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Complexes with halide and other anions of the molybdenum centre of nitrate reductase from *Escherichia coli*

Graham N. GEORGE,* Robert C. BRAY,* Fraser F. MORPETH†‡ and David H. BOXER†
**School of Chemistry and Molecular Sciences, University of Sussex, Brighton BN1 9QJ, U.K. and †Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland, U.K.*

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The interconversion of nitrate reductase from *Escherichia coli* between low-pH and high-pH Mo(V) e.p.r. signal-giving species was re-investigated [cf. Vincent & Bray (1978) *Biochem. J.* **171**, 639–647]. The process cannot be described by a single pK value, since the apparent pK for interconversion is raised by the presence of various anions. The low-pH form of the enzyme exists as a series of complexes with different anion ligands of molybdenum. Each complex has specific and slightly different e.p.r. parameters, but all show strong coupling of Mo(V) to a single proton, exchangeable with the solvent, having $A(^1H)_{av.}$ 1.0 to 1.3mT. Complexes with Cl^-, F^- [$A(^{19}F)_{av.}$ 0.7mT], NO_3^- and NO_2^- give particularly well-defined spectra. The high-pH form of the enzyme is now shown to bear a coupled proton. Like that in the low-pH species, this proton is exchangeable with the solvent, but the coupling is much weaker, with $A(^1H)_{av.}$ 0.3mT. Thus, contrary to earlier assumptions, the proton detectable by e.p.r. is probably not identical with the proton whose dissociation controls interconversion between the two species; the latter proton could be located in the protein rather than on a ligand of molybdenum. Treatment of the enzyme with trypsin [Morpeth & Boxer (1985) *Biochemistry* **24**, 40–46] did not affect its Mo(V) e.p.r. signals.

The structures and mechanisms of action of molybdenum centres in enzymes (Bray, 1980; Bray & George, 1985) are frequently studied by e.p.r. or by extended-X-ray-absorption-fine-structure spectroscopy, and the usefulness of the two methods in this field has recently been compared (Bray, 1984). Studies by the former method of Mo(V) in the molybdoenzymes nitrate reductase from *Escherichia coli* (Vincent & Bray, 1978) and sulphite oxidase from liver (Cohen *et al.*, 1971; Lamy *et al.*, 1980) have been important in characterizing the molybdenum centres of these enzymes at which their primary substrates interact. Both enzymes show a pH-dependent equilibrium between a high-pH and a low-pH species, the low-pH form being distinguished by strong hyperfine coupling in its e.p.r. spectrum of Mo(V) to a single proton, exchangeable with the solvent. The magnitude and the extent of the anisotropy of the coupling of this proton are comparable with those exhibited by a hydroxyl ligand of molybdenum in irradiated crystals of alkylammonium molybdate (Yamase *et al.*, 1981). This, and additional data on coupling to ^{17}O (Bray & Gutteridge, 1982) in sulphite oxidase, make it essentially certain that the proton in the low-pH form of this enzyme, and presumably in the low-pH form of nitrate reductase also, is in the form of a hydroxyl ligand of the metal.

The high-pH forms of these enzymes show apparently simple rhombic spectra, without resolved hyperfine couplings (Vincent & Bray, 1978; Lamy *et al.*, 1980). Bray *et al.* (1983) reported that equilibrium between high-pH and low-pH forms of sulphite oxidase depends not only on the concentration of protons but equally on that of Cl^- ions (a 10-fold change in Cl^- concentration having effects similar to those of a pH change of 1 unit). They concluded that the interconversion between the high-pH and low-pH forms of this enzyme was as shown below:

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

For nitrate reductase from *E. coli* (Vincent & Bray,

‡ Permanent address: National College of Food Technology, University of Reading, Reading RG6 2AP, U.K.

1978), or for the similar enzyme from *Pseudomonas aeuriginosa* (Godfrey *et al.*, 1984), effects of anions on the apparent p*K* for interconversion between the high-pH and low-pH forms have not been reported. For the *E. coli* enzyme the value of this p*K* was given as 8.26 and the suggestion made that the low-pH form was the one involved in catalysis (Vincent & Bray, 1978).

Two assimilatory nitrate reductases, those from spinach (Gutteridge *et al.*, 1983) and *Chlorella* (Solomonson *et al.*, 1984), have also been studied in a preliminary way by e.p.r. Both show a proton-split low-pH signal (referred to as signal A by Gutteridge *et al.*, 1983), spectra of this signal for enzyme from the two sources being quite similar, though rather different from that from the *E. coli* enzyme. Though high-pH forms of the assimilatory enzymes may differ and have not yet been obtained in pure form, nevertheless, significantly, Gutteridge *et al.* (1983) reported that formation of the proton-split signal was favoured by Cl^-.

We therefore thought it important to re-investigate the high-pH and low-pH forms of nitrate reductase from *E. coli* and their interconversion, and the effects of different anions and their concentrations on this process. Our work was facilitated by availability of improved preparations of the enzyme (Morpeth & Boxer, 1985). A preliminary note on parts of this work has been published (Morpeth *et al.*, 1983).

Materials and methods

Nitrate reductase was prepared as described by Morpeth & Boxer (1984). Samples of the enzyme, concentrated by using Minicon B concentrators (from Amicon), were transferred to appropriate media by dialysis or gel filtration. Concentrations of the enzyme were determined by assaying for protein by the method of Lowry *et al.* (1951). pH values in ^2H_2O refer to uncorrected pH-meter readings. Trypsin-modified enzyme was prepared by the procedure of Morpeth & Boxer (1985).

Spectra of Mo(V) in the enzyme were obtained on frozen samples of enzyme either as prepared or after anaerobic addition of $Na_2S_2O_4$ (about 1 e^- per Mo atom). Such additions usually gave an increase in the intensity of the signals but no change in their form. E.p.r. spectra were recorded on a Varian E9 spectrometer linked to a computer (Bray *et al.*, 1978) and were subsequently transferred via a link to the University of Sussex Digital Equipment Company VAX 11/780 computer. E.p.r. simulation routines (Bray & George, 1985) calculated theoretical lineshapes by using a second-order perturbation solution of the appropriate spin Hamiltonian. E.p.r. parameters were obtained by a combination of computer minimization of the sum-of-squares error between experimental and simulated spectra and by inputting new values from the terminal to get a visual fit. E.p.r. running conditions were temperature about 123K, microwave power 10mW, modulation amplitude 0.16mT, and microwave frequency about 9.3GHz.

Results

Effect of halide ions

Fig. 1 shows the substantial effect of Cl^- ions on the apparent p*K* for interconversion between the low-pH and high-pH Mo(V) e.p.r. signal-giving species from *E. coli* nitrate reductase. Fig. 1(*a*) shows the spectrum of the enzyme as isolated, in the absence of added Cl^- ions. Though the pH of the sample (7.0) was much below the p*K* value of 8.26 reported by Vincent & Bray (1978), nevertheless the signal is dominated by the high-pH form (accompanied by about 40% of the low-pH form). On addition of 0.3M-KCl to this sample, the spectrum changed to one (Fig. 1*b*) that was mainly (about 85%) in the low-pH form. Thus clearly interconversion between the low-pH and high-pH species is a more complex process than was

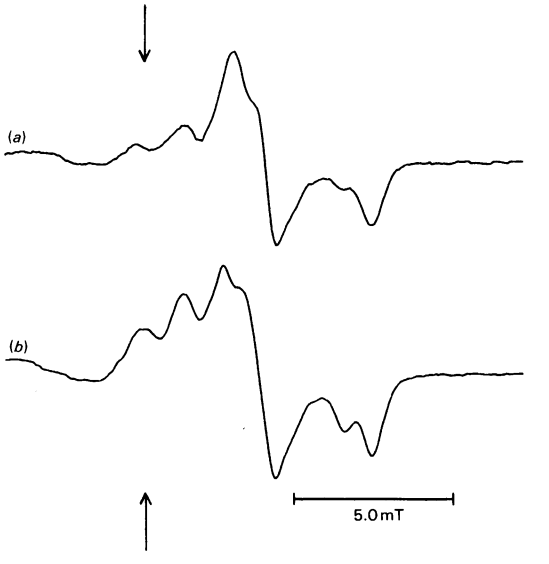

Fig. 1. *Effect of Cl^- ions on the e.p.r. spectrum of nitrate reductase*

(*a*) shows the spectrum in the absence of added Cl^- ions. The sample consisted of enzyme as prepared, after dialysis into 50mM-Hepes/NaOH buffer, pH 7.0. (*b*) shows the same sample after thawing, addition of KCl to a final concentration of 0.3M, and refreezing. The pH of the sample was checked at the end of the experiment and found still to be 7.0. The arrows here and in Figs. 2–4 correspond to $g = 2.0037$.

reported by Vincent & Bray (1978) and cannot be described by a single pK value. Further work is needed on the quantitative nature of effects of Cl^- and other anions on the apparent pK. However, additional experiments (results not shown) indicated that, though the pK increased systematically with increasing Cl^- concentrations, nevertheless the increase was considerably less than the 1 pH unit expected, by analogy with sulphite oxidase (Bray *et al.*, 1983), for a 10-fold increase in Cl^- concentration. Our data suggested, furthermore, that the pK value given by Vincent & Bray (1978) most probably related to a medium containing substantial amounts of Cl^-, though this was not made clear in the original work.

Fig. 2(*a*) shows the pure low-pH signal in the presence of 0.3M-KCl, obtained by subtracting a small amount of the high-pH signal (see below) from the spectrum of Fig. 1(*b*). Bearing in mind the observed dependence of the low-pH signal on Cl^- and the widespread occurrence of Cl^- as a contaminating anion, we refer below (cf. Bray *et al.*, 1983) to this normal low-pH form of nitrate reductase as the low-pH chloride species.

Bray *et al.* (1983) presented evidence for partial conversion of sulphite oxidase into a low-pH fluoride species, showing hyperfine coupling of Mo(V) to fluoride ($I = \frac{1}{2}$). For nitrate reductase, in contrast, we did not have to resort to the use of difference spectra, and complete conversion of signal-giving species into the low-pH fluoride form was readily achieved by adding 0.3M-KF. Figs. 2(*d*) and 2(*g*) show the low-pH fluoride species in ^1H_2O and in ^2H_2O respectively, the corresponding computer simulations being shown in Figs. 2(*e*) and 2(*h*). Figs. 2(*a*) and 2(*b*) show, for comparison, the low-pH chloride species in ^1H_2O and the simulation. The anisotropy of the coupling to fluorine is clearly visible by comparing the A_3 feature in Fig. 2(*d*) (where the fluorine gives a coupling smaller than does the proton) with that in the A_1 orientation in Fig. 2(*g*) (where the strong coupling to fluorine is apparent, despite broadening from the unresolved ^2H triplets).

Effect of NO_3^- and NO_2^- ions

The effect of promoting formation of a low-pH species was found for a number of additional anions, with, as discussed below, each anion forming its own distinct complex with the low-pH form of the enzyme. Perhaps not surprisingly, the most pronounced effect was found with the substrate NO_3^-, where a concentration of 10mM-NO_3^- was sufficient to convert close to 100% of the signal into the low-pH nitrate species (Fig. 3*a*). This is to be compared with Cl^-, for which a concentration of 300mM failed to convert all of the enzyme into the anion-bound form (Fig. 1*b*). In agreement with the conclusions of Morpeth & Boxer (1985) that the dissociation constant for the enzyme–nitrate complex is very low, some samples of the enzyme, examined as prepared without additions to the solution, exhibited e.p.r. spectra attributable in part to the low-pH nitrate signal.

We investigated the spectra of the nitrate (Figs. 3*a–d*) and nitrite (Figs. 3*g–h*) complexes more carefully than did Vincent & Bray (1978), and our parameters for these species (Table 1) are more reliable than the original values. A new finding in the present work was that both NO_3^- and NO_2^- are capable of forming two complexes with the enzyme, each showing slightly different parameters. We refer to these as the low-pH nitrate type 1 and type 2 species (and similarly for the nitrite complex). The species so far referred to are the type 1 complexes. The spectrum of the nitrate type 2 complex is shown in Fig. 3(*e*), with a simulation in Fig. 3(*f*). The main difference from the type 1 spectrum (Fig. 3*a*) is an increase in the value of g_1 by about 0.003, with a decrease in the linewidth of the g_1 features (Table 1). The type 2 nitrite complex shows a similar change of parameters relative to the nitrite type 1 species (results not illustrated; see Table 1 for parameters). We

Table 1. *E.p.r. parameters of Mo(V) species from nitrate reductase of E. coli*

Values given were obtained as described in the text, with the help of computer simulation, and were used for the simulated spectra of Figs. 1–4. A-values and half-linewidths (Δ) are given in mT. g-values are believed to be accurate to ± 0.0003.

Species	g_1 (Δ_1)	g_2 (Δ_2)	g_3 (Δ_3)	$g_{av.}$($\Delta_{av.}$)	Nucleus	A_1	A_2	A_3	$A_{av.}$
High-pH	1.9875 (0.20)	1.9807 (0.22)	1.9617 (0.29)	1.9766 (0.24)	^1H	0.38	0.32	0.33	0.34
Low-pH, nitrate, type 1	2.0024 (0.27)	1.9858 (0.22)	1.9655 (0.25)	1.9846 (0.25)	^1H	1.46	1.11	1.09	1.22
Low-pH, nitrate, type 2	2.0057 (0.16)	1.9858 (0.16)	1.9655 (0.25)	1.9857 (0.19)	^1H	1.52	1.11	1.09	1.24
Low-pH, nitrite, type 1	1.9997 (0.28)	1.9851 (0.23)	1.9642 (0.26)	1.9830 (0.26)	^1H	1.57	1.04	1.04	1.22
Low-pH, nitrite, type 2	2.0031 (0.17)	1.9855 (0.17)	1.9642 (0.22)	1.9843 (0.19)	^1H	1.54	1.19	1.11	1.28
Low-pH, chloride	2.0008 (0.40)	1.9858 (0.25)	1.9642 (0.35)	1.9836 (0.33)	^1H	1.13	0.85	0.90	0.96
Low-pH, fluoride	2.0001 (0.39)	1.9851 (0.25)	1.9652 (0.23)	1.9835 (0.29)	^1H	1.40	0.97	1.00	1.12
					^{19}F	1.10	0.55	0.48	0.71

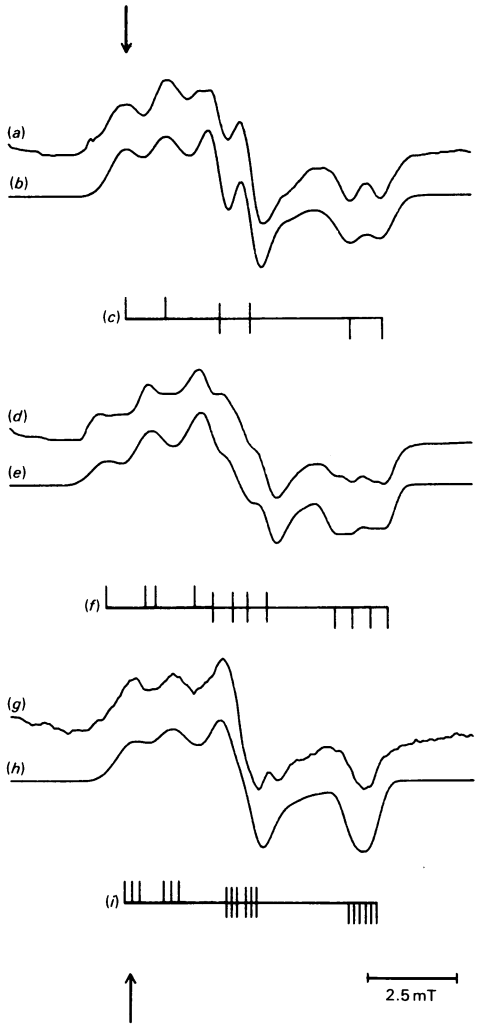

Fig. 2. *E.p.r. spectra of the low-pH chloride and low-pH fluoride species of nitrate reductase in ^1H_2O and ^2H_2O*

Experimental spectra in ^1H_2O are shown in (*a*) and (*d*), and in ^2H_2O in (*g*). All samples were in 50mM-Hepes/NaOH buffer, pH 7.0, with the addition of 0.3M-KCl to (*a*) and of 0.3M-KF to (*d*) and (*g*). (*a*) is a difference spectrum obtained by subtracting a small amount of the high-pH signal (Fig. 4*c*) from the observed spectrum; (*d*) and (*g*) are as recorded. The corresponding simulated spectra, obtained by using the parameters listed in Table 1, are given in (*b*), (*e*) and (*h*) respectively, and stick diagrams showing the principal *g*-values split, as appropriate, by ^1H, ^2H and ^{19}F, are given in (*c*), (*f*) and (*i*).

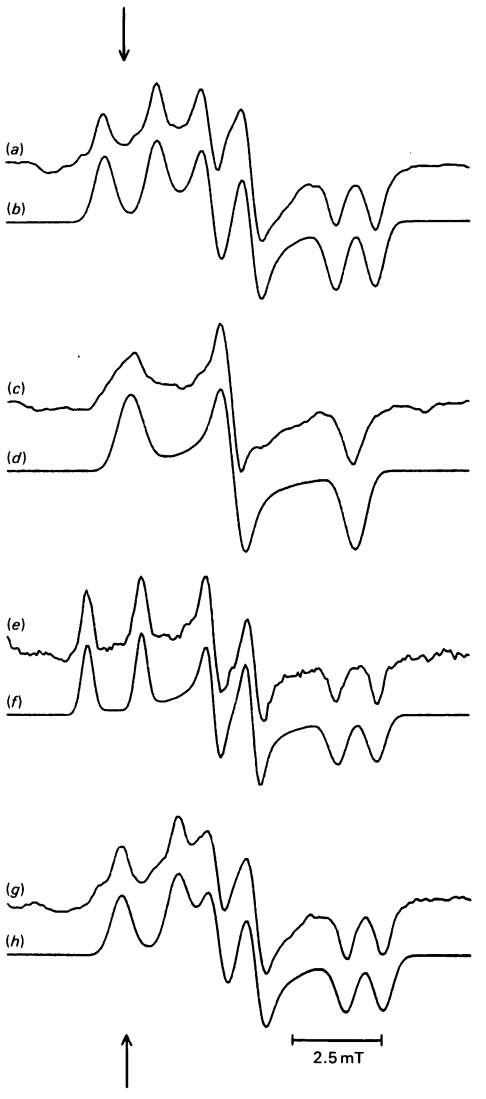

Fig. 3. *E.p.r. spectra of the low-pH nitrate and nitrite complexes of nitrate reductase*

(*a*)–(*f*) show nitrate complexes, and (*g*) and (*h*) nitrite complexes. Experimental spectra are given in (*a*), (*c*), (*e*) and (*g*), and the corresponding simulations, based on the parameters of Table 1, in (*b*), (*d*), (*f*) and (*h*) respectively. The solvent was ^1H_2O, except in (*c*), for which it was ^2H_2O. In all cases the buffer was 50mM-Hepes/NaOH, pH 7.0. (*a*) and (*c*) show the type 1 form of the spectrum, and were obtained in the presence of 10mM-$NaNO_3$. (*e*) shows the spectrum from the type 2 nitrate complex and was obtained under the same conditions as in (*a*), except that enzyme of lower specific activity was employed. [Note that in (*c*) a small amount of the type 2 spectrum may be present, in addition to the type 1 spectrum.] (*g*) shows the type 1 nitrite complex signal and was obtained with 10mM-$NaNO_2$.

have not so far defined fully conditions leading to generation of the type 2 rather than the type 1 spectra. However, appearance of the type 2 forms appeared to be associated with partial loss of nitrate reductase activity.

Effect of other anions

In addition to the above anions, we also studied effects of several others on the form of the low-pH spectrum. In each case we obtained evidence for a specific complex having its own distinct e.p.r. spectrum, with, in every case, strong coupling to a single proton. The anions studied included the substrates ClO_3^- and BrO_3^- (Morpeth & Boxer, 1985) as well as $H_2PO_4^-$ and HCO_2^-. In the case of Br^-, though the spectrum was not well resolved, there were distinct indications of hyperfine coupling to bromine [^{79}Br and ^{81}Br, both with $I=\frac{3}{2}$; $A_1(Br)$ about 0.2mT].

Nature of the high-pH species

Though Vincent & Bray (1978) reported that the high-pH signal was unchanged in form when developed in ^2H_2O, we found that replacing ordinary water by this solvent resulted in a considerable decrease in the linewidths of the signal (Fig. 4). This indicates that in the signal-giving species at least one exchangeable proton must be coupled to molybdenum. Simulation (assuming a single coupled proton) of the spectrum in ^1H_2O (Figs. 4*c* and 4*d*), in comparison with that in ^2H_2O (Figs. 4*a* and 4*b*), indicates near-isotropic coupling to a proton with $A(^1H)_{av.}$ 0.34mT (see Table 1).

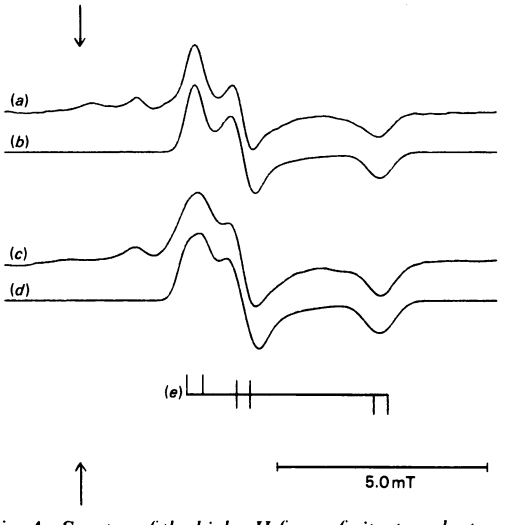

Fig. 4. *Spectra of the high-pH form of nitrate reductase in ^2H_2O and in ^1H_2O*

(*a*) shows the spectrum in ^2H_2O and (*c*) that in ^1H_2O, and (*b*) and (*d*) are the corresponding simulations based on the parameters given in Table 1. (*e*) is a stick diagram corresponding to (*d*). The buffer was Hepes/NaOH, pH7.0, in (*a*), and Bicine/NaOH, pH8.0, in (*c*). [Effects of ^2H_2O comparable with those illustrated were also observed in other buffers.]

Mo(V) e.p.r. signals from the trypsin-modified enzyme

Morpeth & Boxer (1985) have shown that treatment of nitrate reductase with trypsin removes cytochrome *b* and part of the β-subunit from the enzyme, and modifies its catalytic properties in relation to interaction with quinol-reducing substrates. In agreement with the conclusion of these workers that the site at which nitrate interacts is not affected by this treatment, we found that Mo(V) e.p.r. signals from trypsin-treated enzyme were indistinguishable from those from the untreated enzyme. Signals examined included particularly the high-pH signal and the low-pH nitrate type 1 signal.

Discussion

Our data reveal hitherto unsuspected complications in the structures of the Mo(V) signal-giving species from nitrate reductase from *E. coli*. Some of these complications we are not immediately in a position to interpret. Thus further work by analysis of extended-X-ray-absorption-fine-structure spectroscopy or by using substitution with ^{17}O will be required to substantiate the proposal that the proton of the low-pH species is in the form of a hydroxyl ligand of molybdenum, as in sulphite oxidase. On the other hand, our data provide clear evidence that the low-pH species of nitrate reductase bears an obligatory anion ligand, the nature of which can be varied, with each anion giving rise to a complex having distinct, though essentially similar, e.p.r. parameters. Particularly for the fluoride complex, the magnitude of the splitting [$A(^{19}F)_{av.}$ 0.7mT] leaves no room for doubt that the anion is a ligand of the metal, rather than simply being bound at a nearby site in the protein. The presence of such an anion ligand provided by the phosphate buffer that was used (together with incomplete oxidation of the sample used) might provide an explanation for the anomalous extended-X-ray-absorption-fine-structure results described by Cramer *et al.* (1984) for *E. coli* nitrate reductase.

Further work will be required on the relation of the anion complexes to the activity of the enzyme. Since the Mo(V) signals we studied are derived (cf. Vincent, 1979) from enzyme molecules containing 1 reducing equivalent only per molecule, the observed nitrate complex must be on a side path rather than on the main catalytic pathway. The latter presumably involves (cf. Berg & Holm, 1984) molybdenum cycling between the Mo(IV) and Mo(VI) oxidation states. Nevertheless the structure of this complex is no doubt highly relevant to that in the true Michaelis complex. That other anions may be inhibitory to the enzyme is indi-

cated by studies with Cl^-. This has recently been found (F. F. Morpeth & D. H. Boxer, unpublished work) to be a weak competitive inhibitor of the enzyme with K_i about 0.5M; inhibition by Cl^- of NO_3^- reduction from *Suaeda maritima* has also been reported (Billard & Boucaud, 1982).

It is noteworthy that sulphite oxidase forms a specific complex, detectable by e.p.r., with the substrate SO_3^{2-} (Bray *et al.*, 1982), as well as with the inhibitor, phosphate (Lamy *et al.*, 1980). Neither of these complexes shows the normal coupling to protons of the low-pH forms of the enzyme. (Indeed, such forms of sulphite oxidase seem to be limited to the complexes with halide ions.) It has been suggested that the complex with sulphite (and the spectroscopically analogous complex with phosphate) is in some way related to the catalytic reaction of sulphite oxidase. In this connection it is noteworthy that we have not so far seen analogous complexes of nitrate reductase; the phosphate complex of this enzyme shows an e.p.r. spectrum only slightly different from that of the chloride complex.

Clearly the nature of the interconversion between the low-pH and high-pH species in nitrate reductase from *E. coli* is more complex than was thought by Vincent & Bray (1978), and is not of the relatively simple form proposed for sulphite oxidase by Bray *et al.* (1983). Thus we now know that both low-pH and high-pH forms bear an exchangeable proton coupled to Mo(V). A similar situation has recently been found in sulphite oxidase, where a coupled proton in the e.p.r. spectrum of the high-pH species has been detected (G. N. George, unpublished work) by the observation of proton spin-flip transitions.

These observations bring into question the assumption, explicitly or implicitly accepted from the work of Cohen *et al.* (1971) onwards, that the proton 'seen' in the low-pH spectrum of these enzymes, and which is replaceable by ^2H, is identical with the proton that dissociates on raising the pH, and thus controls conversion into the high-pH species. It now becomes simpler to assume, on the contrary, that the coupled proton is the same in the high-pH and low-pH species, with dissociation of an auxiliary proton, not detectably coupled to Mo(V) and whose location is uncertain, controlling the pK. Subramanian *et al.* (1984) have reported, for Mo(V) model compounds, pH-dependent interconversion between protonated and non-protonated species of the forms $[MoOL]^-$ and $[MoOLH_2]^+$, which are clearly distinguishable by e.p.r. In this case the protons are located on nitrogen ligands of molybdenum but are apparently not detectably coupled to the metal (though fully detailed e.p.r. results were not presented by these workers). In the case of nitrate reductase, the e.p.r.-undetectable proton whose dissociation controls the pK might be located in an amino acid side chain or possibly in the pteridine cofactor. Such a scheme would bring sulphite oxidase and nitrite reductase into line with xanthine oxidase and other molybdenum-containing hydroxylases, with the high-pH–low-pH transition in the former enzymes becoming somewhat analogous to the Rapid type 2–Rapid type 1 transition in the latter. The type 2–type 1 change is now believed (Bray *et al.*, 1984) to involve an anion- and pH-mediated change of ligand geometry, without any conversion of −Mo−OH (or Mo−SH) species into −Mo=O (or −Mo=S).

Although such a scheme is attractive for *E. coli* nitrate reductase, considerable further work will be required to establish it fully and to explain the greater effects of anions on the e.p.r. spectrum of this enzyme in comparison with sulphite oxidase. Additional complexities to be explained for nitrate reductase concern existence of two low-pH complexes with nitrate and nitrite and the finding that the low-pH–high pH transition is much less sensitive to Cl^- concentrations than in sulphite oxidase.

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