-containing sample of xanthine oxidase giving the Resting II signal, but prepared at pH 12.0, a pH that is expected to denature many proteins and to change

Fig. 7. High- and low-temperature forms of the Resting II Mo(V) spectrum of deflavo xanthine oxidase

The sample was reduced with dithionite. (a) was recorded at 120 K and 5 mW microwave power, and (b) at 39 K and 0.1 mW microwave power. Field modulation was 0.2 mT at 100 kHz. The microwave frequency was 9.176 GHz.

Fig. 8. Effect of high pH on the Resting II Mo(V) e.p.r. signal from xanthine oxidase

The sample was reduced with dithionite, then adjusted to pH 12.0 (see the Materials and Methods section for details). (a) and (b) show the high- and low-temperature Resting II Mo(V) signals measured at 120 K and 45 K respectively. Microwave powers were 10 mW and 0.1 mW for (a) and (b). (c) shows the Fe/S I centre spectrum. This was recorded at 45 K and 10 mW microwave power with 0.5 mT field modulation at 100 kHz. The microwave frequency was 9.163 GHz.

the conformation of groups such as tyrosine residues (Tanford, 1968). As shown in Figs. 8(a) and 8(c), the Mo(V) and the Fe/S I spectra are unaltered (cf. Figs. 4a and 6a respectively). Fig. 8(b) shows that the interaction has again been slightly altered (cf. Fig. 5a).

Results of these experiments are summarized in Table 2. In the absence of measurements at 35 GHz, changes in the anisotropies caused by the above treatments cannot be estimated, but are presumably significant. On the other hand the splittings seem to be little affected, since the overall widths of the low-temperature molybdenum spectra (Figs. 7b and 8b) are virtually unchanged.

Discussion

The results fully confirm that the splitting of the molybdenum signals is due to interaction with the Fe/S I centre, since the expected splitting of the Fe/S I signal by the molybdenum has now been observed. Furthermore, the change in the Mo(V) spectrum observed near to 4.2 K confirms that the splitting is basically isotropic in character, with a smaller anisotropic component superimposed. There is no evidence from the present work for interactions other than that between Mo(V) and reduced Fe/S I.

For the sake of simplicity we shall assume that the interaction occurs within single subunits of the enzyme, though the possibility of intersubunit interactions is not excluded by our data. That the molybdenum and iron-sulphur chromophores can be modified separately confirms that they are not in close proximity to one another. The relative lack of sensitivity of the interaction to removal of the flavin shows that the rather large flavin molecule is not important in a bridging capacity as far as the coupling is concerned between molybdenum and the iron-sulphur centre (see Scheme 1). The sensitivity of the interaction (both the splitting and the anisotropy) to modification of either of the centres is not particularly surprising, if both dipolar and exchange terms are contributing. Modification of one chromophore is almost certain to result in a shift of its magnetic axes, and this could readily account for the change in the coupling, even if the distance between the chromophores remained completely unaltered. From the parameters of the coupling we may place limits on the distance between molybdenum and centre Fe/S I. If the absolute value of the interaction is equated to a dipolar effect, then the largest interaction (that of signal Resting II) gives a point-dipole-to-point-dipole distance of 1.0–1.2 nm; the mechanism by which a dipolar interaction can give rise to an apparently isotropic interaction with the same sign at all g values is not, however, clear. If the largest anisotropy observed (0.4 mT for signal Resting II) is taken as the dipolar component (a theoretically more satisfying

assumption), then a Mo-to-Fe/S I distance of 2.0–2.5 nm is given; the large size of this latter distance, however, seems to be in conflict with the required existence of an exchange pathway and with the relative lack of sensitivity to removal of flavin, which has dimensions of the order of 1.0 nm. It should, however, be noted in this context that the energy of the largest interaction is only $2 \times 10^{-3} \text{ cm}^{-1}$. That the interaction changes only slightly at pH 12.0 would suggest that any special pathway that may exist lies in a particularly stable part of the protein. Since the subunit (mol.wt. 140 000) will have a diameter of about 4.0 nm, there are clearly no problems in accommodating a Mo-to-Fe/S I distance of 2.0–2.5 nm.

In connection with the distance estimates, a special case of the relative locations of the Mo and Fe/S I has to be mentioned. If it happened that the vector joining Mo to Fe/S I was equally inclined (at an angle of $54^\circ 44'$, the 'magic angle') to the three principal axes of the g tensor, then it can be shown that the dipolar coupling would disappear completely along these principal axes. [A somewhat analogous situation was considered by Leigh (1970).] However, for a number of reasons we do not consider it likely that this is the case here; in particular, it can be shown that small deviations (about 1°) from the angle of $54^\circ 44'$ result in the appearance of significant anisotropies. This is incompatible with several different Mo(V) signals all presumably with different g -tensor principal axes (Garner *et al.*, 1977) all giving apparently isotropic interactions.

It is noteworthy that changes in interaction produced by modifying the chromophores are much greater than any that we have achieved in attempted modifications of the protein. It was disappointing in this regard to find that the relevant part of the protein was apparently less sensitive to guanidinium chloride than were iron-sulphur chromophores. Although there is little direct evidence for the pathway, it remains, however, extremely difficult to interpret the data unless it is assumed that exchange interaction takes place via a pathway consisting of at least several atoms that intervene between the ligands of the two metal atoms. Thus the original paradox concerning the interaction still remains, though we are able to put a smaller upper limit on the distance than the earlier one of Lowe *et al.* (1972). Further interpretation of the magnetic interaction in xanthine oxidase and reconciliation of the paradox will require a full analysis of the effects of dipolar interactions on Mo(V), which is expected to have a large spin-orbit coupling constant, and also additional spectroscopic data, such as the study of single crystals, if these become available.

Finally, we note that there is no positive correlation between magnitude of the magnetic interaction and the rates of intramolecular electron transfer, at room

temperature, from other constituents to or from Mo(V) in the milk enzyme molecule. Electron-transfer rates (Bray *et al.*, 1975; R. C. Bray, M. J. Barber & R. Cammack, unpublished work) decrease drastically in the series Rapid>Slow>>Resting II, whereas the splittings decrease in the reverse sequence for the milk and turkey enzymes. Thus the factors determining the splitting are not those that limit the electron-transfer rates.

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