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# Electron-Paramagnetic-Resonance Studies on the Molybdenum of Nitrate Reductase from *Escherichia coli* K12

By ROBERT C. BRAY, STEPHEN P. VINCENT and DAVID J. LOWE
*School of Molecular Sciences, University of Sussex, Brighton BN1 9QJ, U.K.*
and ROGER A. CLEGG and PETER B. GARLAND
*Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, U.K.*

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Studies on the respiratory nitrate reductase (EC 1.7.99.4) from *Escherichia coli* K12 by electron-paramagnetic-resonance spectroscopy indicate that its molybdenum centre is comparable with that in other molybdenum-containing enzymes. Two Mo(V) signals may be observed; one shows interaction of Mo(V) with a proton exchangeable with the solvent and has: $A$ ($^1$H) 0.9–1.2mT; $g_1 = 1.999$; $g_2 = 1.985$; $g_3 = 1.964$; $g_{av.} = 1.983$. Molybdenum of both signal-giving species may be reduced with dithionite and reoxidized with nitrate.

Assimilatory nitrate reductase from *Neurospora crassa* (EC 1.6.6.3) was one of the first molybdenum-containing enzymes to be studied (Nicholas & Nason, 1954), and the presence in its samples of molybdenum in the quinquivalent state was detected in the enzyme from *Pseudomonas aeruginosa* by electron-paramagnetic-resonance (e.p.r.) spectroscopy as long ago as 1961 (Fewson & Nicholas, 1961). However, despite some more recent work on the respiratory enzyme from *Escherichia coli* by using this method (DerVartanian & Forget, 1975), e.p.r. has not so far provided definitive information on the nature of the molybdenum active centre of nitrate reductases, or on similarities between them and other molybdenum-containing enzymes. In view of the extensive information on the molybdenum of xanthine oxidase (EC 1.2.3.2) furnished by e.p.r. (Bray, 1975), it seemed important to carry out more e.p.r. work on a nitrate reductase. For such studies we selected the enzyme purified from *E. coli* K12 (Clegg, 1976).

There are a number of points in the earlier e.p.r. literature on molybdenum in nitrate reductase that are not readily reconcilable with recent work on xanthine oxidase. We therefore thought it particularly important to look for new evidence relating to the following. Very high $g$-values ($g_{\|}$ up to 2.09) have been reported for Mo(V) in nitrate reductase from *Micrococcus denitrificans* (Forget & DerVartanian, 1972). In contrast, with xanthine oxidase and xanthine dehydrogenase (EC 1.2.1.37) the highest $g$-value observed is that of the $g_z$ component of the Very Rapid signal, at $g = 2.025$ (Bray & Meriwether, 1966; Barber *et al.*, 1976). E.p.r. signals attributed to Mo(III) have been reported from reduced samples of both the *M. denitrificans* (Forget & DerVartanian, 1972) and the *E. coli* (DerVartanian & Forget, 1975) enzymes. Yet, for xanthine oxidase, reduction of molybdenum to the tervalent level has not been achieved (Bray *et al.*, 1975; Bray, 1976). Finally, with the enzyme from *E. coli* a seven-line e.p.r. spectrum has been reported and provisionally attributed to nitrogen hyperfine structure from an NO complex, perhaps with iron in the enzyme (DerVartanian & Forget, 1975). Nitrogen hyperfine structure has not apparently been reported elsewhere from any molybdenum or iron–sulphur centre in an enzyme.

## Materials and Methods

Nitrate reductase was prepared from *E. coli* K12 by the method of Clegg (1976), after release by Triton X-100 from the membrane. Such preparations are homogeneous by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Enzyme samples were concentrated to about 5mg/ml by adsorption on DEAE-cellulose and subsequent desorption. Specific activity at the time of the experiments was about 60$\mu$mol of $NO_2^-$/min per mg of protein, measured at 30°C either spectrophotometrically with Benzyl Viologen (Kemp *et al.*, 1975) or by $NO_2^-$ formation (MacGregor *et al.*, 1974). All samples were at a pH or pD of 7.8 in 10mM-potassium phosphate buffer containing 400mM-NaCl and 0.1mM-mercaptoethanol. Transfer to $^2H_2O$ was accomplished by dialysis and pD values were obtained by the method of Glasoe & Long (1960). Sample preparation by manual freezing and e.p.r. measurements were as described by Lowe *et al.* (1972); e.p.r. tubes of 4mm internal diameter were employed.

## Results and Discussion

The e.p.r. spectrum of a sample of nitrate reductase, as prepared, in ordinary $^1H_2O$ is shown in Fig. 1(*a*). The spectrum is well-defined and is attributable to a single chemical species giving rise to a rhombic type of spectrum, split into a doublet at each of the principal features. Doublets with splittings of the observed magnitude (0.9–1.2mT) may arise from interaction with protons, and furthermore two Mo(V) e.p.r. signals from milk xanthine oxidase (Rapid and Slow) are known (Bray, 1976) to show interaction with protons that are exchangeable with those of the solvent water, and a similar phenomenon is seen with a Mo(V) signal for sulphite oxidase (EC 1.8.3.1) (Cohen *et al.*, 1971). We therefore tried the effect of dialysing nitrate reductase into $^2H_2O$. The resultant e.p.r. spectrum is shown in Fig. 1(*b*) and shows very clearly that the splittings in Fig. 1(*a*) are due to interaction with an exchangeable proton. Parameters of the signal measured from Figs. 1(*a*) and 1(*b*) are: $g_1 = 1.999$; $g_2 = 1.985$; $g_3 = 1.964$; $g_{av.} = 1.983$; $A_1 = 1.2$mT; $A_2 = 0.9$mT; $A_3 = 0.9$mT. Although the spectra have not been rigorously shown to be due to Mo(V) by the identification of $^{95}$Mo and $^{97}$Mo hyperfine structure, nevertheless other possibilities may safely be excluded, from the known composition of the preparations, as well as from the *g*-values, the linewidths and the appearance of proton splittings analogous to those from other molybdenum-containing enzymes. Our signal is particularly like the low-pH signal from sulphite oxidase (Cohen *et al.*, 1971). The preparation of the nitrate reductase used for this work was not analysed for molybdenum; however, the enzyme has been reported to have a content of about 1 Mo atom/230000mol.wt. (MacGregor *et al.*, 1974; Forget, 1974). Though intensity of the signals was weak, corresponding to about 10% of this assumed Mo content, we note that with xanthine oxidase relatively low intensity of the molybdenum signals is attributable (Olson *et al.*, 1974) to equilibria among the quadri-, quinqui- and sexi-valent states of the metal.

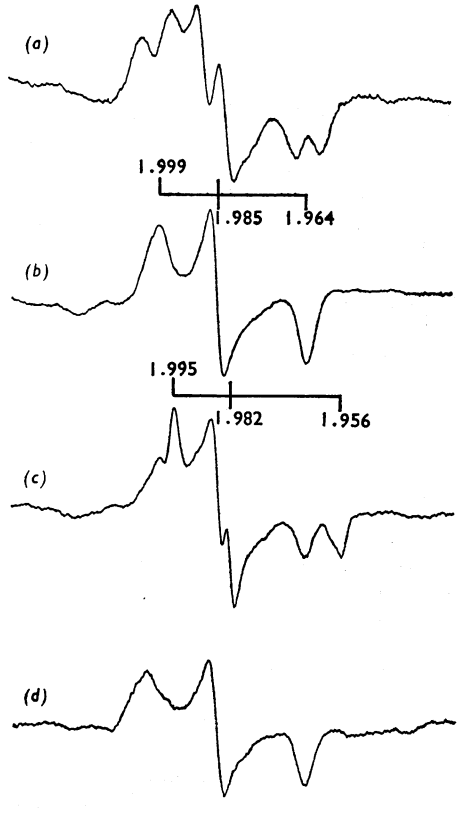


Fig. 1. *E.p.r. spectra of Mo(V) in nitrate reductase samples*

In (*a*) the solvent was $^1H_2O$ and in (*b*)–(*d*) it was $^2H_2O$. (*a*) and (*b*) are untreated enzyme; (*d*) is enzyme in the presence of 1M-$KNO_3$; in (*c*) the sample was first reduced with 10mM-$Na_2S_2O_4$, then reoxidized for 1min with 20mM-$KNO_3$. The stick diagrams show the $g_1$, $g_2$ and $g_3$ positions for the two Mo(V) species in $^2H_2O$. Spectra were recorded at 120K with 20mW microwave power and 0.25mT modulation, at a frequency of 9.10–9.15GHz.

For further studies on nitrate reductase, to simplify the e.p.r. spectra and to improve signal/noise ratios, we worked exclusively with preparations in $^2H_2O$. Anaerobic addition of dithionite or nitrate to solutions of the enzyme resulted in complex changes. Addition of nitrate resulted in small shifts in the *g*-values, but in little change in the intensity of the signal. The most apparent change was a shift of the $g_1$ value from 1.999 to 2.003, accompanied by some broadening of this feature (compare Figs. 1*b* and 1*d*). Whether this effect of nitrate is a specific one or whether other anions produce similar effects has not so far been ascertained. Addition of small amounts of dithionite (1–2mM final concentration) to the enzyme, in the absence of nitrate, had little effect on the intensity of the existing signal, but caused the appearance of a new species, also presumed to be due to Mo(V), with: $g_1 = 1.995$; $g_2 = 1.982$; $g_3 = 1.956$; $g_{av.} = 1.978$. On further addition of dithionite (10mM) both the new signal and the original one disappeared. Subsequent addition of small amounts of nitrate restored both the signals (Fig. 1*c*), and finally a larger addition of nitrate caused the new signal to disappear and the original signal to change its parameters slightly, as already discussed (see Fig. 1*d*).

Of the common valencies of molybdenum, Mo(V) and Mo(III) give e.p.r. signals, whereas Mo(VI) and Mo(IV) do not. Compounds having the metal in the lower valencies are usually unstable in the presence of air, though there are some stable Mo(V) compounds (see, e.g., Lowe *et al.*, 1976). It is clear that

molybdenum occurs in at least two different types of chemical environment in our preparations of nitrate reductase. Both sorts of molybdenum atoms undergo reduction by dithionite and reoxidation by nitrate. However, whereas the one giving the first signal is in the quinquivalent state in the untreated enzyme and is reduced to Mo(IV) by dithionite, in contrast that of the second signal must contain Mo(VI) in the untreated state, so that Mo(V) appears as a transient during both reduction by dithionite and reoxidation by nitrate. The significance of the two types of molybdenum in the preparations is not clear. Fast kinetic studies would be necessary to establish which (if either) of the signals is kinetically competent to serve as an intermediate in the enzyme-catalysed reaction. Of the six Mo(V) e.p.r. signal-giving species that are known for molybdenum-containing hydroxylases, only two are attributable to the functional enzymes. The other four are due to chemically modified forms of the enzyme, and, of these, three can occur naturally (Bray, 1976; Barber *et al.*, 1976; Dalton *et al.*, 1976; Lowe *et al.*, 1976); comparison of the modified forms with the native form has provided information about the structures of the active centres. Clearly, for nitrate reductase, the possibility of non-functional forms of the enzyme in our preparation has to be considered. Its specific activity, though lower than the best preparations obtained by Clegg (1976), was nevertheless comparable with those reported by other workers (Enoch & Lester, 1975; MacGregor *et al.*, 1974). Hence the possibility cannot be excluded that non-functional forms are present in all nitrate reductase preparations.

The *g*-values for the two molybdenum signals we have observed contrast with the very high values (*g* up to 2.09) reported by others for the metal in nitrate reductases (DerVartanian & Forget, 1975; Forget & DerVartanian, 1972). The *g*-values of molybdenum compounds are particularly sensitive to the nature of the ligand atoms (Marov *et al.*, 1972), but such high *g*-values seem to be outside the range expected with biologically possible ligands (Meriwether *et al.*, 1966). It therefore seems likely that the reported high-field features were not in fact due to molybdenum.

From our results we conclude that the molybdenum centre of nitrate reductase is much more like that of other molybdenum-containing enzymes, such as the molybdenum-containing hydroxylases and sulphite oxidase, than has had previously been supposed. The presence of a proton interacting with Mo(V) in the enzyme is particularly noteworthy. There is evidence for direct hydrogen transfer from reducing substrates to a site in the xanthine oxidase molecule interacting with molybdenum (Bray, 1971). Further, Stiefel (1973) has discussed the coupling of proton and electron transfers in molybdenum-containing enzymes generally, pointing out that nitrogen ligands of molybdenum in its lower valencies protonate more readily than do the same ligands when the metal is in the higher oxidation states.

Our work has revealed no evidence for Mo(III) in nitrate reductase preparations, nor has it revealed any evidence for nitrogen hyperfine structure. It seems that the structure attributed to this by DerVartanian & Forget (1975) could readily be explained in terms of proton interactions with multiple molybdenum species.

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