Biochem. J. (1983) **211**, 227–236
Printed in Great Britain

Equilibria amongst different molybdenum (V)-containing species from sulphite oxidase

Evidence for a halide ligand of molybdenum in the low-pH species

Robert C. BRAY, Steven GUTTERIDGE,* M. Teresa LAMY† and Teresa WILKINSON
School of Chemistry and Molecular Sciences, University of Sussex, Brighton BN1 9QJ, U.K.

(*Received 17 September 1982/Accepted 18 November 1982*)

The interaction of chloride, fluoride and phosphate ions with the molybdenum centre of sulphite oxidase in the pH range 6.2 to 9.6 has been studied by e.p.r. of Mo(V) in the enzyme reduced by sulphite. Detailed studies were made from e.p.r. spectra recorded at about 120 K and more limited studies from spectra of liquid samples at about 295 K and also from enzyme activity measurements. Interconversion between low-pH and high-pH Mo(V) e.p.r. signal-giving species [described by Lamy, Gutteridge & Bray (1980) *Biochem. J.* **185**, 397–403] is influenced by chloride concentration, a 10-fold increase in concentration (in the range of about 1 mM to 100 mM) causing an increase of about 1 pH unit in the apparent pK for the conversion. This suggests that chloride is a constituent of the low-pH species. In support of this, high concentrations of fluoride modified the e.p.r. spectrum. Partial conversion to a Mo(V) species, in which F^- has presumably replaced Cl^- and showing hyperfine coupling of $A(^{19}F)_{av.}$ 0.5 mT, is indicated. It is proposed that interconversion between high-pH and low-pH species is of the form:

$$\text{Enzyme—Mo=O} + H^+ + Cl^- \rightleftharpoons \text{Enzyme}\begin{matrix}\diagup\text{OH}\\ \diagdown\text{Cl}\end{matrix}$$

No evidence that Cl^- is essential for enzymic activitity was found. Data relating to equilibria amongst low-pH, high-pH and also the phosphate species are presented. Depending on pH and on concentrations of Cl^- and $H_2PO_4^-$, one, two, or all three species may be present. Qualitatively, under appropriate conditions, the phosphate species tends to replace some or all of the low-pH species. Quantitative analysis by a computer procedure permitted an appropriate scheme to be deduced and equilibrium constants to be evaluated. Studies on the e.p.r. signals at 295 K indicated that similar equilibria applied in liquid solution, but with some changes in the values of the constants. The structure of the molybdenum centre in its various states and the nature of the enzymic reaction are discussed.

Studies by e.p.r. of molybdenum(V) in molybdenum-containing enzymes provide important information bearing on their structures and catalytic mechanisms (Bray, 1980*a*). In such work it is possible to distinguish and study individually different chemical species of molybdenum(V), each of which arises from a different chemical state of the molybdenum centre of the enzyme molecule. In the case of sulphite oxidase, four molybdenum(V) e.p.r. signals have been described, and in accordance with previous work (Lamy *et al.*, 1980; Bray *et al.*, 1982), these will be referred to as 'high-pH', 'low-pH', 'phosphate' and 'sulphite', respectively. Except for that from the sulphite form of the enzyme, the signals have all been simulated both for 9 and for 35 GHz spectra, giving some assurance that only single chemical species are involved (Lamy *et al.*, 1980; Bray, 1980*a*). The low-pH form shows coupling to molybdenum of a single exchangeable proton. This is believed to arise from a Mo—OH group. The other species show no superhyperfine splittings.

Molybdenum(V) e.p.r. spectra are routinely obtained by reducing the molybdenum(VI)-containing oxidized enzyme with the substrate, sulphite. This

* Present address: Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, U.K.
† Present address: Instituto de Quimica, Universidad de Sao Paulo, Sao Paulo 20.780, Brazil.

gives partial reduction of the metal to the molybdenum(V) state. Except under specific and rather special conditions, spectra so obtained are mixtures of two or more of the different species. Cohen *et al.* (1971) studied the effect of pH variation on the equilibrium between the high- and low-pH forms and concluded that these are interconverted by reversible uptake of a proton (this proton being presumed to be the one detected in the low-pH signal). The pK value for this interconversion (in a medium containing variable amounts of chloride) was reported as 8.2. Conversion of the enzyme to the phosphate form is obviously favoured by the presence of inorganic phosphate in the medium (Lamy *et al.*, 1980; Gutteridge *et al.*, 1980) though other conditions favouring this process have not been studied in detail.

We now report results of a systematic investigation of the effects of pH, of chloride concentration and of phosphate concentration on the signals from sulphite oxidase. The results provide important information on the structures of the signal-giving species and are thus relevant to an understanding of the mechanism of the enzymic reaction.

Materials and methods

The enzyme was partially purified from frozen chicken livers as described by Lamy *et al.* (1980). Enzymic activity was measured by following reduction of cytochrome *c* in the presence of sulphite, generally as described by these workers. Absence of contamination of our preparations by xanthine dehydrogenase or aldehyde oxidase was indicated by failure of purine to elicit Mo(V) e.p.r. signals. Unless otherwise stated the buffer employed for all experiments and activity assays was a mixture of 25mM-Pipes (1,4-piperazinediethanesulphonic acid) and 25mM-Bicine [*NN*-bis-(2-hydroxyethyl)glycine], adjusted to the required pH value with NaOH. Colorimetric analysis for molybdenum using toluene-3,4-dithiol was carried out as described by Hart *et al.* (1970). Analysis of buffers, etc. for chloride present as a contaminant was carried out by the method of Iwasaki *et al.* (1952), determining the ferric thiocyanate produced from the mercuric thiocyanate reagent by measurement at 460nm.

Enzyme samples for e.p.r. measurement

The enzyme was concentrated to give a Mo concentration of about 20–40μM, after dialysis against buffer at the appropriate pH value. Additions of concentrated sodium chloride, sodium fluoride or sodium phosphate buffer were then made as required and finally sodium sulphite was added to a concentration of 5mM to reduce the enzyme. In all cases, the pH values quoted refer to measurements made with a microelectrode on the sample removed from the e.p.r. tube after the spectrum had been recorded.

Most e.p.r. measurements were made on manually frozen samples, in which case the sodium sulphite addition was made in the e.p.r. tube and the solution was frozen about 1min later. In some cases, measurements were made on liquid samples at about 22°C by using an e.p.r. flat cell. In this case exposure of the enzyme to the substrate was considerably longer. A few measurements were made by rapid freezing with isopentane (Gutteridge *et al.*, 1978) and here a reaction time of 50ms was employed.

E.p.r. measurements

E.p.r. spectra were recorded on a Varian E9 spectrometer linked to a computer and visual display system (Bray *et al.*, 1978). Recording conditions were generally approx. 120K, microwave power 10mW, modulation amplitude 0.16mT. The microwave frequency was about 9.3GHz. For measurement on liquid samples the microwave power was raised to 50mW and the modulation to 2.5mT. Absolute quantification of intensities of molybdenum(V) e.p.r. signals was obtained by double integration with corrections as described by Lamy *et al.* (1980). These values for concentrations of e.p.r.-detectable Mo(V) were divided by the total molybdenum content of the samples, determined colorimetrically on portions of the solutions removed from the tubes after the e.p.r. measurements. Computer simulation of e.p.r. spectra was carried out according to Lowe (1978). Difference spectra were obtained as described previously (Bray *et al.*, 1978; Bray & Gutteridge, 1982).

Relative quantification of intensities of the three e.p.r. signals, namely low-pH, high-pH and phosphate, when these were present together, was carried out as follows by using the computer and visual display systems. The experimental spectrum to be examined, and spectra of the three pure species required for comparison, were first adjusted in amplitude so as to have identical integrated intensities. Increasing proportions of the phosphate signal were then subtracted from the experimental spectrum, until features due to this species were judged just to have disappeared from the difference spectrum. The fraction of the phosphate spectrum in the original experimental spectrum was then given by the fractional amplitude of the phosphate signal subtracted. Further subtraction of the high-pH signal from the experimental spectrum minus the phosphate signal, by the same procedure, yielded the fractional intensity of the high-pH species. The final difference spectrum, if the subtractions had been carried out correctly and if no additional species

were present, then corresponded to the pure low-pH species.

Computer fits to equilibrium schemes

Combined data from a series of experiments of the types illustrated in Figs. 1, 3 and 4 (see below) were used. Data were in the form of fractional intensities of each of the three e.p.r. signals which were studied, each set of intensities corresponding to a different combination of the variables pH, chloride concentration and phosphate concentration. The computer program allowed comparison of these experimental data with the corresponding calculated values of the signal intensities. These were deduced, for example from Scheme 1(*b*) (see below), by calculation after solving the five simultaneous equations governing the overall equilibrium. Goodness of fit was judged by the sum of the squares of the differences for each point between the calculated and the experimental signal intensities. The program permitted variation of the form of the equilibrium scheme which was assumed. For each scheme used an iterative fitting routine varied the values of the equilibrium constants, until a minimum value was achieved for the sum of the squares of the errors.

The program treated the concentration of chloride ions present as a contaminant as an additional variable, assuming it to be constant throughout all the experiments, and adding it where appropriate to concentrations of chloride which had been added deliberately. This contaminating chloride level was varied along with the equilibrium constants, in seeking the best fit to the experimental data.

Where the presence of an additional species of sulphite oxidase, not detectable by e.p.r. [as in Scheme 1(*b*) below], was assumed, the program constrained concentrations of this species towards zero by treating them as errors, which were squared and added into the sum of squares of errors in the fitting procedure.

The effect of formation of a sulphite complex detectable by e.p.r. was tested for by modifying the equations for Scheme 1(*b*) so as to allow for this additional species, as indicated in Scheme 2 (see below).

Results

Effect of variations in the concentration of chloride ions on transition between the high-pH and low-pH species

Fig. 1 shows an investigation of the effects of chloride ion concentration and of pH on the transition between the high-pH and the low-pH species of sulphite oxidase. At a chloride concentration of 100mM the apparent p*K* for this conversion was about 9.0 whereas at 10mM-chloride the apparent p*K* had decreased to 8.0–8.2.

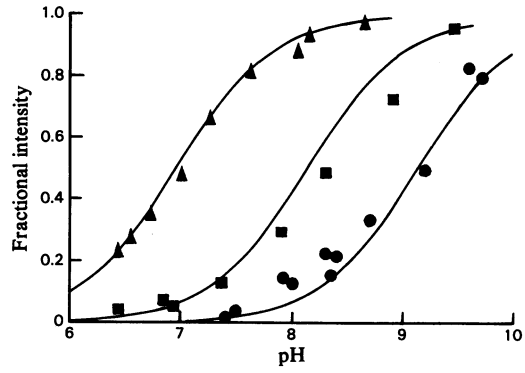


Fig. 1. *Effect of concentration of chloride ions on interconversion between the high-pH and the low-pH (chloride) Mo(V) e.p.r. signal-giving species*

Fractional intensity of the high-pH signal (estimated as described in the Materials and methods section from spectra recorded at 120K) is plotted as a function of pH for three experiments carried out at different chloride concentrations: ▲, no chloride added (but contaminating chloride concentration believed to be 0.1–1.0mM: see the text); ■, 10mM-NaCl added: ●, 100mM-NaCl added. The curves through the experimental points are theoretical ones computed as described in the text.

This observation of a 10-fold change in chloride concentration producing a change in the relative amounts of the signals comparable with that of a 10-fold change in hydrogen ion concentration at once suggests the participation of chloride as well as of protons in the structure of the low-pH signal-giving species.

The third curve in Fig. 1, for which the apparent p*K* was 7.0, was obtained in the absence of added chloride. When due allowance was made for the presence in some experiments at low pH values of traces of the sulphite signal (see Bray *et al.*, 1982), then the form of the low-pH signal in the absence of added chloride could not be distinguished from that in experiments where this anion was added.

Chloride is a common contaminant in chemical and biochemical work. We therefore wondered whether it could be present in these experiments at a concentration sufficiently high as to be a constituent of the low-pH signal giving species. Analysis for chloride, as described in the Materials and methods section, of a sample buffer similar to that used in the experiments indicated a chloride concentration of about 0.3mM. This concentration (which applied to one batch only of the buffer) is considerably higher than that of the molybdenum centres, in agreement with the hypothesis that chloride is a constituent of the low-pH species.

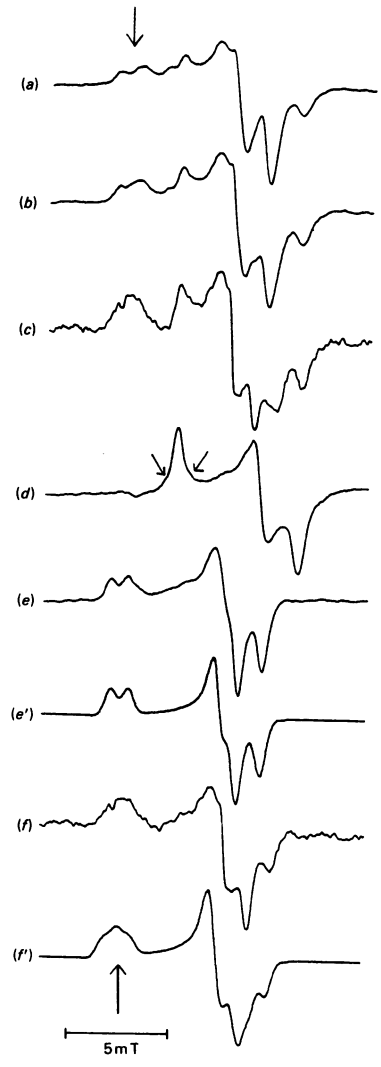


Fig. 2. *Comparison of the effects of fluoride and of chloride ions on the e.p.r. spectrum of sulphite oxidase*

(*a*) shows the spectra of a sample of sulphite oxidase at pH 7.0 and (*b*) and (*e*) respectively are similar samples containing in addition 100mM-NaF or 100mM-NaCl. The latter is the pure low-pH (chloride) species, whose simulation is shown in (*e'*). The high-pH spectrum is shown in (*d*) and was generated in 100mM-Caps [3-(cyclohexylamino)-1-propanesulphonic acid] buffer, pH 9.2. Low-pH (chloride) and high-pH species were successively eliminated by difference techniques from the spectrum of the sample containing F^-. Thus, (*c*) corresponds to spectrum (*b*) after subtracting 0.85 × spectrum (*a*) and increasing the vertical scale 3.85-fold. Similarly, (*f*), which is believed to represent the pure low-pH (fluoride) species, corresponds to spectrum (*c*) after subtracting 0.60 × spectrum (*d*) and increasing the vertical scale 0.80-fold. (*f'*) is a simulation of (*f*). It was obtained by adding fluoride splittings to the parameters of Lamy *et al.* (1980), as used in the simulation of the low-pH (chloride) species (*e'*). Coupling to a single F^- nucleus was assumed in (*f'*), with A $(^{19}F)_{1,2,3}$ 0.52, 0.31, 0.75mT. The arrow corresponds to $g = 2.0037$.

Confirmation of the sensitivity of the signals to low concentrations of chloride was provided by additional experiments, which will not be described in detail, in which chloride at concentrations of 0.1–1mM was added to the enzyme. Our first experiments gave inconsistent results, which was traced to contamination by KCl leaking into the enzyme samples from the pH microelectrode. Conductivity measurements indicated that chloride contamination from this source could easily reach 1mM. Subsequently, we measured pH values of samples only on thawing, after their e.p.r. spectra had been recorded.

Effect of fluoride on the e.p.r. spectrum

Since the above experiments suggested that chloride was a constituent of the low-pH signal-giving species, we tested whether it could be replaced by fluoride. Fluorine has a magnetic nucleus with $I = \frac{1}{2}$ and tends to give much larger hyperfine coupling than does chlorine. Thus, if the halide was coupled to molybdenum, then hyperfine structure might be detectable in the e.p.r. spectrum of the enzyme when fluoride replaced chloride. Fig. 2 (spectra *a*, *b* and *e*) shows that addition of 100mM-sodium fluoride to a sample of sulphite oxidase at pH 7.0 had a much smaller effect on the spectrum than did addition of the same concentration of sodium chloride. However, by appropriate use of difference techniques, the spectrum of Fig. 2(*f*) was obtained, and is believed to represent a low-pH species in which a fluoride ion has replaced a chloride ion. In confirmation of the significance of the fluoride effect, the main features of Fig. 1(*f*), particularly those in the $g_\perp$ region, were reproduced in difference spectra from replicate samples. With fluoride in place of chloride as a ligand of molybdenum, then g and $A(^1H)$ values might be changed somewhat, though probably only slightly. We therefore attempted a preliminary simulation [Fig. 2(*f'*)] of the spectrum of Fig. 2(*f*) by assuming that these parameters were as in the low-pH chloride species (Lamy *et al.*, 1980), but with moderately strong anisotropic hyperfine coupling of a single fluorine nucleus to molybdenum. The agreement between Figs. 2(*f*) and 2(*f'*) supports these assumptions.

The data thus indicate that, in the presence of high concentrations of fluoride, a relatively small proportion of the enzyme molecules are converted into a species, analogous to the normal low-pH species, having a fluorine coupled to molybdenum and presumably replacing a chlorine, with an essentially unchanged ligand geometry.

Equilibria amongst the high-pH, low-pH (chloride) and phosphate species

We next studied conditions required for conversion of the enzyme into the phosphate species. As is shown in Fig. 3, with 10mM-phosphate at pH values in the region of 6.5 and in a medium without added chloride, the enzyme was converted almost quantitatively to the phosphate species. On raising the pH this was transformed progressively into the high-pH form. Fig. 4 shows a similar experiment at a lower concentration of phosphate (1mM). Here both the phosphate and the low-pH (chloride) species were present at low pH values but both of these were replaced by the high-pH species as the pH was increased. Further experiments (results not shown) at fixed pH values confirmed progressive and parallel elimination of both the high-pH and the low-pH (chloride) species and their replacement by the phosphate species, as the concentration of phosphate was increased. In other experiments, competition between chloride and phosphate for the enzyme was confirmed (see also the data of Kessler & Rajagopalan, 1972). Thus, e.g., contaminating chloride at a level of about 1mM in a sample containing 2mM-phosphate at pH6.8 was sufficient to change the spectrum from the pure phosphate form to one corresponding to about 80% phosphate and 20% low-pH (chloride).

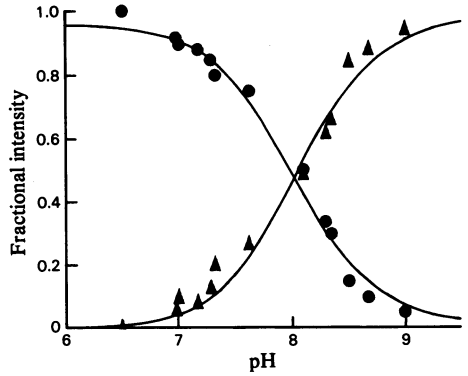


Fig. 3. *Effect of pH on the equilibria amongst Mo(V) species from sulphite oxidase observed in the presence of 10mM-phosphate*

Fractional intensity of the high-pH species (▲) and the phosphate species (●) (estimated as described in the Materials and methods section from spectra recorded at 120K) are plotted as a function of pH. The low-pH species could not be detected in this experiment. The curves through the experimental points are theoretical ones computed as described in the text.

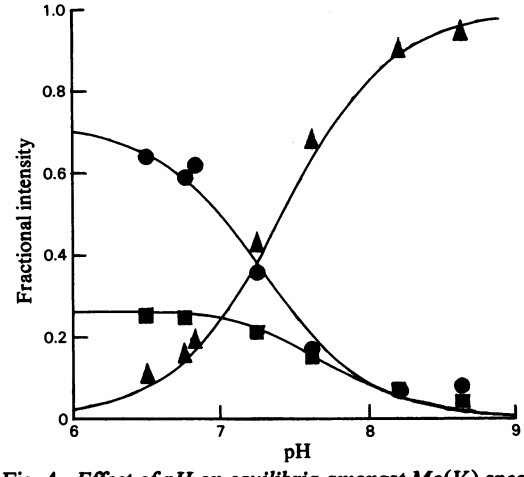


Fig. 4. *Effect of pH on equilibria amongst Mo(V) species from sulphite oxidase observed in the presence of 1.0mM-phosphate*

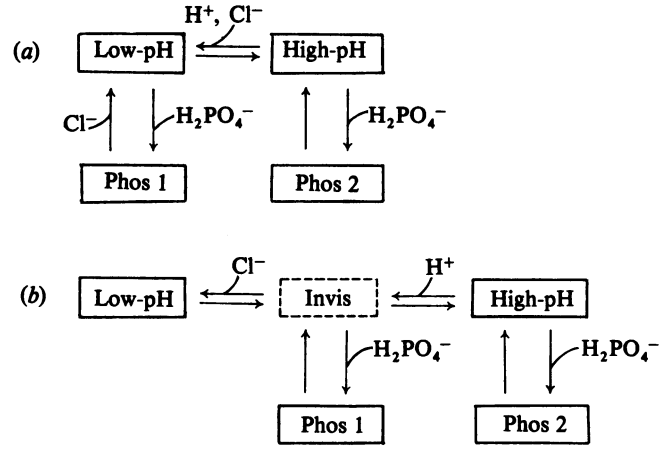
Fractional intensity of the following species: low-pH (■), high-pH (▲) and phosphate (●) (estimated as described in the Materials and methods section from spectra recorded at 120K) are plotted as a function of pH. The curves through the experimental points are theoretical ones computed as described in the text.

The above data, together with those in Fig. 1, confirm the equilibrium nature of the relationship to one another of the three signal-giving species. We employed a computer analysis, as described in the Materials and methods section, of all our data to establish the form of the equilibrium relationship. The final schemes which were considered are illustrated in Schemes 1(*a*) and 1(*b*). After the computer program had optimized the variables, these two schemes gave equally good fits of the computed signal intensities to experimental intensities, as judged by the minimum sum of squares error estimate over all the experiments. Replacing these schemes by a number of simplifications or other variants of Scheme 1(*a*) gave substantially worse minimum error estimates, as summarized in Table 1.

Table 1. *Comparison of the quality of computer fits to the experimental data when using different equilibrium schemes*

Experimental data are those illustrated in Figs. 1, 3 and 4 (together with one additional similar experiment which is not illustrated). Schemes used were Scheme 1(*b*) or Scheme 1(*a*), with or without the variations indicated. For each of these, computer fitting was carried out as described in the Materials and methods section. Goodness of fit for each scheme is indicated by the minimum error value.

Scheme	Variations	Minimum error
1(*b*)	None	0.20
1(*a*)	None	0.20
1(*a*)	Replacing $H_2PO_4^-$ by $(H_2PO_4^- + HPO_4^{2-})$	0.44
1(*a*)	Omitting 'Phos 2'	0.48
1(*a*)	Omitting 'Phos 1'	1.50



Scheme 1. *Possible equilibria governing interconversions among the three signal-giving species*
For computer fitting, the observed phosphate signal was taken to be the sum of the species 'Phos 1' and 'Phos 2'. 'Invis' in (*b*) denotes a hypothetical species not detected by e.p.r.

Because of uncertainties (and possible variations from one experiment and from one sample to another) in the contamination of our samples with chloride, the program treated as variables to be optimized, not only the equilibrium constants in the various schemes, but also the concentration of chloride assumed present as a contaminant in all samples, over and above concentrations which were added deliberately. For reasons discussed below, we prefer Scheme 1(*b*) to Scheme 1(*a*). The solid lines through the experimental points in Figs. 1, 3 and 4 were calculated by using Scheme 1(*b*), with the optimum values of the parameters (as summarized in Scheme 2 below). Clearly these lines are a good fit to the experimental data, with only minor and largely random deviations. The concentration of contaminating chloride calculated in this analysis and employed in the plots was 0.64 mM. This is within the range of possible values, as indicated above, which we take to be about 0.1–1.0 mM. (Note that the value of 0.64 mM could be weighted by one or two critical samples which happened to be particularly heavily contaminated.)

Additional species

Scheme 1(*b*) contains a species not detected by e.p.r. and referred to as 'Invis'. The possible nature of this will be considered below. Two additional e.p.r.-detectable species have to be considered. The first is the sulphite complex (Bray *et al.*, 1982). In agreement with that work, we were able to detect this species at the lowest pH values, provided that chloride concentrations were low (e.g. traces of the sulphite species are revealed on close inspection of the spectra of Figs. 2*a* and 2*b*). However, the sulphite complex was not apparent in substantial concentrations in any of the experiments which we submitted to the quantitative analysis described above.

The other species we have to consider is apparent on close inspection of the spectrum of high-pH species (Fig. 2*d*). The g_1 feature of this spectrum invariably shows small 'shoulders' indicated by the arrows in the Figure. As discussed elsewhere, these suggest very strongly the presence of relatively small amounts of an additional species (cf. Lamy *et al.*, 1980; Lamy Freund, 1981), whose nature is as yet uncertain. Amounts of this species were unchanged (as shown by the g_1 shoulders remaining constant) on adding Cl^- (0.1 M), sulphate (25 mM) or extra sulphite (to 25 mM) to samples showing the high-pH species.

We did not detect any new species either in the presence of ions such as NO_3^- (in agreement with Lamy *et al.*, 1980, but in contrast to claims made by Kessler & Rajagopalan, 1974), or additionally, in the presence of I^- or pyrophosphate. Presumably, these ions are too large to enter into the site occupied by Cl^- or phosphate.

E.p.r. studies in liquid solution at room temperature

Despite their potential importance, few e.p.r. studies have been carried out in liquid solution at ambient temperatures on Mo(V) in enzymes (Bray, 1980*b*), because both of reduced instrument sensitivity and of instability of some of the signal-giving species. Since signals obtained by reduction of sulphite oxidase with excess sulphite generally change little with time this enzyme appeared particularly well suited to room temperature e.p.r. studies. We therefore carried out semi-quantitative experiments at 295 K, with the aim of obtaining an

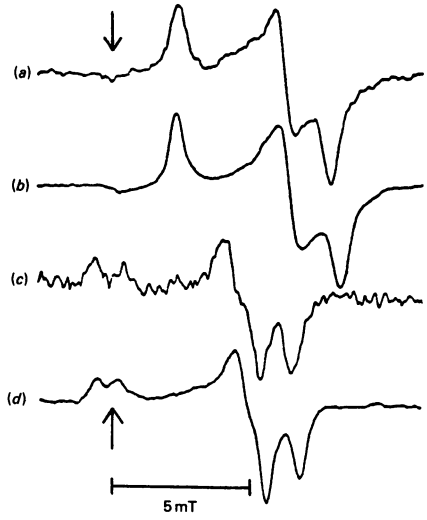


Fig. 5. *Comparison of e.p.r. spectra from pure species from sulphite oxidase in liquid solution at about 295 K (a and c) and in frozen solution at about 120 K (b and d)*
(*a*) and (*b*) show the high-pH species and (*c*) and (*d*) the low-pH (chloride) species. Samples for use at the two temperatures were prepared similarly to those used for Figs. 2(*d*) and 2(*e*). (Note that whereas low-temperature spectra were recorded directly, those at 295 K are difference spectra obtained after subtracting small amounts of the low-pH from the high-pH species and *vice versa*.) The arrow corresponds to $g = 2.0037$.

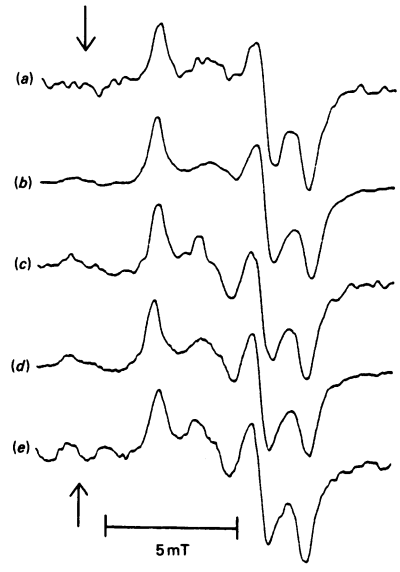


Fig. 6. *E.p.r. spectra consisting of mixtures of the high-pH and the low-pH (chloride) species obtained from samples prepared at different pH values and in the presence of different Cl^- concentrations, and recorded either at 295 K (a, c, and e) or at 120 K (b and d)*
pH values (at 20–25°C) and concentrations of NaCl were as follows: (*a*) 6.55, 0.1–1.0mM; (*b*) 7.62, 0.1–1.0mM; (*c*) 6.72, 10mM; (*d*) 8.91, 10mM; (*e*) 7.60, 100mM. The arrow corresponds to $g = 2.0037$.

indication of the extent to which the equilibria which we had studied in detail in frozen solution were applicable to the enzyme in the liquid state.

Fig. 5 demonstrates that the low-pH and the high-pH species could each be observed at about 295 K, with essentially no change in form from that seen at 120 K. This applied also to the phosphate signal (results not shown). (We did not attempt to measure g values or hyperfine couplings precisely at room temperature because of signal-to-noise problems.)

Changes in apparent pH occurring on freezing samples for e.p.r. spectroscopy have been discussed by Williams-Smith *et al.* (1977) (see also Orii & Morita, 1977). These workers noted that, with appropriate choice of buffer, the state of ionization of an e.p.r.-detectable group in a macromolecule could remain about the same in a frozen sample at 120 K as when in liquid solution at 295 K. We tested the buffer used in the present work, with or without addition of chloride or phosphate, by the indicator method recommended by these workers and found that apparent pH changes on freezing were in all cases small (about 0.3 pH units or less).

Fig. 6 illustrates a few typical experiments in which we compared effects of chloride concentration and of pH (measured at 20–25°C) on e.p.r. spectra of sulphite oxidase samples recorded at about 120 or 295 K. The spectra fall into two groups; thus (*a*) and (*b*) correspond to about 20% of the low-pH form and 80% of the high-pH and (*c*)–(*e*) correspond to about 40% of the low-pH and 60% of the high-pH. To judge the effect of freezing on the apparent pK, we may compare (*a*) with (*b*) (0.1–1.0mM-Cl^-) or alternatively (*c*) with (*d*) (10mM-Cl^-). Such comparisons suggest that in the low temperature samples, the apparent pK for the high-pH/low-pH (chloride) transformation is raised relative to that in the room temperature samples, by 1 to 2 pH units.

Fig. 6 also confirms that chloride has effects at room temperature as well as at low temperature. Thus, comparison of (*b*) with (*d*) (for 120 K) and of (*a*) with (*c*) (for 295 K) shows that 10mM-Cl^- gives an increase in the proportion of the low-pH (chloride) signals as compared with 0.1–1mM-Cl^- samples, even though, here, pH values were in both cases higher for the samples at the higher chloride concentration. Finally, and perhaps more definitively, comparison of (*c*) with (*e*) indicates that, at 295 K, a 10-fold increase in Cl^- concentration was countered by a pH increase of 1 unit.

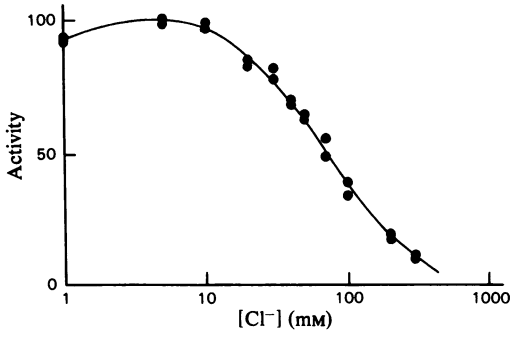


Fig. 7. *Effect of chloride ions on sulphite:cytochrome c reductase activity of sulphite oxidase at pH 6.8*
Activity measurements were carried out as described in the Materials and methods section. Concentration of added KCl is plotted on a logarithmic scale.

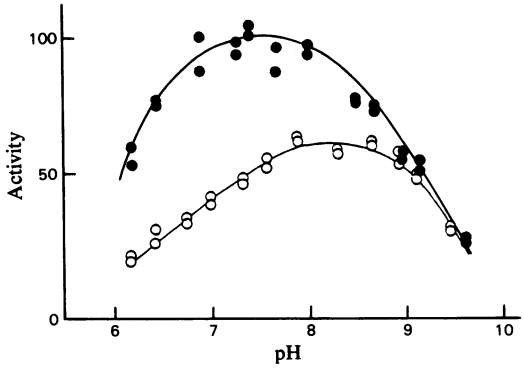


Fig. 8. *Comparison of pH–activity curves for sulphite oxidase in the presence of 10 mM-Cl⁻ (filled circles) or of 10 mM-phosphate (open circles)*
The sulphite:cytochrome *c* reductase assay was employed as described in the Materials and methods section. All activities are expressed as percentages of the activity with Cl^- at the pH optimum.

We also carried out a few experiments in which samples were prepared by the rapid freezing method (Bray, 1961). Apparent p*K* values, in so far as these could be estimated, tended either to be intermediate between those obtained in the liquid state and those obtained by manual freezing, or else to approximate to the liquid solution values.

Enzymic activity measurements

For comparison with the e.p.r. results, we carried out a few experiments on effects of chloride, phosphate and pH on enzymic activity in the sulphite:cytochrome *c* reductase assay. Fig. 7 shows the effect of chloride on activity at pH 6.8 and is in general agreement with the data reported by Kessler & Rajagopalan (1974) for a different buffer. Fig. 8 shows pH/activity curves in the presence of 10 mM-chloride or 10 mM-phosphate.

The small increase in activity with increasing chloride at low concentrations (Fig. 7), seemed of particular interest in relation to our finding, above, that chloride is a constituent of the low-pH e.p.r. signal-giving species, as it might indicate that chloride was essential for enzymic activity. Assays at carefully determined chloride concentrations in the range 0.1–1 mM, in an imidazole/nitrate buffer (which we found to be much less contaminated by Cl^-), however, did not produce lower activities. Thus, presumably, there is no requirement for chloride ions for enzymic activity, although because of the low dissociation constants which might be involved, it is difficult to exclude this rigorously.

Discussion

Our findings on the effect of variations in chloride concentration on the transition between the low-pH and high-pH species are completely at variance with the statement by Cohen *et al.* (1971) that "KCl up to 0.2 M had no effect on the e.p.r. spectrum of sulphite oxidase plus sulphite, whether the pH was 7.0 or 9.2". On the other hand the quantitative data presented by these workers is in fact fully consistent with our conclusion that chloride is an integral part of the low-pH signal-giving species. Thus, Cohen *et al.* (1971) show a titration curve in "0.1 M Tris-HCl" buffer. Inspection of this curve indicates a span, from 10% conversion to 90% conversion, of about 1.0 pH unit, whereas for a simple ionization governed by the Henderson–Hasselbach equation this span should be 1.9 pH units. Presumably Cohen *et al.* (1971) adjusted the pH value of samples of 0.1 M-Tris base by adding HCl. Thus they would have added hydrogen ions and chloride ions simultaneously and in these circumstances their result is fully consistent with our interpretation.

It is not surprising that, within experimental error, all our data on effects of pH, chloride concentration and phosphate concentration on intensities (measured at low temperature) of the three main e.p.r. signals, namely low-pH, high-pH and phosphate, can be accounted for by alternative equilibria, Schemes 1(*a*) and 1(*b*). No doubt other more complex forms are also possible. It is interesting that phosphate appears to react as $H_2PO_4^-$. Of these two schemes, we prefer 1(*b*) to 1(*a*), despite its greater complexity, on the grounds that the latter involves an improbable termolecular reaction of H^+ and of Cl^-, simultaneously, with the high-pH species.

Though we never succeeded in converting more than a small proportion of our enzyme samples into the low-pH (fluoride) species, the data we obtained on its spectrum are in accordance with a fluoride

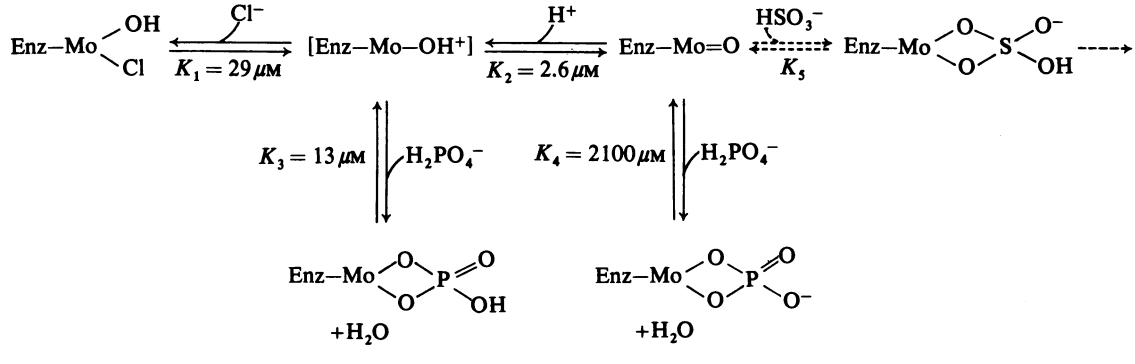
ligand of molybdenum. Our value (Fig. 2*f'*) of A $(^{19}F)_{av.}$ 0.53 mT may be compared, e.g., with one of 2.3 mT reported for this parameter in $(MoOF_4)^-$ by Sunil & Rogers (1981). Why the apparent dissociation constants for fluoride from the enzyme should be, as seems implied by our data, some 100–1000 times higher than that for chloride is not certain. Perhaps a scheme analogous to Scheme 1(*b*) in which the halide ion binds to the high-pH species before rather than after its protonation ought to be considered. If this applied, then failure of the enzyme to be converted completely to a low-pH (fluoride) species, in comparison with ready conversion to the low-pH (chloride) form, might be accounted for by a difference in pK between the two 'invisible' halide-containing species.

Returning to Scheme 1(*b*), it must be emphasized that though this is capable, formally, of explaining our data on relative intensities of the Mo(V) e.p.r. signals, it does not take any account of the well-documented phenomenon amongst molybdenum-containing enzymes of equilibria amongst Mo(IV), Mo(V) and Mo(VI) species. In order to do so, it would be necessary to add to Scheme 1(*b*) equilibria, for each of the Mo(V) species depicted, with corresponding Mo(IV) and Mo(VI) species. Though we did not investigate such equilibria in detail, we did, nevertheless, determine the proportion of total molybdenum in the Mo(V) state in a number of samples under different conditions of pH, etc. Values found ranged from 22 to 46%. This suggests that equilibrium constants for conversion to the higher and lower oxidation states are fairly close to unity (cf. Bray, 1980*a*).

E.p.r. measurements on molybdenum-containing enzymes are much more readily carried out on frozen samples than on liquid samples. It is therefore encouraging to find that the limited data which we obtained on liquid samples are consistent with similar equilibria applying as in the frozen state. The shift in apparent pK which we observed of 1 to 2 pH units between the two sets of conditions is in no sense unexpected.

Scheme 2 shows a suggested chemical interpretation of the equilibria of Scheme 1(*b*). The high-pH form of the enzyme is shown as having a terminal oxygen ligand of molybdenum. Cramer *et al.* (1981) have presented evidence from e.x.a.f.s. work for such ligands, both in Mo(VI) and in Mo(IV) forms of sulphite oxidase, though caution needs to be exercised in comparing their results with ours, since they appear to have used phosphate buffer and hence, presumably, were studying the phosphate complex of the enzyme. Scheme 2 implies that protonation of the oxygen of the high-pH species is immediately followed by an increase in the co-ordination number of the molybdenum, as the chloride ligand is taken up to yield the low-pH signal-giving species. Though we have not found an exact analogy in the chemistry of low molecular weight molybdenum complexes, nevertheless protonation of oxygen ligands in reduced states of the metal is well-known (Stiefel, 1973) and, e.g., Divand *et al.* (1976) have shown that reaction with HCl increases the metal's co-ordination number to seven, in converting a Mo=O group to $MoCl_2$ in Mo(VI) dithiocarbamato complexes.

Scheme 2 suggests a cyclic structure for the phosphate complex analogous to that of the Inhibited species from xanthine oxidase (Bray & Gutteridge, 1982). However, as pointed out by these workers, further work (cf. Gutteridge *et al.*, 1980) is needed with ^{17}O on the phosphate complex. A possible mode of interaction of sulphite with the enzyme is also included. An extension of our computing studies, with the sulphite concentration set at 5 mM



Scheme 2. *Proposed chemical interpretation of Scheme 1(b)*

The values of the equilibrium constants given are the ones which gave the best fit to the experimental data and which were used in computing the theoretical curves in Figs. 1, 3 and 4.

as in the e.p.r. work, and with K_s arbitrarily set at 10mM, showed that Scheme 2 then predicts appearance of the sulphite species roughly in accordance with the data reported by Bray *et al.* (1982). Thus, the computing indicated that the sulphite species would be detectable (i.e., 5% or more of the total signal-giving species), only if the pH was below 8, and that it would be suppressed at all pH values by quite low concentrations of phosphate or of chloride.

A cyclic complex formed, as indicated in Scheme 2, by addition of the HSO_3^- ion to Mo=O could presumably break down by rupture of the two molybdenum–oxygen bonds to liberate the product, HSO_4^-. It was tempting to extend the computing to activity data, on the assumption that this is indeed the catalytic pathway for the enzyme. However, there is a disappointing lack of data from steady-state turnover studies on the enzyme for comparison with the present e.p.r. work, i.e. no attempt seems to have been made to determine $k_{cat.}$ and $V_{max.}$ as a function of pH, with a view to establishing whether HSO_3^- or SO_3^{2-} is the substrate. Nevertheless, one point of agreement, even if this should eventually prove fortuitous, between our activity data and computing based on Scheme 2 with the added assumption that enzyme turnover is proportional to the concentration of the sulphite species, may be mentioned. This is that the Scheme predicts, in agreement with Fig. 8, that phosphate should raise the pH optimum of the enzyme.

We thank Dr. D. J. Lowe for supplying the iterative computer routine, Dr. F. Morpeth for advice on analysis for chloride and Dr. J. A. Broomhead for discussions on molybdenum chemistry. R. C. B. thanks Dr. C. Balny and INSERM U128, Montpellier, France, for hospitality during the preparation of the paper. The work was supported by the M.R.C. and the S.E.R.C. M. T. L. was in receipt of a studentship from the Conselho Nacional de Desenvolvimiento Cientifico e Technologico, Brazil.

References

Bray, R. C. (1961) *Biochem. J.* **81**, 189–193
Bray, R. C. (1980*a*) *Adv. Enzymol. Relat. Areas Mol. Biol.* **51**, 107–165
Bray, R. C. (1980*b*) in *Biological Magnetic Resonance* (Berliner, L. J. & Reuben, J., eds.), vol. 2, pp. 45–84, Plenum Press, New York
Bray, R. C. & Gutteridge, S. (1982) *Biochemistry* **21**, 5992–5999
Bray, R. C., Barber, M. J. & Lowe, D. J. (1978) *Biochem. J.* **171**, 653–658
Bray, R. C., Lamy, M. T., Gutteridge, S. & Wilkinson, T. (1982) *Biochem. J.* **201**, 241–243
Cohen, H. J., Fridovich, I. & Rajagopalan, K. V. (1971) *J. Biol. Chem.* **246**, 374–382
Cramer, S. P., Wahl, R. & Rajagopalan, K. V. (1981) *J. Chem. Soc.* **103**, 7721–7727
Divand, J., Ricard, L. & Weiss, R. (1976) *J. Chem. Soc. Dalton* 278–282
Gutteridge, S., Tanner, S. T. & Bray, R. C. (1978) *Biochem. J.* **175**, 869–878
Gutteridge, S., Lamy, M. T. & Bray, R. C. (1980) *Biochem. J.* **191**, 285–288
Hart, L. I., McGartoll, M. A., Chapman, H. R. & Bray, R. C. (1970) *Biochem. J.* **116**, 851–864
Iwasaki, I., Utsuni, S. & Ozawa, T. (1952) *Bull. chem. Soc. Japan* **25**, 226
Kessler, D. C. & Rajagopalan, K. V. (1972) *J. Biol. Chem.* **247**, 6566–6573
Kessler, D. C. & Rajagopalan, K. V. (1974) *Biochim. Biophys. Acta* **370**, 389–398
Lamy Freund, M. T. (1981) D.Phil. Thesis, University of Sussex
Lamy, M. T., Gutteridge, S. & Bray, R. C. (1980) *Biochem. J.* **185**, 397–403
Lowe, D. J. (1978) *Biochem. J.* **121**, 649–651
Orii, Y. & Morita, M. (1977) *J. Biochem.* (*Tokyo*) **81**, 163–169
Stiefel, E. I. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 988–992
Sunil, K. K. & Rogers, M. T. (1981) *Inorg. Chem.* **20**, 3283–3287
Williams-Smith, D. C., Bray, R. C., Barber, M. J., Tsopanakis, A. D. & Vincent, S. P. (1977) *Biochem. J.* **167**, 593–600