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Comparison of the Molybdenum Centres of Native and Desulpho Xanthine Oxidase

THE NATURE OF THE CYANIDE-LABILE SULPHUR ATOM AND THE NATURE OF THE PROTON-ACCEPTING GROUP

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The non-functional form of xanthine oxidase known as the desulpho enzyme was compared with the functional enzyme in various ways, to obtain information on the structure of the molybdenum centre and the mechanism of the catalytic reaction. The desulpho enzyme, like the functional one, possesses a site for the binding of anions, presumably as ligands of molybdenum. Evidence is presented that in the Mo(V) e.p.r. signal from the desulpho-enzyme, as in that from the functional enzyme, a weakly coupled proton, in addition to a strongly coupled proton, interacts with the metal. Measurements were carried out by e.p.r. on the rate at which the proton strongly coupled to molybdenum exchanged, on diluting enzyme samples with ^2H_2O. For the desulpho enzyme the exchange rate constant was $0.40 s^{-1}$, at pH 8.2 and 12°C, and for the functional enzyme it was $85 s^{-1}$. It is shown that the great majority of reported differences between the enzyme forms are consistent with functional enzyme containing an (Enzyme)–Mo=S grouping, replaced in the desulpho form by (Enzyme)–Mo=O. Protonation of these groups, with pK values of about 8 and 10 respectively, would give (Enzyme)–Mo–SH and (Enzyme)–Mo–OH, these being the forms observed by e.p.r. The accepting group in the functional enzyme, for the proton transferred from the substrate while molybdenum is reduced in the catalytic reaction [Gutteridge, Tanner & Bray (1978) *Biochem J.* **175** 869–878], is thus taken to be Mo=S.

Conversion of native xanthine oxidase into a non-functional form, given the name desulpho xanthine oxidase, by Bray (see Section II, B, 1, b, Bray, 1975), is a reaction that is difficult to prevent entirely in normal storage and handling of the enzyme. Massey & Edmondson (1970) showed that the reaction can be brought about more rapidly by treating the enzyme in the oxidized state with CN^-. They showed that in the inactivation process a sulphur atom was liberated from the enzyme in the form of thiocyanate. Reincorporation of sulphur into the desulpho-enzyme with partial restoration of enzymic activity could be brought about by treatment with sulphide ions (Massey & Edmondson, 1970; Edmondson *et al.*, 1972). Compounds that are reducing substrates, reacting at the molybdenum site of native xanthine oxidase, generally lack the ability to reduce desulpho xanthine oxidase at an appreciable rate, thus accounting for the lack of activity of this form of the enzyme.

The conversion of the native into the desulpho form is accompanied by changes in several properties of the enzyme. Thus, on appropriate reduction of molybdenum to the five-valent state, the desulpho-enzyme gives the e.p.r. signal designated Slow by Bray & Vänngård (1969), whereas the functional enzyme gives the Rapid signal. Parameters of these signals differ from one another in important ways. In the Slow signal, in comparison with the Rapid signal, $g_\perp$ $[\frac{1}{2}(g_x+g_y)]$ has become greater than $g_\parallel$ (g_z), $g_{av.}$ $[\frac{1}{3}(g_x+g_y+g_z)]$ has decreased slightly, and the magnitude of the hyperfine splitting, due to a strongly coupled exchangeable proton, has increased somewhat (see Section II, B, 4, d, Bray, 1975). Redox potentials at pH 8.2 for the systems Mo(VI)/Mo(V) and Mo(V)/Mo(IV) are decreased from −355 mV and −355 mV in the functional enzyme to −440 mV and −480 mV, respectively in the desulpho form (Cammack *et al.*, 1976). Probably related to this, and as is implied by the name of the Slow signal, rates both for reduction by dithionite and reoxidation by oxygen of molybdenum in the desulpho enzyme are sluggish in comparison with those for the functional enzyme (Bray *et al.*, 1975). Finally, relative to the functional enzyme, the desulpho form has decreased absorption in the near-u.v. ($\Delta\varepsilon$ per molybdenum

atom about 4000 at 320nm) (Edmondson *et al.*, 1972; Cleere *et al.*, 1974).

Despite availability of the above information, the nature of the cyanide-labile sulphur atom of native xanthine oxidase and the details of the reaction involved in its loss have remained controversial. Massey & Edmondson (1970) originally proposed that the sulphur was present in the form of a persulphide group, RSS^-, its properties modified, however, by other groups in the enzyme. Bray & Swann (1972) suggested that the sulphur might be a ligand of molybdenum. More specifically, Coughlan (1977) proposed cysteine sulphur liganded to molybdenum. However, evidence that this could give rise to thiocyanate, on treatment with cyanide, seems slight. Furthermore, the improbable assumption was made, without spectroscopic data to support it, that naturally arising desulpho enzyme is fundamentally different in structure from that produced by cyanide treatment. Rejection of this theory therefore seems justified. In its stead, we return to an earlier suggestion (Bray, 1973) that in the desulpho enzyme an unspecified sulphur atom has become replaced by an oxygen atom. Independently, analogy with the inorganic chemistry of molybdenum had suggested to others that xanthine oxidase may contain a terminal sulphur ligand (Mo=S) (Williams & Wentworth, 1973; Stiefel, 1977). This could react with cyanide (cf. Drew *et al.*, 1976), presumably (though this is not explicitly stated by any of these workers) to give a terminal oxygen, Mo=O, thus:

$$\text{(Enzyme)–Mo=S}+CN^-+H_2O \rightarrow$$

$$\text{(Enzyme)–Mo=O}+CNS^-+2e^-+2H^+$$

The above scheme provides an attractive hypothesis as to the nature of the cyanide-labile sulphur atom. To test it, as well as to obtain further information on the catalytic mechanism of the enzyme, we carried out the work described below. The experiments involved comparisons of further properties of desulpho xanthine oxidase with those of the functional enzyme.

Materials and Methods

Xanthine oxidase samples

The procedures and materials used were generally as described by Gutteridge *et al.* (1978) and in the references cited by these workers. Desulpho xanthine oxidase was prepared from the functional enzyme by treatment with cyanide (Massey & Edmondson, 1970). We found, however, when working at an enzyme concentration of about 0.1–0.5mM [determined from ε_{450} (per half-molecule) = 36] and with 30mM-cyanide, that the reaction time had to be extended to about 20h to decrease activity to the required level. Such samples, after isolation by gel filtration on Sephadex G-25, were found to be less than 1% functional.

Final concentrations of enzyme, used in the different experiments, ranged from 0.2 to 0.5mM. When functional enzyme was employed, functionality was about 70%.

Bicine [*NN*-bis-(2-hydroxyethyl)glycine], adjusted with NaOH, was the buffer chosen for almost all experiments, to avoid large variations of apparent pH on freezing (Williams-Smith *et al.*, 1977). Where ^2H_2O was used as solvent, enzyme samples were dialysed against several changes of ^2H_2O/Bicine until residual ^1H_2O content was less than 4%. A Varian T60 n.m.r. spectrometer was used to determine ^1H_2O content in all ^2H_2O solutions. pH values are expressed as $pD_{app.}$ for the ^2H_2O solutions, i.e. as uncorrected readings obtained with a glass electrode.

Rapid-freezing procedure

The hydrogen/deuterium exchange experiments were carried out with the new ram-drive rapid-freeze equipment described by Gutteridge *et al.* (1978). The dead-time for this apparatus, determined by the myoglobin–azide reaction (Ballou & Palmer, 1974), was about 3ms (M. J. Barber & R. C. Bray, unpublished work). For experiments with active enzyme a three-syringe double-push system was used. The first push allowed reduction of the enzyme with enough dithionite to produce maximum Rapid signal, with no interference from the Slow species. At pH8.2, 12°C and in the presence of 200mM-$NaNO_3$, the optimum time was found to be 10s. The second push diluted this Rapid species with ^2H_2O from the third syringe, and, after predetermined reaction times, the samples were then frozen. Reduction of the desulpho enzyme with dithionite produced the Slow signal, which was stable for some hours (Bray *et al.*, 1975). Only a two-syringe system was therefore required to dilute the reduced desulpho species with ^2H_2O buffer, before freezing after appropriate reaction times. Double-push operation was used when these times were extended above 0.50s.

E.p.r. and computer procedures

E.p.r. spectra were recorded under conditions similar to those described by Gutteridge *et al.* (1978) and computer-generated difference spectra were obtained by the procedures of Bray *et al.* (1978). Computer simulation of e.p.r. spectra was as described by Lowe (1978). When it was appropriate, complete replacement of hyperfine splitting from ^1H by that from ^2H was allowed for in the simulations (cf. Gutteridge *et al.*, 1978). Incomplete replacement was simulated by adding, in suitable proportions, the simulated ^1H and ^2H spectra.

Results

Evidence for an anion-binding site associated with molybdenum in desulpho xanthine oxidase

The Slow signal from desulpho xanthine oxidase was first described by Bray *et al.* (1968). Though simulations have not been published, spectra reported at 9 and at 35GHz, on samples in ^1H_2O or in ^2H_2O, appeared consistent with a single chemical species having Mo(V) coupled to a single exchangeable proton.

Gutteridge *et al.* (1978) have shown that NO_3^- modifies the Rapid signal from functional xanthine oxidase. They attributed this to binding of the ion as a ligand of the metal in the enzyme, the complex having a dissociation constant of about 5mM, at pH8.2. Earlier work on the desulpho enzyme (R. T. Pawlik & R. C. Bray, unpublished work, quoted by Cammack *et al.*, 1976) had already shown that changing the nature of the buffer could likewise modify slightly the lineshape of the Slow signal.

To obtain further information on ion effects on the Slow signal, we carried out the experiments illustrated in Fig. 1, the signal being generated by reduction with dithionite in various media, all in ^1H_2O at pH8.2, with spectra recorded at 9.3GHz. Most of the work was in Bicine buffer (see the Materials and Methods section). Fig. 1(*a*) shows the Slow signal in 50mM-pyrophosphate, in the form originally described by Bray *et al.* (1968). We found that changing to Bicine buffer (Fig. 1*c*) or to Tris (not illustrated) caused quite small, though reproducible, spectral changes. However, more dramatic effects were produced by nitrate, nitrite and, to a lesser extent, perchlorate (Figs. 1*d*, 1*e* and 1*b*). These spectra provide clear evidence for interactions of various ions with the molybdenum centre of desulpho xanthine oxidase.

Analysis of such effects was developed further, by using spectroscopy at 35GHz on samples prepared in either ^1H_2O or ^2H_2O. Fig. 2 illustrates the changes in the Slow spectrum caused by NO_3^-. Fig. 2(*b*) is the Slow spectrum in the presence of 300mM-nitrate in ^2H_2O, and Figs. 2(*c*) and 2(*d*) correspond to the spectra of the pure nitrate complex in ^2H_2O and in ^1H_2O, respectively. For comparison, the typical Slow spectrum of the aquo complex formed in ^2H_2O [see Gutteridge *et al.* (1978) for nomenclature] is shown in Fig. 2(*a*). Clearly the effect of the anion has been to shift appreciably the g_1 feature to higher field until it is almost coincident with the g_2 region. The g_3 has also changed position, but to lower field. The computer simulations of Figs. 2(*a'*), 2(*c'*) and 2(*d'*) show that all these spectra may be simulated quite satisfactorily. Parameters for the aquo complex (Fig. 2*a'*) are comparable with, but more reliable than, those reported by Bray (1973).

As is detailed in the caption to Fig. 2 (and as will be discussed below), we used difference spectra to eliminate other contributions and obtain the pure spectra of the nitrate complex. However, further support for our interpretation of spectra in the presence of nitrate is provided by success in simulating comparable nitrate difference spectra recorded at 9GHz (Fig. 3), when making use of the same parameters as those employed at 35GHz. Figs. 3(*a*) and 3(*b*) show the difference spectra recorded in ^1H_2O and ^2H_2O respectively; Figs. 3(*a'*) and 3(*b'*) are the corresponding simulations.

Comparison of Fig. 1(*d*) with Fig. 3(*a*) indicates the effect of increasing concentrations of NO_3^- on the Slow spectrum. The former is mainly from a mixture

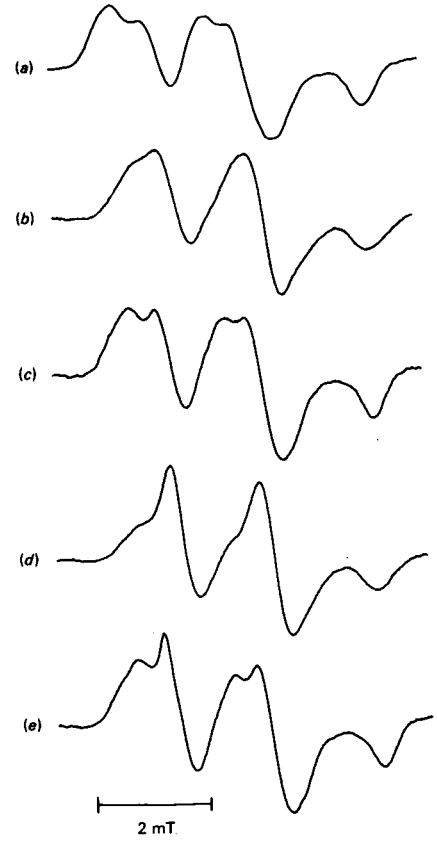

Fig. 1. *Effects of nitrate and other ions on the Slow Mo(V) e.p.r. signal in ^1H_2O, recorded at 9.3GHz*

The Slow signal was generated by reduction of the desulpho enzyme in various media at pH8.2 in ^1H_2O at 20–25°C for 10–20min with 1–5mM-dithionite. Buffers were as follows: (*a*) pyrophosphate (50mM); (*b*) Tris (5mM); (*c*) Bicine (100mM); (*d*) and (*e*) Bicine (10mM). In addition the following anions were present (added as sodium salts): (*b*) perchlorate (100mM); (*d*) nitrate (115mM); (*e*) nitrite (1.0M).

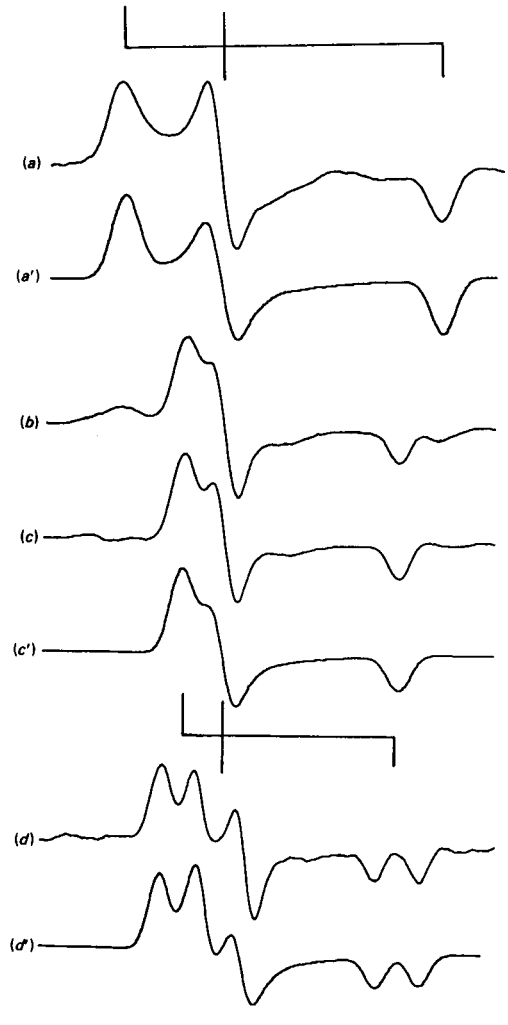

Fig. 2. *Experimental and simulated spectra at 35.2 GHz of the Slow signal in its nitrate and aquo complex forms*
Spectra (*b*)–(*d'*) show the nitrate complex and (*a*) and (*a'*) the aquo form. Spectra (*a*), (*b*), (*c*) and (*d*) are experimental; spectra (*a'*), (*c'*) and (*d'*) are computer simulated. Spectra (*d*) and (*d'*) correspond to solution in ^1H_2O and the remainder to solution in ^2H_2O. Stick diagrams show the g_1, g_2 and g_3 positions for the two forms. Signals were generated as described in Fig. 1, in 10mM-Bicine, pH8.2; (*b*) was obtained in the presence of 300mM-nitrate, but other experimental nitrate spectra were obtained as difference spectra (see the text); (*c*) is (300mM-NO_3^-) minus (50mM-NO_3^-); (*d*) is (250mM-NO_3^-) minus (6mM-NO_3^-). Simulation (see the Materials and Methods section) in spectra (*c'*) and (*d'*) used the following parameters (with allowance for ^2H replacing ^1H as appropriate): g_1, 1.9689; g_2, 1.9666; g_3, 1.9571; A_1 (^1H), 1.50; A_2 (^1H), 1.48; A_3 (^1H), 1.52; half-linewidths Δ_1 0.34, Δ_2 0.35, Δ_3 0.35; in spectrum (*a'*), parameters were g_1, 1.9721; g_2, 1.9669; g_3, 1.9550; A_1 (^1H), 1.70; A_2 (^1H), 1.66; A_3 (^1H), 1.56; and half-linewidths Δ_1 0.42, Δ_2 0.37, Δ_3 0.37. Widths and splittings are in mT.

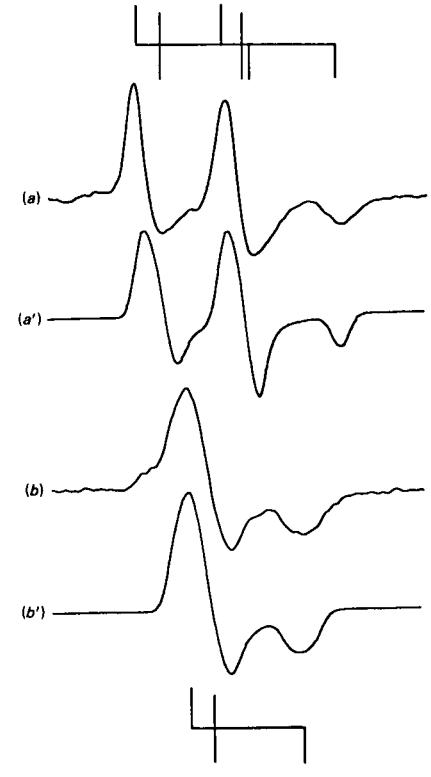

Fig. 3. *Experimental and simulated spectra at 9.3 GHz of the Slow signal in ^1H_2O and ^2H_2O in its nitrate complex form*
Spectra (*a*) and (*b*) are experimental in ^1H_2O and ^2H_2O respectively and spectra (*a'*) and (*b'*) are the corresponding computer simulations. Stick diagrams show the g_1, g_2 and g_3 positions and the ^1H splittings. Signals were generated as described in Fig. 1, in 10mM-Bicine, pH8.2; experimental spectra were obtained as difference spectra (see the text); spectrum (*a*) is (100mM-NO_3) minus (20mM-NO_3^-); spectrum (*b*) is (400mM-NO_3^-) minus (50mM-NO_3^-). Simulations employed the parameters of the nitrate complex used for Figs. 2(*c'*) and 2(*d'*), except that half-linewidths were all decreased by 0.2mT.

of the aquo and nitrate complexes. Spectra at various intermediate nitrate concentrations (not illustrated) provided an estimate of the dissociation constant of the complex of about 20mM. However, unexpectedly, even with nitrate concentrations as high as 1.0M, we were not able to obtain complete conversion of the

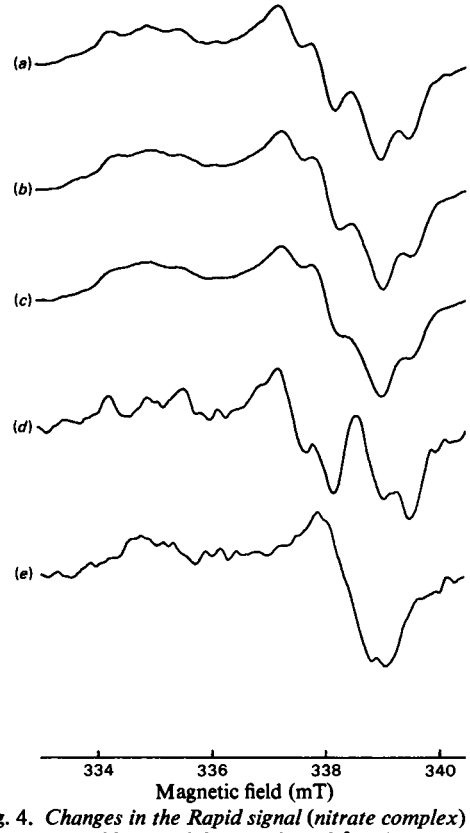

Fig. 4. *Changes in the Rapid signal (nitrate complex) after dilution of the sample with ^2H_2O*

The Rapid signal was generated by reduction of functional xanthine oxidase in ^1H_2O in Bicine buffer, pH8.2, at 12°C, with dithionite (3.5mol per mol of total enzyme active centres), in the presence of 200mM-$NaNO_3$, for 10s. The sample was then mixed with ^2H_2O buffer to a final concentration of 69% (v/v). E.p.r. spectra (at 9.308GHz) in spectra (*a*)–(*c*) correspond respectively to samples frozen 3, 10 and 32ms after this dilution with ^2H_2O. Spectra (*d*) and (*e*) are difference spectra obtained (see the text for details) by suitable subtraction of (*b*) from (*a*) and vice versa. Thus spectrum (*d*) corresponds to $4.5\times[(a)-0.85(b)]$ and spectrum (*e*) to $4.5\times[(b)-0.8(a)]$

spectrum into the nitrate form, with elimination of minor features from the aquo complex or other residual species. Whether this was due to competition for the anion site, perhaps by small amounts of nitrite produced enzymically, or whether it indicated, e.g., minor heterogeneity in the desulpho enzyme, is not certain. Whatever the cause, it in no way affects our main conclusions about nitrate binding to the desulpho enzyme.

Rates of exchange in ^2H_2O, of protons of the Rapid and Slow signals, from enzyme reduced with dithionite

With functional xanthine oxidase, a proton transferred from the substrate, 1-methylxanthine, to the enzyme in the catalytic reaction exchanges out again with a first-order rate constant of $27s^{-1}$ at pH8.2 and 12°C (Gutteridge *et al.*, 1978). The process could be followed by e.p.r., because of coupling of the proton to molybdenum in the Rapid signal, with $A_{av.}$ 1.4mT.

An alternative method of studying the rate of hydrogen/deuterium exchange would be to prepare the Rapid signal by suitable reduction of functional enzyme in ^1H_2O (for example with dithionite), then to dilute the sample with ^2H_2O, and follow the subsequent exchange process by freezing samples at intervals for observation of their e.p.r. spectra. Without the substrate occupying the anion site, it should now be possible to follow the exchange kinetics with any desired ion in this site. Further, it should in principle be possible to study the exchange rate, not only of the proton giving the large splitting of the Rapid signal, but also that of the proton giving the small splitting. According to Gutteridge *et al.* (1978), only the stongly coupled proton is transferred from the substrate to the enzyme in the catalytic reaction. Finally, and perhaps most importantly of all, such a procedure should be applicable to

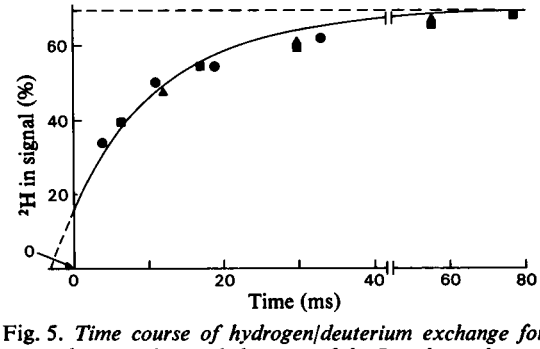

Fig. 5. *Time course of hydrogen/deuterium exchange for the strongly coupled proton of the Rapid signal*

The experiment is the one described in Fig. 4, carried out at pH8.2 and 12°C. The spectrum at each reaction time was resolved into its two components (as illustrated in Figs. 4*d* and 4*e*) and integrated intensities of these were estimated. The proportion of the ^2H form (Fig. 4*e*) as a percentage of total signal intensity was then calculated and is shown plotted as a function of time from the addition of ^2H_2O. The broken line corresponds to the final proportion of ^2H_2O in the solution. Results of three independent experiments are shown. The curve through the experimental points corresponds to a first-order process with a rate constant of $85s^{-1}$, with a dead-time of 3ms (see the Materials and Methods section).

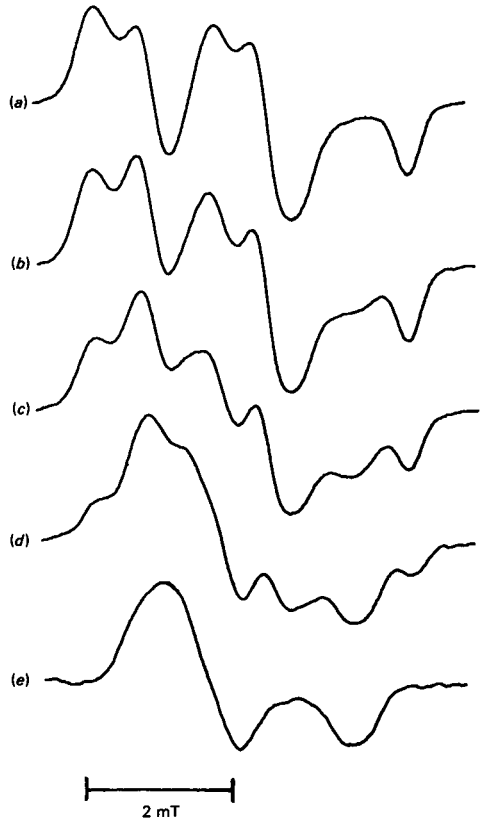

Fig. 6. *Changes in the Slow signal after dilution with ^2H_2O*

The Slow signal was generated by reduction of desulpho enzyme in ^1H_2O in 100mM-Bicine buffer, pH8.2, for approx. 1h with 5mM-dithionite. Samples were then mixed at 12°C with ^2H_2O buffer at a final concentration of 69% (v/v). E.p.r. spectra (at 9.3GHz) in spectra (*a*)–(*d*) correspond to the following times after addition of the ^2H_2O: (*a*) 0.05s; (*b*) 0.5s; (*c*) 1.0; (*d*) 10s. Spectrum (*e*) corresponds to the difference spectrum obtained on subtracting spectrum (*b*) from spectrum (*d*) (see the text for details), i.e. (*d*) −0.4 (*b*)

measurements of the exchange rate for the proton of the Slow signal, thus making possible comparison of a further important property of the desulpho and the functional enzymes.

We first used the ^2H_2O dilution procedure to study exchange rates of the strongly coupled proton of the Rapid signal generated in the presence of nitrate ions. Spectra illustrating the exchange process are shown in Fig. 4. At short reaction times (Fig. 4*a*) well-resolved splitting of the molybdenum signal due to the proton (^1H) can be seen, particularly in the g_2, g_3 region. However, with increasing time (Figs. 4*b* and 4*c*) more deuterium appears in the strongly coupled site. The spectrum at each reaction time could be resolved by difference techniques into the two component spectra (Bray *et al.*, 1978) illustrated in Figs. 4(*d*) and 4(*e*). Fig. 4(*d*) is the ^1H form and Fig. 4(*e*) is the ^2H form. The proportion of the ^2H-form in the spectrum could thus be estimated, and is shown plotted against time in Fig. 5. The exchange rate constant obtained from this plot was $85s^{-1}$. Note that exact first-order kinetics are not necessarily expected for an exchange reaction of this type, which presumably proceeds via a deprotonated species (Frost & Pearson, 1961; Harris, 1951). Nevertheless, within the limits of our data, no deviation from first-order kinetics was observed. The observed rate constant of $85s^{-1}$ will approximate to the rate constant for ^1H dissociation from the active centre.

For the Rapid signal, developed in the presence of NO_3^-, a weakly coupled proton, with $A_{av.}$ (^1H) about 0.3mT, is observable in the spectrum in addition to the strongly coupled one (Gutteridge *et al.*, 1978). The second proton splits the g_1 features and broadens the g_2, g_3 region. Unfortunately, noise, particularly in the g_1 region, in the difference spectrum of Fig. 4(*d*) makes it impossible to obtain a reliable estimate of the exchange rate for this proton. However, comparison of the g_2 and g_3 features of Fig. 4(*d*) with spectra of

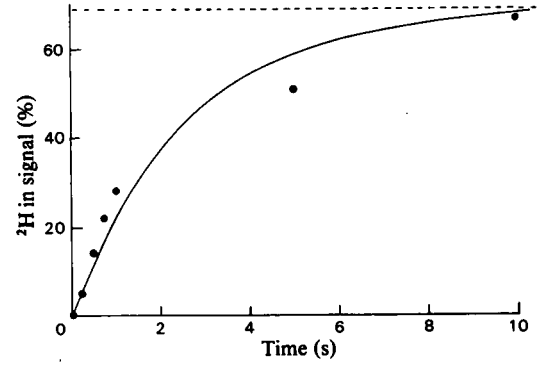

Fig. 7. *Time course of hydrogen/deuterium exchange for the strongly coupled proton of the Slow signal*

The experiment is the one described in Fig. 6, carried out at pH8.2, 12°C. Computer subtraction confirmed that spectra such as those of Figs. 6(*b*)–6(*d*) were all made up from the unexchanged spectrum (^1H form, cf. Fig. 6*a*) and the fully exchanged spectrum (^2H form, cf. Fig. 6*e*). The spectrum at each reaction time was resolved into these components and integrated intensities were estimated. The proportion of the ^2H form, as a percentage of total signal intensity, was calculated, and is shown plotted as a function of time from the addition of ^2H_2O. The line through the experimental points corresponds to a rate constant of $0.40s^{-1}$ and the broken line corresponds to the proportion of ^2H in the solution.

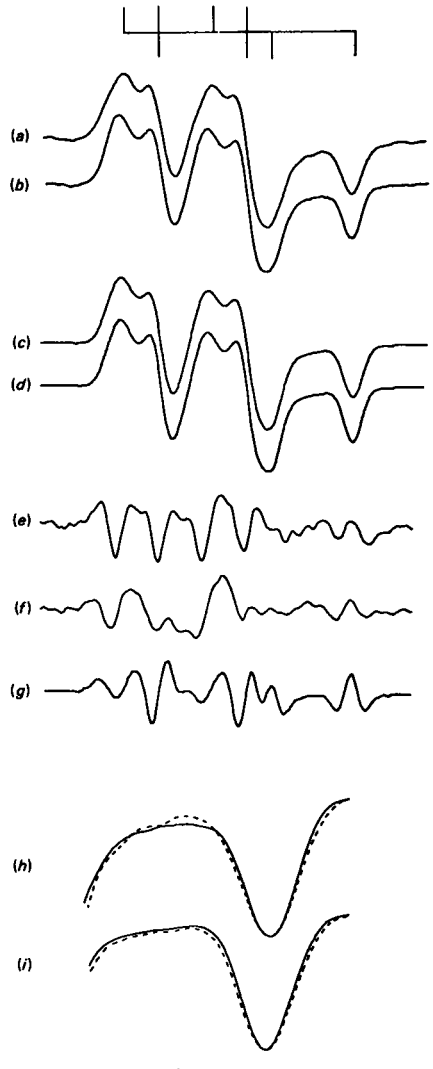

Fig. 8. *Evidence, from ^2H_2O exchange, that lineshape of the Slow signal depends on a weakly coupled, as well as on a strongly coupled, proton*

The spectra all correspond to the Slow signal prepared by reduction of desulpho enzyme in ^1H_2O in 100mM-Bicine buffer, pH8.2, with 5mM-dithionite. Spectrum (*a*) is such a sample prepared without addition of ^2H_2O. Spectrum (*b*) is one frozen 0.05s after dilution with ^2H_2O to a final concentration of 69% (v/v) (this sample is the one of Fig. 6*a*). Spectrum (*h*) shows an enlargement of the high-field portion of spectrum (*a*) and spectrum (*b*) (the full line corresponds to the sample to which ^2H_2O was added). Spectra (*c*) and (*d*) are computer simulations of spectra (*a*) and (*b*) respectively (see the Materials and Methods section for details of simulation procedures). Spectrum (*i*) shows enlargements of the high-field portion of the simulated spectra (again the full line corresponds to the sample to which ^2H_2O had been added). Spectra (*e*)–(*g*) correspond to difference spectra (with the vertical scale suitably expanded); spectrum (*e*) corresponds to (*a*) minus (*b*), and spectrum (*g*) to (*c*) minus (*d*); spectrum (*f*) is the difference spectrum between another pair of experimental samples and is analogous to spectrum (*e*) (see the text for details). Parameters used in the simulations were as follows: g_1, 1.9719; g_2, 1.9671; g_3, 1.9551; A_1, 1.66, 0.16; A_2, 1.66, 0.16; A_3, 1.56, 0.16. Half linewidths were Δ_1 0.22, Δ_2 0.17, Δ_3 0.17. When appropriate, 69% occupancy of the site giving the small splitting by ^2H was allowed for, as discussed in the text. The stick diagram corresponds to the positions of the g_1, g_2 and g_3 features, split by the strongly coupled proton.

Rapid species Hd (see Gutteridge *et al.*, 1978) suggests that the weakly coupled proton may perhaps exchange even faster than does the strongly coupled one.

We next applied the ^2H_2O dilution procedure to the Slow signal. Results are presented in Figs. 6 and 7 and show an exchange process very much slower than that for the main proton of the Rapid signal. At the shortest time employed (0.05s) virtually no exchange had taken place (Fig. 6*a*) and about 10s was required (Fig. 6*d*) for the process to approach completion. Spectra at intermediate times could all be resolved by computer subtraction into the starting spectrum (cf. Fig. 6*a*) and a spectrum corresponding to the aquo Slow signal in pure ^2H_2O (Fig. 6*d*). Fig. 7 shows, for this experiment, the percentage of the deuterium form in the observed Slow signal, as a function of time. The data indicate an exchange rate constant of $0.40s^{-1}$.

Evidence for a second exchangeable proton in desulpho-enzyme, weakly coupled to molybdenum in the Slow signal

As already discussed, the Rapid signal shows two exchangeable protons, one strongly and the other more weakly coupled to molybdenum. In the Slow signal, evidence has, hitherto, been presented only for a strongly coupled proton. However, closer examination of the spectra from the experiment of Figs. 6 and 7, described above, provide evidence implicating a second weakly coupled proton in the lineshape of the Slow signal, as it is in the Rapid. The evidence is summarized in Fig. 8. Unlike the second proton of the Rapid signal, which gives partly resolved splittings, that in the Slow signal causes broadenings only. Its effects are small, as may be seen by comparing Figs. 8(*a*) with 8(*b*). The latter is identical with Fig. 6(*a*) and corresponds to the Slow signal, shortly after dilution with ^2H_2O, before the main proton has exchanged significantly. Fig. 8(*a*) is a control sample with no ^2H_2O present. Differences between the two spectra are apparent, mainly in the greater resolution,

in ^2H_2O (Fig. 8*b*), of structure at the tops of the main upward features of the spectrum. There is also a decrease in ^2H_2O in the linewidth of the downward feature at highest field (the 'γ peak' in the nomenclature of Palmer *et al.*, 1964); this feature is shown enlarged in Fig. 8(*h*) (^2H_2O spectrum, solid line, and ^1H_2O spectrum, broken line.

We succeeded in obtaining satisfactory computer simulations of Figs. 8(*a*) and 8(*b*). These are shown respectively in Figs. 8(*c*) and 8(*d*). Parameters used are shown in the legend [they differ very slightly from those employed for the aquo Slow signal in Fig. 2(*a'*); this is no doubt the result of our having changed the buffer concentration]. The basis of the simulation was to assume that the decrease in linewidth in ^2H_2O was due to replacement of a weakly coupled ^1H by ^2H. For simplicity, isotropic splitting by this proton was assumed. The strongly coupled proton was taken to be 100% in the ^1H form in both spectra. However, for the second proton it was necessary to take into account the ^2H_2O concentration of 69% in Fig. 8(*b*). Occupancy of this site was therefore taken, in the simulation of Fig. 8(*d*), as 69% ^2H and 31% ^1H, with, of course, 100% occupancy by ^1H in Fig. 8(*c*). The final simulations, illustrated, assumed A (^1H) 0.16mT for the second proton. They are shown in Figs. 8(*c*) and 8(*d*); the high-field region is shown enlarged in Fig. 8(*i*), with the spectrum in ^2H_2O solid and that in ^1H_2O broken.

Though these simulations seemed quite satisfactory, we were very conscious that the effects attributed to the secondary proton were indeed small. To obtain more information, we therefore once again resorted to difference spectra. Figs. 8(*e*)–8(*g*) show, after suitable expansion, difference spectra obtained by subtracting spectra in ^2H_2O from the corresponding ones in ^1H_2O. Fig. 8(*e*) corresponds to the difference between the experimental spectra of Figs. 8(*a*) and 8(*b*), and Fig. 8(*g*) is the difference between the simulated spectra of Figs. 8(*c*) and 8(*d*). Finally, Fig. 8(*f*) is another difference spectrum from separate experimental samples (original spectra not shown), obtained quite independently of those used in Fig. 8(*e*). [In fact one of the spectra used for Fig. 8(*f*), that corresponding to Fig. 8(*b*), was itself a difference spectrum obtained between samples having reaction times of 0.75 and 5s.]

Comparison of the three difference spectra (Figs. 8*e*–8*g*) provides a very stringent test, both of the reproducibility of the effects of ^2H_2O attributed to the secondary proton and of the goodness of the simulation. In view of the noise in the experimental spectra, perfect agreement amongst the difference spectra could not be expected, nor is it found. Nevertheless, close inspection reveals that all the features of the simulated difference spectrum (Fig. 8*g*) are reproduced qualitatively if not quantitatively, in one or other, or in both, of the experimental difference spectra (Figs. 8*e* and 8*f*). Thus, for example, sharpening of the high-field γ peak leads to an upward feature flanked by downward features and this is present in all three difference spectra.

We conclude that effects we have discussed, observable immediately on dilution with ^2H_2O of samples showing the Slow signal, are reproducible and that they are indeed explicable in terms of coupling of molybdenum to the secondary proton, with $A_{av.}$ (^1H) about 0.16mT. Unlike the strongly coupled proton, the weakly coupled proton of the desulpho-enzyme clearly exchanges rapidly with solvent protons. Such data as we have indicate its exchange rate constant to be greater than about $20s^{-1}$.

Discussion

Our experiments provide additional data supporting earlier more tentative conclusions (cf. Section II, B, 4, d, Bray, 1975) that differences between desulpho and native xanthine oxidase are minimal. Thus we have now shown that in both species there is a site for the binding of anions, presumably as ligands of molybdenum. Furthermore, both species show interaction of molybdenum with two exchangeable protons. The similarities are emphasized still further by the finding (Tanner & Bray, 1978) that ethylene glycol is a substrate, though an extremely slowly reacting one, for the desulpho as well as for the native enzyme.

We now consider the exchange rates of the e.p.r.-detectable protons with solvent protons. Though precise data are not available, the one giving the smaller splitting appears to exchange quite rapidly, both in the functional and in the desulpho enzyme. For the proton giving the larger splitting, we obtained more reliable information. In the functional enzyme its exchange was some 3 times faster in the absence of substrate than it is in its presence. Thus the nature of the species in the anion site does have some effect on the exchange rate. Of considerably greater interest, however, is the much slower exchange of the strongly coupled proton associated with the Slow signal, in comparison with that from the Rapid signal. The protons involved are presumably bound to oxygen, nitrogen or sulphur atoms. To understand the exchange, we have to consider the rate constants, both for proton association with the binding group in the enzyme, and for the corresponding proton-dissociation reaction. It is reasonable to assume the rate of the association reaction to be near to the diffusion-controlled limit, with a rate constant of perhaps about $5\times10^9 M^{-1}\cdot s^{-1}$ (Eigen & Hammes, 1963; Crooks, 1975). The measured exchange rate constants at pH 8.2 are 85 (or 27) s^{-1} for the functional enzyme and $0.40s^{-1}$ for the desulpho enzyme. We may, therefore, calculate pK values for the proton-binding groups to be about 8 for functional enzyme and 10 for the desulpho form. Possible reasons for the deprotonated enzyme forms not apparently giving

any detectable Mo(V) e.p.r. signals will be considered below.

In attempting to account for the difference in pK values we note that the one structural difference between the two forms that is definitely established is the loss of the sulphur atom from the functional enzyme. On conversion to the desulpho form this has presumably been replaced by some other atom, and as considered in the introduction the most likely candidate is an oxygen, derived from water. The pK difference then immediately brings to mind the greater acidity of protons bound to sulphur in comparison with those bound to oxygen (for example, compare pK values of 4.8 for acetic acid and 3.3. for thioacetic acid). This in turn points to the proton in the enzyme being bound directly to this sulphur or oxygen atom. Therefore, combining the pK data with the proposal that we have (Enzyme)–Mo=S in the functional enzyme and (Enzyme)–Mo=O in the desulpho form, our data indicate the protonation–deprotonation reactions to be of the forms:

$$\text{(Enzyme)–Mo=S} \overset{\pm H^+}{\rightleftharpoons} \text{(Enzyme)–Mo}^+\text{–SH} \quad (1)$$

$$\text{(Enzyme)–Mo=O} \overset{\pm H^+}{\rightleftharpoons} \text{(Enzyme)–Mo}^+\text{–OH} \quad (2)$$

There is much evidence in support of these schemes. Indeed, their possible importance in molybdenum enzymes has already been suggested by Williams & Wentworth (1973). Such reactions are consistent with properties of known molybdenum compounds. Though little is known about transition-metal–SH compounds generally (De Vaira *et al.*, 1978; Dilworth *et al.*, 1978), on the other hand it is known that molybdenum-linked oxygen atoms (Mo=O) can become protonated. For example, in a cyanide complex of Mo(IV) (Robinson *et al.*, 1975), the pK value corresponding to eqn. (2) is about 12.2 (Stiefel, 1977; Murmann & Robinson, 1975).

This proposed location of the strongly-coupled protons, bound respectively in the functional and desulpho enzyme, to sulphur and oxygen ligands of the molybdenum atom, with these becoming terminal ligands on deprotonation, appears consistent with all but one of the known properties of the enzyme species. We will discuss the inconsistency first. It seems rather likely that a terminal sulphur ligand atom would, even in the protein, be acid-labile. However, Edmondson *et al.* (1972) and Massey & Edmondson (1970) reported that desulpho-(xanthine oxidase) possessed a full complement of acid-labile sulphur. Since most, if not all, the acid-labile sulphur in xanthine oxidase comes from the iron–sulphur centres and since no analytical data were presented by these workers, the matter would perhaps bear re-investigation.

On the other hand, a large body of evidence is readily reconciled with our structural proposals. Thus a sulphur ligand in the functional enzyme could account (see Phillips & Williams, 1966) for the higher redox potentials of its molybdenum. Similarly, the loss of absorbance at 320nm, on conversion into the desulpho form, could be accounted for in a variety of ways such as, for example, a ligand-to-metal charge-transfer band moving to shorter wavelengths (see also Müller *et al.*, 1973).

With regard to the e.p.r. parameters, we first note that the proton hyperfine splitting for the strongly coupled proton of both enzyme forms is relatively large. This suggests either that the proton is bound directly to molybdenum as a hydride (Edmondson *et al.*, 1973) or that there is no more than a single atom intervening between them (Bray & Vänngård, 1969). Paramagnetic transition metal hydrides may have proton splittings ranging from rather less (Brintzinger, 1967; Henrici-Olivé & Olivé, 1968) to considerably more (Henrici-Olivé & Olivé, 1969) than those in the enzyme. Because of the proximity of the proton to the metal in a hydride, a strong anisotropic dipolar contribution to the coupling would be expected. Unfortunately, in the references cited, only solution e.p.r. data are presented, precluding evaluation of the dipolar term. However, very recently, information has become available on a vanadium hydride (A. G. Evans, J. C. Evans, P. H. Morgan & J. Mortimer, personal communication) and for this, as expected, the proton splitting is highly anisotropic. For the enzyme, the apparently nearly isotropic proton splittings, observed in both the Rapid and Slow signals, thus argue very strongly against a hydride (Section II, B, 4, d, Bray, 1975; Bray & Vänngård, 1969). [From the parameters given by Gutteridge *et al.* (1978) and in Fig. 8, the maximum variation in $|A|$ for the three different orientations, is no more than 0.10mT for both signals.]

Bray & Vänngård (1969) suggested oxygen or sulphur as the atom intervening between the proton and the metal, but Stiefel and co-workers (Pariyadath *et al.*, 1976) preferred nitrogen. Unfortunately, we are aware of no Mo–OH or Mo–SH model compounds to help to elucidate the enzyme structure. We note, however, that nitrogen hyperfine structure was observed, with A^N 0.24mT, in a Mo–NH model compound by Pariyadath *et al.* (1976). Rather larger A^N values are given by some Mo–N compounds (Stiefel, 1977). No resolved nitrogen hyperfine splitting is seen with xanthine oxidase and nitrogen splittings of such a magnitude could scarcely be accommodated within the relatively small linewidths used in our simulations. This argues against a proton-bearing nitrogen ligand in the enzyme, at least in the case of the strongly coupled proton.

We therefore return to the hypothesis of Mo–SH in the functional enzyme and Mo–OH in desulpho, and consider further supporting e.p.r. evidence.

No definitive interpretation is possible in the absence of knowledge of ligand geometry. However, so far as the relative magnitudes of the proton hyperfine splittings are concerned, simple theory suggests that decreased covalency of a molybdenum–oxygen bond, in comparison with one to sulphur, would account for increased contact terms (Goodman & Raynor, 1970). Our splittings are presumably governed mainly by these terms. Thus, the increase in $A_{av.}$ (^{1}H), from 1.4mT in the Rapid signal to 1.6mT in the Slow signal, is in accordance with expectations. All components of the ^{95}Mo hyperfine splittings have not been reported. However, a trend similar to that in the $A_{av.}$ (^{1}H) values, is observed also for A_z (^{95}Mo), which was reported to increase from 6.4mT in the Rapid signal to 7.0mT in the Slow signal (Bray *et al.*, 1968). Decrease of covalency could also, perhaps, account for the decrease in $g_{av.}$, from 1.975 in the Rapid signal to 1.966 in the Slow signal. However, here, the decrease seems more likely to be related to increased spin-orbit coupling to sulphur, when this is the ligand atom. This coupling might also be involved in the change from $g_\perp > g_\parallel$ in the Slow signal to $g_\parallel > g_\perp$ in the Rapid signal (see Marov *et al.*, 1972).

Though the identity of the group carrying the strongly coupled proton thus seems moderately well established, on the other hand that of the group carrying the weakly coupled proton is much less certain. For this proton, $A^{H}_{av.}$ is 0.30mT in the 1-methylxanthine Rapid signal and 0.16mT in the Slow signal, relatively low pK values being indicated in both cases. A nitrogen ligand of molybdenum is a possible site for this proton.

We now return to the proposals of Stiefel (1973), that the mechanisms of molybdenum-containing enzymes involve coupled proton and electron transfers. These proposals were based largely on the reasonably well-established greater tendency of ligands of molybdenum to protonate, when the metal is in lower rather than higher valency states. Accepting this, we would expect Mo=S to be favoured in the oxidized state and Mo–SH to be favoured in reduced forms of functional xanthine oxidase. Three further conclusions follow from this. First, if Mo=S is present only in the oxidized enzyme and not to any large extent in the reduced form, then the hitherto unexplained failure of cyanide to inactivate the enzyme while this is in the reduced state (Massey & Edmondson, 1970) becomes understandable in terms of the absence of the enzyme species required to react with the reagent. Secondly, it has long been considered that there is a thiol group in reduced xanthine oxidase, which is not present in the oxidized form, and which on reduction becomes available for reaction with mercurial reagents (Fridovich & Handler, 1958; Massey *et al.*, 1969). Clearly this observation could be accounted for, also, by the present proposals. Finally, and most importantly of all, the catalytic mechanism of the enzyme has been shown to involve proton abstraction from the 8-position of the xanthine molecule, the substrate being bound initially in the anion-binding site (Gutteridge *et al.*, 1978). The present work leads to the conclusion that the proton-abstracting group must be Mo=S.

In the desulpho enzyme the Mo=O group should also have proton-abstracting ability, so we must seek further reasons for its lack of catalytic efficiency. Substrate binding to this form of the enzyme has not been studied and might be weak. [Edmondson *et al.* (1972) observed reasonably tight, though apparently slow, inhibitor binding by desulpho-enzyme; data on purine binding by Pick & Bray (1969) put an upper limit of a few millimolar on its dissociation constant from a possible complex.] Also, the results of Cammack *et al.* (1976) and of Barber *et al.* (1977) make it clear that the redox potential of molybdenum in the desulpho enzyme is not favourable for reduction by xanthine. Finally, apart from these problems, slow proton dissociation from the desulpho enzyme would in any case limit its turnover rate for xanthine (if indeed it does carry out this function at all) to a small fraction of the turnover rate for functional enzyme.

In conclusion we have to consider why no e.p.r. signals have been observed in our work corresponding to deprotonated Mo(V) in either functional or desulpho enzyme. This may appear particularly surprising in the case of the functional enzyme, for which the proton-exchange experiments, carried out at pH8.2, indicated a pK value in the region of 8. Tsopanakis *et al.* (1978) showed conclusively that the important, but unique, Very Rapid Mo(V) signal is not the deprotonated form for which we should seek. We suggest that equilibrium constants are such that, on deprotonation, Mo(V) reverts mainly to Mo(VI), with the resultant disappearance of signals, the electron passing on to the iron–sulphur or flavin centres. However, more information on signal intensities (and proton exchangeability) at differing pH values and also for different states of reduction of the enzyme would be required to settle this pcint.

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