Biochem. J. (1978) **171**, 639–647
Printed in Great Britain

Electron-Paramagnetic-Resonance Studies on Nitrate Reductase from *Escherichia coli* K12

By STEPHEN P. VINCENT and ROBERT C. BRAY
School of Molecular Sciences, University of Sussex, Brighton BN1 9QJ, U.K.

(*Received* 12 *September* 1977)

Nitrate reductase was purified from anaerobically grown *Escherichia coli* K12 by a method based on the Triton X-100 extraction procedure of Clegg [(1976) *Biochem. J.* **153**, 533–541], but hydrophobic interaction chromatography was used in the final stage. E.p.r. spectra obtained from the enzyme under a variety of conditions are well resolved and were interpreted with the help of the computer-simulation procedures of Lowe [(1978) *Biochem. J.* **171**, 649–651]. Parameters for five molybdenum(V) species from the enzyme are given. The low-pH species ($g_{av.}$ 1.9827) is in pH-dependent equilibrium with the high-pH species ($g_{av.}$ 1.9762), the pK for interconversion of the species being 8.26. Of a variety of anions tested, only nitrate and nitrite formed complexes with the enzyme (in the low-pH form), giving modified molybdenum(V) e.p.r. spectra. These complexes, as well as the low-pH form of the free enzyme, showed interaction of molybdenum with a single exchangeable proton. The fifth molybdenum(V) species, sometimes detected in small amounts, appears not to be due to functional nitrate reductase. After full reduction of the enzyme with dithionite, addition of nitrate caused reoxidation of molybdenum to the quinquivalent state, in a time less than the enzyme turnover. Activity of the enzyme in the pH range 6–10 is controlled by a pK of 8.2. It is suggested that the low-pH signal-giving species is the form of the enzyme involved in the catalytic cycle. Iron–sulphur and other e.p.r. signals from the enzyme are briefly described and the enzymic reaction mechanism is discussed.

Of about six molybdenum-containing enzymes known at the present time (Bray, 1977), two, namely formate dehydrogenase and nitrate reductase, are found associated with one another in the anaerobic respiratory chain of *Escherichia coli*. This pathway has been extensively studied (Ruiz-Herrera & DeMoss, 1969; Enoch & Lester, 1974; Garland, 1975). The pathway involves successive electron transfer from formate via formate dehydrogenase to a *b*-type cytochrome, to coenzyme Q_6, to another *b*-type cytochrome and finally to the terminal electron acceptor, nitrate, this being reduced to nitrite by nitrate reductase. The pathway is only present when the organism is grown under anaerobic conditions and in the presence of nitrate.

Nitrate reductase from *E. coli* has been purified by a number of workers (Forget, 1974; Enoch & Lester, 1975; MacGregor *et al.*, 1974; Clegg, 1976; Lund & DeMoss, 1976) and has been shown to contain iron–sulphur centres in addition to molybdenum. Depending on the isolation procedure, the enzyme may or may not contain also some cytochrome *b*.

E.p.r. spectroscopy is capable of giving very detailed information about molybdenum-containing enzymes (Bray, 1975, 1977). Early e.p.r. work on nitrate reductase from *E. coli* (Dervartanian & Forget, 1975) or from related organisms (Forget & Dervartanian, 1972; Van't Riet *et al.*, 1975) yielded only poorly defined molybdenum spectra and thus provided limited information. It led, nevertheless, to considerable speculation about features in the enzyme that would have seemed improbable had an analogy been assumed with more extensively studied molybdenum enzymes such as xanthine oxidase (Bray, 1975). In contrast, as reported by Bray *et al.* (1976), nitrate reductase prepared by the method of Clegg (1976) yields well-resolved e.p.r. spectra from molybdenum(V). These showed the molybdenum centre of the enzyme to be related to that in xanthine oxidase and still more closely related to that in sulphite oxidase. We have therefore extended our investigations on nitrate reductase prepared from *E. coli* by the method of Clegg (1976) and now report more detailed studies on the molybdenum centre, together with some work on the iron–sulphur centres, as well as some kinetic studies.

Materials and Methods

Growth of Escherichia coli

Escherichia coli K12 strain A 1002 (K12Y

mel,ato⁻,fad R^c,ilv⁻,lac I⁻,met^cE⁻,rha⁻) was kindly provided by Professor P. B. Garland & Dr. R. A. Clegg (Medical Sciences Institute, University of Dundee). Growth was in the anaerobic medium described by Forget (1974), supplemented with molybdate (0.1 μM), selenite (0.1 μM) and azide (0.2mM), in a 400-litre glass fermentation vessel (K. Baker, unpublished work) at 37°C. Inoculation was by a 20-litre culture grown in the same medium. Harvesting was by a continuous centrifuge (Westfalia Seperater, Old Wolverton, Milton Keynes, Bucks., U.K.). Cells were stored in a liquid-N_2 refrigerator until required.

Enzyme enriched with ^{95}Mo was prepared from a 40 litre culture of *E. coli* grown in the standard growth medium supplemented with 3.8mg of $^{95}MoO_3$ (isotopic purity 97%).

Purification of the enzyme

The purification procedure finally adopted was based on the method of Clegg (1976), and was as follows.

All buffers, except where stated, contained 0.1mM-EDTA and 0.1mM-dithiothreitol, and all operations were carried out at 0–4°C. Frozen cells (50g wet wt.) were thawed in 50mM-potassium phosphate buffer, pH7.8, containing 5mM-$MgCl_2$ and 7.5mM-EDTA, giving 350ml of cell suspension. Cells were broken by passage of the suspension through a French pressure cell (Amicon, High Wycombe, Bucks., U.K.) at 29MPa (4000lb/in^2). The broken cells were then centrifuged for 20min at 14000$g_{av.}$. The supernatant was decanted and centrifuged for 2h at 110000$g_{av.}$. The precipitate, designated the membrane fraction, was resuspended in the minimum possible volume of 10mM-potassium phosphate buffer, pH7.8, and frozen rapidly in liquid N_2 until required for use.

The membrane fraction was thawed and sufficient 10mM-potassium phosphate buffer, pH7.8, added to give a volume of 81ml. Triton X-100 (20%, v/v) was added to a final concentration of 1%. The suspension was stirred at 20°C for 30min, then rapidly cooled, and insoluble material was removed by centrifuging at 110000$g_{av.}$. The supernatant was applied to a column (32.cm×14cm) of DEAE-cellulose and washed successively with 100ml each of the above buffer containing 0.1% Triton X-100, the same buffer containing 0.1% Triton X-100 and 0.1M-NaCl, and finally 50mM-phosphate buffer, pH7.8, containing 0.4M-NaCl and 0.1% Triton X-100. Nitrate reductase was eluted in the final step. The enzyme solution was then applied to a column (2.2cm×25cm) of octyl-Sepharose 4B-Cl [Pharmacia (G.B.), London W5 5SS, U.K.], equilibrated with the last-named buffer, but without Triton X-100. The column was washed successively with 200ml of 50mM-potassium phosphate buffer, pH7.8, containing 0.4M-NaCl and 0.1% Triton and then with 100ml of the same buffer, but containing 0.2M-NaCl, and finally with 400ml of the same buffer, but lacking NaCl. Nitrate reductase was slowly eluted in this step; it was collected, and concentrated by ultrafiltration with an Amicon PM 10 membrane. The enzyme was frozen in the form of beads in liquid N_2 and stored over liquid N_2 until required for use.

In some earlier preparations the last purification stage was replaced by chromatography on Sepharose 6B-CL. There was no essential difference in the e.p.r. spectra of enzyme prepared by the two different methods.

The enzyme appeared to be mildly air-sensitive at certain stages during the purification. Accordingly the preparation was carried out as far as possible under N_2, and in some cases in the presence of sodium dithionite (either 100mg/l of buffer, or sufficient to give a blue colour with Methyl Viologen paper, whichever was the greater). Again, there were no differences in the e.p.r. spectra of such anaerobic preparations as compared with aerobic preparations.

The yield of nitrate reductase from 50g (wet wt.) of cells was about 2000 units, corresponding to recovery of 20% of the activity of the initial extract. Specific activity was usually about 60 units/mg of protein.

Enzyme assays and analytical procedures

Nitrate reductase assays were carried out at 30°C in open test tubes in the medium described by Kemp *et al.* (1975), with reduced Benzyl Viologen as the electron donor. Nitrite was determined as described by MacGregor *et al.* (1974). Units of enzyme activity are expressed as μmol of NO_2^- produced/min. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Molybdenum and iron analyses were carried out colorimetrically as described by Hart *et al.* (1970). Cytochrome *b* concentration was determined from reduced–oxidized difference spectra, assuming $\varepsilon_{560} = 19$ litre·mmol^{-1}·cm^{-1}. Polyacrylamide-gel electrophoresis was carried out in the buffer system described by Laemmli (1970).

Ultracentrifugation was performed in 0.05M-phosphate buffer, pH7.8, containing 0.4M-NaCl and 0.1% Triton at 20°C with a Beckman model E analytical ultracentrifuge and an An-D rotor.

E.p.r. spectroscopy

Preparation of samples and recording of e.p.r. spectra was as described by Lowe *et al.* (1972). Rapid freezing was as described by Bray *et al.* (1973). The use of buffers for which large changes of apparent pH occur on freezing (Williams-Smith *et al.*, 1977) was avoided where appropriate. A dual-sample

e.p.r. cavity with manganese and diphenylpicrylhydrazyl reference standards was used for *g*-value determinations. Computer manipulation of spectra was as described by Bray *et al.* (1978). Simulation of e.p.r. spectra was as described in the following paper (Lowe, 1978).

Buffers

In addition to the phosphate buffers and 50mM-Tris/HCl, pH7.9, the following buffers were used and adjusted with NaOH: Mes (4-morpholine-ethanesulphonic acid); Pipes (1,4-piperazinediethanesulphonic acid); Mops (4-morpholinepropanesulphonic acid); Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulphonic acid; Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]: Hepps [4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid]; Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}; Ches (cyclohexylaminoethanesulphonic acid); Caps (3-cyclohexylaminopropanesulphonic acid).

Results and Discussion

Properties of purified nitrate reductase

Examination of several samples of the enzyme, prepared as described in the Materials and Methods section, by polyacrylamide-gel electrophoresis and in the ultracentrifuge indicated that it was of reasonable purity, though considerably degraded. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate showed two well-defined bands, corresponding to the α- and β-bands reported by MacGregor *et al.* (1974), the latter being split into two (Clegg, 1976). Multiple additional, but generally much weaker, bands were presumably due to proteolysis. In the ultracentrifuge a well-defined coloured peak sedimented as a single component with a sedimentation coefficient, s_{20}, of 10–16S (measured in the presence of 0.1% Triton X-100), which apparently depended on the protein concentration, and that no doubt corresponded to the enzyme (cf. Lund & DeMoss, 1976). Additional, slower-sedimenting, polydisperse material probably included products of proteolysis as well as the Triton micelle (Becher, 1967).

The highest specific activity we obtained was 95μmol of nitrite produced/min per mg of protein. This is comparable with activities reported by MacGregor *et al.* (1974) and by Enoch & Lester (1975). Although others have reported that the specific activity can be up to 3 times this value (Forget, 1974; Clegg, 1976), nevertheless, according to D. Boxer (personal communication), such activities are not regularly obtained for enzyme prepared by the method of Clegg (1976).

It may be more meaningful to express activity of the enzyme on the basis of the molybdenum content of the samples rather than on the protein content. The presence of non-functional molybdenum-containing molecules, such as those present in xanthine oxidase preparations (Bray, 1975), might thus be revealed. On this basis our samples had activity of 35 ± 5 units/nmol of molybdenum. This compares with literature activity:molybdenum ratios of 55 (Forget, 1974), 24 (Lund & DeMoss, 1976) and 19 (MacGregor *et al.*, 1974). Unfortunately not all workers report their molybdenum content. Recovered samples of the enzyme that were used for some work had activity:molybdenum ratios of about 50% of the highest values we found.

The nitrate reductase monomer appears to have a molecular weight in the region of 220000 (Clegg, 1976; Lund & DeMoss, 1976). A number of workers have reported molybdenum contents close to 1 atom/monomer (MacGregor *et al.*, 1974; Forget, 1974; Lund & DeMoss, 1976). However, nitrate reductase samples from *E. coli* (Taniguchi & Itagaki, 1960) or from other sources (Stouthamer, 1976) can have a molybdenum content as low at p.2 atom/220000 mol.wt. Our molybdenum analyses on our preparation from *E. coli* indicated 0.2–0.4 atom/220000 mol.wt. Whether this is due to some loss of the metal during purification or dialysis (Lund & DeMoss, 1976) or whether it indicates the presence of demolybdo-nitrate reductase, analogous to the well-known demolybdo form of xanthine oxidase (Bray, 1975), is not clear. MacGregor *et al.* (1974) reported that inactive enzyme appeared to be separated from active enzyme by Na_2SO_4 precipitation. Similarly, Lund & DeMoss (1976) found that the molybdenum content varied across the elution peak during chromatography on Bio-Gel.

We found about 5 atoms of iron/220000 mol.wt., which is comparable with values reported for the enzyme from *Klebsiella aërogenes*, though lower than those quoted for the enzyme from *E. coli* (Stouthamer, 1976; Lund & DeMoss, 1976).

The cytochrome *b* content of nitrate reductase preparations depends on the precise conditions that are used during purification (Clegg, 1976). Our samples contained from 0.03 to 0.4 mol of cytochrome/220000g of protein.

Obtaining from a protein e.p.r. signals that can be shown to correspond to single chemical species can provide a good criterion for protein purity. On this basis, our preparations of nitrate reductase are the best that have been reported. All the e.p.r. work described below was reproducible from one sample of the enzyme to another, except that the molybdenum(V) signal, given the name 'non-functional', seemed to vary somewhat in intensity from one sample to another.

Description of molybdenum(V) e.p.r. signals from nitrate reductase

Our preparations of nitrate reductase from *E. coli* gave under appropriate conditions five different signals which we ascribe to molybdenum(V). Each of these signals corresponded, within the limitations of our various studies, to a single chemical species. To avoid confusion the names by which we shall refer to these signals are: the 'low-pH signal', the 'high-pH signal', the 'nitrate complex signal', the 'nitrite complex signal' and the 'non-functional signal'.

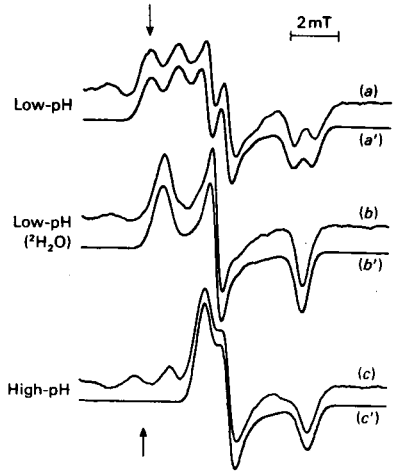

Fig. 1. *Mo(V) signals from resting oxidized nitrate reductase: (a) low-pH form, (a') simulation of (a), (b) low-pH form in ^2H_2O, (b') simulation of (b), (c) high-pH form, (c') simulation of high-pH form*

Buffers used were: (*a*) 50mM-potassium phosphate, pH7.8; (*b*) as (*a*), but in ^2H_2O (p^{2}H7.8); (*c*) 50mM-Caps (sodium salt), pH10.8. All buffers contained 0.4M-NaCl. The spectrum of (*c*) was unchanged by transfer into ^2H_2O. Conditions of e.p.r. running were: microwave frequency, 9.3GHz; microwave power, 20mW; modulation amplitude, 0.25mT; temperature, 120K. Here, and in other Figures, the arrow indicates the resonance of diphenylpicrylhydrazyl (*g* 2.0036). Simulations were performed by using the parameters shown in Table 1. Half-linewidths were taken as 0.34, 0.20 and 0.26mT for each of the three principal *g*-features in spectra (*a'*) and (*b'*), and 0.295, 0.265 and 0.33mT in spectrum (*c'*). In addition a three-line hyperfine splitting was incorporated into spectrum (*b'*), with *A*-values one-seventh those in (*a'*), to allow for the magnetic interaction of Mo(V) with ^{2}H. Some discrepancies between the experimental and simulated spectra exist. It should be noted that the simulations are for a nucleus with spin *I* 0, and the small experimental peaks corresponding to nuclei (^{95}Mo and ^{97}Mo) with spin *I* 5/2 are thus not accounted for in the simulations.

Resting oxidized enzyme gave a spectrum the exact form of which depended on the pH of the sample. Figs. 1(*a*) and 1(*c*) show the limiting spectra obtained at low and high pH values respectively. Both spectra are rhombic in form. That they correspond to single chemical species is confirmed by comparison with the computer-simulated spectra shown in Figs. 1(*a'*) and 1(*c'*). The low-pH signal has been described by Bray *et al.* (1976) and shows a splitting due to a single exchangeable proton. Transfer of the enzyme into ^2H_2O yields the unsplit rhombic signal shown in Fig. 1(*b*) and simulated in Fig. 1(*b'*). Transfer into ^2H_2O had no effect on the high-pH signal. Parameters of the signals are given in Table 1.

Transition between the high- and low-pH forms could readily be effected by altering the pH of the solution. The transition is characterized by a p*K* of 8.26 (Fig. 2). [Complications in the determination of this p*K* caused by large changes in apparent pH on freezing certain buffer solutions have been discussed by Williams-Smith *et al.* (1977).] Computer additions of spectra (not shown) confirmed that all spectra obtained at intermediate pH values could be accounted for as mixtures of the high-pH and low-pH forms of Fig. 1.

Absolute intensity of the high-pH and low-pH signals was determined for several samples of the enzyme. Intensity corresponded to about 25% of the molybdenum present in the samples. Changing the pH of a sample did not change the intensity of the signal significantly.

Many Mo(V) complexes have a co-ordination position that can be occupied either by water or by a

Table 1. *Parameters of the molybdenum(V) signals from nitrate reductase*

Spectra other than anion complexes were obtained in a dual-sample cavity using a Mn/diphenylpicrylhydrazyl standard. These *g*-values are believed to be accurate to 0.0005. A^H values are in mT. The relevant spectra are illustrated in Figs. 1(*a*), 1(*c*), 3(*a*), 3(*b*) and 3(*c*) respectively.

	Low-pH	High-pH	Nitrate complex	Nitrite complex	Non-functional
g_1	1.9989	1.9870	2.002	1.999	1.9958
g_2	1.9855	1.9805	1.986	1.985	1.9844
g_3	1.9628	1.9612	1.964	1.964	1.9560
$g_{av.}$	1.9827	1.9762	1.984	1.983	1.9787
A_1^H	1.11	—	1.35	1.56	—
A_2^H	0.84	—	1.04	1.06	—*
A_3^H	0.81	—	1.05	1.01	—
$A_{av.}^H$	0.92	—	1.15	1.21	—

* Fig. 3(*c*) suggests that there is a splitting of approx. 1mT on the g_2-feature. Whether this is a genuine feature, perhaps due to splitting by a single non-exchangeable proton, is uncertain at the present time.

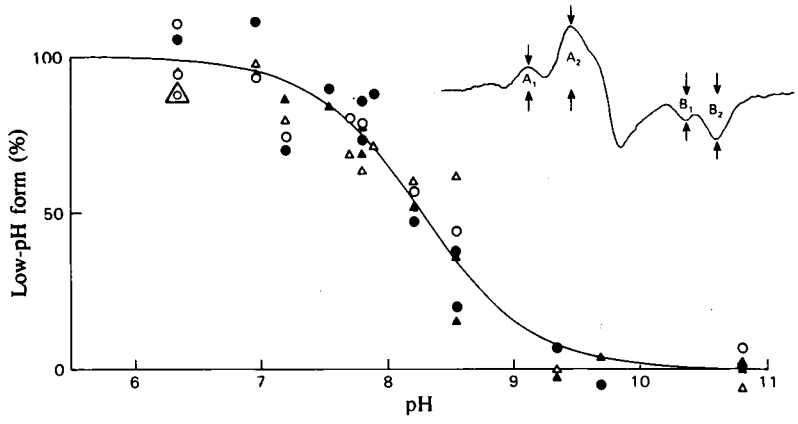

Fig. 2. *Percentage of low-pH form in the Mo(V) signal as a function of pH*
The parameters used to measure the percentage of low-pH form are shown in the inset. Values of the ratios A_1/A_2 and B_1/B_2, measured on the experimental spectra, were compared with ratios from a series of simulated spectra obtained by computer addition of known amounts of the pure high-pH to the pure low-pH spectrum. The curve is a theoretical dissociation curve for an acid with a pK of 8.26; pH values refer to 0°C. Buffers used were at a concentration of 50mM, with the addition of 0.4M-NaCl in some cases. Buffers were: Mes (sodium salt), pH6.36; Pipes (sodium salt), pH6.97; Mops (sodium salt), pH7.21; Tris/HCl, pH8.55; Tes (sodium salt), pH7.70; Hepes (sodium salt), pH7.81; Tris/HCl, pH7.90; Heppes (sodium salt), pH8.21; Tricine (sodium salt), pH8.60; Tris/HCl, pH9.34; Ches (sodium salt), pH9.69; Caps (sodium salt), pH10.80. ○, Percentage of the low-pH form measured from A_1/A_2 values; ●, as above, but in the presence of 0.4M-NaCl; △, percentage of the low-pH form measured from B_1/B_2 values; ▲, as above, but in the presence of 0.4M-NaCl.

relatively weakly bound anion (see, e.g., Cotton, 1973; Marov *et al.*, 1972). We therefore studied the effects of various anions on the Mo(V) e.p.r. spectra of nitrate reductase. Anions having no specific effect on either the low- or high-pH signals, other than the pH effects discussed by Williams-Smith *et al.* (1977), included phosphate, zwitterionic buffers related to those of Good *et al.* (1966), and also Tris, chloride and acetate. Cyanide binds to and inhibits the reduced enzyme (S. P. Vincent & R. C. Bray, unpublished work), rendering the oxidized Mo(V) signal unobservable.

We found that only two anions, nitrate and nitrite, affected the low-pH Mo(V) spectrum substantially (Fig. 3*a* and 3*b*). We have not carried out computer simulations of these spectra and it is possible that, in the spectra illustrated, the anion complexes may not be completely formed. It is clear, however, in both cases, that the A values are increased somewhat compared with the low-pH spectrum (see Table 1). Nitrite also increased the intensity by a factor of approx. 1.5, presumably by reducing Mo(VI). Although the spectra are referred to as the nitrate and nitrite complex spectra, it is not excluded that some intermediate in the enzymic reaction, e.g. NO_2, might be bound in place of the anion. Azide, a competitive inhibitor of the enzyme with respect to nitrate, also had some minor effects on the signal (spectrum not shown). The effects included minor increases in the A values as for nitrate and nitrite. Neither of the above three ligands had any effect on the high-pH spectrum, in agreement with the hypothesis (see

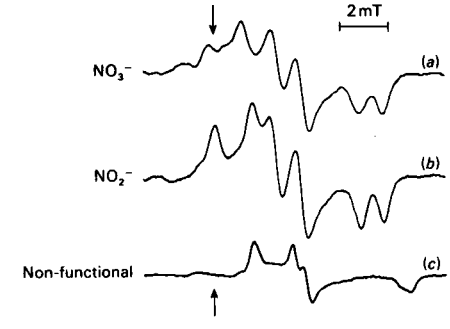

Fig. 3. *Additional Mo(V) spectra from nitrate reductase*
(*a*) Nitrate complex: conditions as in Fig. 1(*a*), but with the addition of 10mM-KNO_3. (*b*) Nitrite complex: as in Fig. 1(*a*), but with the addition of 1M-$NaNO_2$. (Reaction time was approx. 1 min.) (*c*) Non-functional signal: enzyme as in Fig. 1(*b*): reduced for 1 min with 10mM-sodium dithionite, then reoxidized for 1 min with 25mM-nitrate; the signal is shown as a difference spectrum obtained by subtracting Fig. 1(*b*) (above) from Fig. 1(*c*) of Bray *et al.* (1976).

below) that the species responsible for the low-pH spectrum is the one involved in the catalytic cycle.

Another signal obtained from some samples of the enzyme is illustrated in Fig. 3(*c*). This signal we refer to as the non-functional signal. Though its features were noted by Bray *et al.* (1976), the difference spectrum shown has not been presented previously. Parameters are given in Table 1. The origin of the signal is uncertain; we suggest that it is derived from a non-functional form of the enzyme.

To demonstrate unequivocally that the signals are from molybdenum, nitrate reductase enriched in ^{95}Mo was studied. The spectra of the high-pH form at both 9 and 35 GHz are illustrated in Fig. 4, together with a computer simulation at the lower frequency. It appears from the spectra that enrichment of the enzyme with this isotope was at least 95%. Thus all the lines observed are due to hyperfine structure from the ^{95}Mo, which has spin *I* 5/2 and so gives six-line spectra. [Only first-order terms (Lowe, 1978) were included in the simulation illustrated; however, inclusion of second-order terms did not modify the spectrum substantially, no peak being shifted by more than 0.2 mT.] The simulation was only partly satisfactory in that only the low-field part of the spectrum was accurately reproduced, and in that *g*-values significantly different from the experimental one had to be assumed. Presumably this indicates that the axes of *g* and *A* are not parallel. Comparable results (not shown) were obtained for the low-pH signal from ^{95}Mo-enriched enzyme.

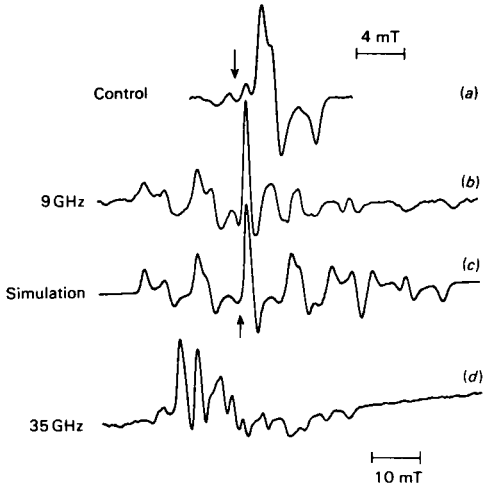

Fig. 4. *High-pH Mo(V) signal from nitrate reductase enriched in ^{95}Mo*

(*a*) Unenriched enzyme (cf. Fig. 1*c*). (*b*) ^{95}Mo-enriched enzyme in 50 mM-Ches (sodium salt), pH 9.69, containing 0.4 M-NaCl. E.p.r. running conditions were as for Fig. 1(*c*). (*c*) Simulation of (*b*). The parameters used were: $g_{1,2,3}$ 1.987, 1.9839, 1.953; $A^{Mo}_{1,2,3}$ 3.45, 4.48, 3.50 mT. (*d*) ^{95}Mo-enriched enzyme in the same buffer as (*b*), but at a microwave frequency of 35.05 GHz. The 4 mT field-scale marker and the arrows at *g* 2.0036 refer to (*a*), (*b*) and (*c*) only.

Evidence that the low-pH molybdenum(V) signal is due to functional nitrate reductase and represents a species involved in the catalytic cycle

To determine whether the molybdenum(V) e.p.r. signals from nitrate reductase are kinetically competent to serve as active enzyme species we carried out limited rapid-freezing studies. Experiments were performed by first reducing the enzyme with an excess of $Na_2S_2O_4$, which caused complete disappearance of the signals, in agreement with Bray *et al.* (1976). Reduced enzyme was then caused to react with nitrate, so putting the enzyme into a steady state. Molybdenum(V) signals were clearly observed at the shortest reaction time with nitrate that we used, which was 9 ms (Fig. 5). The large signal near to the diphenylpicrylhydrazyl marker at *g* 2.0036 will be considered below. Though the molybdenum(V) signal (Fig. 5*b*) was only partly developed in the steady state, comparison with the computer-simulated spectrum (Fig. 5*c*) shows clearly that it is due to the expected mixture of the high- and low-pH signals. We found that the enzyme was nearly two orders of magnitude less efficient in assays with dithionite alone than with Benzyl Viologen: thus the turnover time would be approx. 60 ms. The observed rapid reappearance of molybdenum(V) caused by nitrate is thus fully consistent with the high- and low-pH e.p.r. signals representing functional enzyme. On the other hand, the non-functional molybdenum(V) signal was not observed, in rapid-freezing experiments of the type illustrated in Fig. 5, until much longer reaction times (e.g. 2 min) had elapsed. Hence our conclusion is that it cannot be due to functional enzyme.

pH-dependence of nitrate reductase activity of the enzyme is shown in Fig. 6. We were unable to extend measurements to lower pH values than 6.0. Activity appears to be controlled by a p*K* of 8.2 at 30°C, with the high-pH form having a negligible activity. The closeness of the above p*K* value to the one for transformation from the low-pH protonated form of the enzyme to the high-pH non-protonated form (Fig. 2) strongly suggests that the low-pH form is the one involved in the catalytic cycle.

Other e.p.r. signals from nitrate reductase

Nitrate reductase resembles nitrogenase (Eady & Postgate, 1974) insofar as it contains both molybdenum and several different iron–sulphur centres.

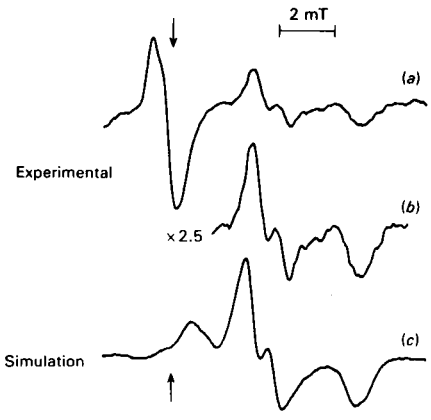

Fig. 5. *E.p.r. signals obtained in a rapid-freezing experiment*
Enzyme (in 50mM-Tris/HCl in ^2H_2O, p^{2}H7.7) was reduced for 1h with 10mM-sodium dithionite, mixed anaerobically with an equal volume of 25mM-KNO_3 in the above buffer, and then frozen 9ms later. The spectrum is shown in (*a*). (*b*) Part of (*a*) with the gain increased by a factor of 2.5 to show the Mo(V) signal. (*c*) Computer addition of 50% each of low- and high-p^{2}H experimental Mo(V) spectra from the functional enzyme. (The presence of nitrate would affect the lineshape only very slightly.)

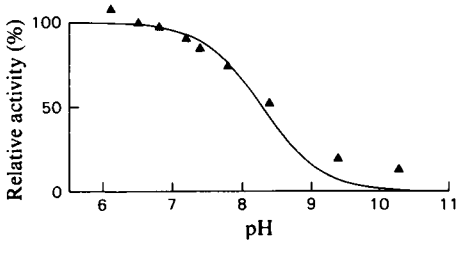

Fig. 6. *Nitrate reductase activity as a function of pH*
Activity was measured as described in the Materials and Methods section, but with the substitution for phosphate buffer, pH6.8, of the various buffers listed in Fig. 2, each at a concentration of 50mM.

Spectra observed from the reduced enzyme at low temperature are shown in Fig. 7(*a*). There appear to be two major species, a rhombic signal and a near-axial signal, whose *g*-values are indicated by the stick diagrams. The two species may be separated by power saturation (the rhombic spectrum saturates more easily at low temperatures), as well as potentiometric titration (S. P. Vincent & R. C. Bray, unpublished work).

The oxidized enzyme at low temperatures shows the rather unusual signal seen in Fig. 2(*b*). A similar signal has been reported from the oxidized nitrate reductase of *Micrococcus denitrificans* (Forget & Dervartanian, 1972). It presumably arises from an oxidized iron–sulphur centre.

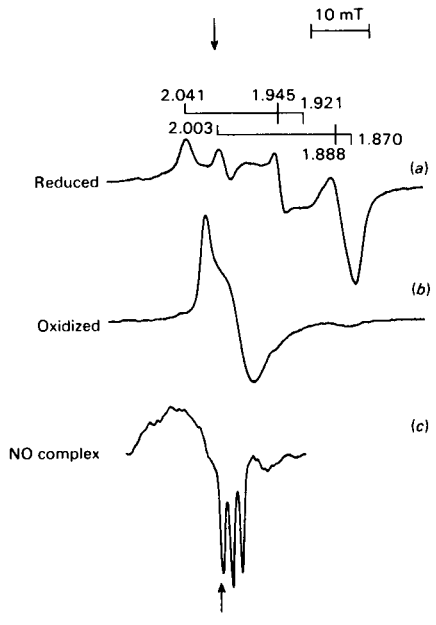

Fig. 7. *Non-molybdenum signals from nitrate reductase*
(*a*) Enzyme reduced with 5mM-dithionite for 2min. Running conditions: power 100mW, temperature 20K, modulation amplitude 1mT. Two major presumed reduced iron–sulphur signals are shown, as indicated by the stick diagrams. (*b*) Enzyme oxidized with 5mM-nitrate: power 100mW, temperature 12K, modulation amplitude 1mT. The signal is presumed to arise from an oxidized iron–sulphur centre. (*c*) Nitric oxide–haemoprotein signal: power 20mW, temperature 120K, modulation amplitude 1mT.

Our preparations contained variable amounts of cytochrome *b*. The only cytochrome-like signal seen to date has been the nitric oxide–haemoprotein signal shown in Fig. 7(*c*). Such signals are well known in other systems (see, e.g., Kon, 1968), but in our case the precise origin of the NO and whether the signal-giving species is present in functional nitrate reductase are unknown. However, we have recently observed the presence of this e.p.r. signal in *E. coli* membranes (S. P. Vincent & R. C. Bray, unpublished work); this may be significant in the mechanism of the enzyme.

Finally, a signal of unknown origin at *g* 2.006 was obtained in the rapid-freezing experiment shown in Fig. 5(*a*), but has been seen so far in one experiment only. It is presumably due to a free radical. Although $SO_2^{-\bullet}$ has not yet been excluded, one wonders whether the signal could be due to a free-radical intermediate in the enzyme reaction.

Conclusions

Nitrate reductase is clearly like hepatic sulphite oxidase (Cohen *et al.*, 1971) in having a high-pH non-protonated molybdenum(V)-containing form and a low-pH protonated one. Indeed, the pK governing conversion between the two forms is virtually identical in the two enzymes. Work on model compounds by Stiefel *et al.* (1977) suggests strongly that the proton on the low-pH form may be on a nitrogen ligand of molybdenum. Anion effects on the molybdenum(V) spectrum of nitrate reductase are reminiscent of those reported for sulphite oxidase by Kessler *et al.* (1974). However, it seems likely that pH changes on freezing (Williams-Smith *et al.*, 1977) were at least partly responsible for the effects reported by these workers. On the other hand, the uniqueness of the effects of the anions nitrate and nitrite on the e.p.r. spectrum of nitrate reductase are in keeping with the enzyme's function. Our experiments (results not shown) indicate dissociation constants for the ions from the molybdenum(V) complexes to be of the order of 4mM for the nitrate complex and 60mM for the nitrite complex. These values are considerably higher than the K_m value 0.20mM for nitrate measured in our laboratory, which confirmed the data of Garland (1975). Possibly the discrepancy might be explained by kinetic considerations.

Our results provide no evidence for molybdenum(III) in nitrate reductase, though this species has been widely postulated in relation to the enzyme. A problem concerning the mechanism of action of the enzyme is whether the NO_3^- reacts with molybdenum by a one-electron or a two-electron mechanism (see for example Durant *et al.*, 1977). Unfortunately our data are not adequate to answer this question. Oxidation–reduction-potential measurements (S. P. Vincent & R. C. Bray, unpublished work) indicate that thermodynamically either molybdenum(V) or molybdenum(IV) in the enzyme should be capable of reducing nitrate. Though oxidation of molybdenum(IV) to molybdenum(V) in the enzyme by nitrate in the rapid-freezing experiment, at a reaction time shorter than the turnover time, is consistent with a one-electron mechanism, nevertheless it is equally possible that rapid intramolecular equilibration of molybdenum and iron–sulphur in the enzyme followed a two-electron reaction (compare fast intramolecular electron transfer in xanthine oxidase: see Olson *et al.*, 1974; also Bray, 1975).

We thank Mr. K. Baker and Professor J. R. Postgate for much help with growing *E. coli*, Professor P. B. Garland and Dr. R. Clegg for supplying the cultures, Mr. L. Sarcoe for carrying out the ultracentrifuge work, and Dr. D. J. Lowe for advice and help with the computing and with the e.p.r. system. The work was supported by the Medical Research Council. S. P. V. was supported by a Medical Research Council studentship.

References

Becher, P. (1967) in *Non-Ionic Surfactants* (Shick, M. J., ed.), p. 478, Dekker, New York
Bray, R. C. (1975) *Enzymes 3rd Ed.* **12**, 299–419
Bray, R. C. (1977) in *Chemistry and Uses of Molybdenum* (Mitchell, P. C. H. & Seaman, A., eds.), pp. 271–275, Climax Molybdenum Co., London
Bray, R. C., Lowe, D. J., Capeillère-Blandin, C. & Fielden, E. M. (1973) *Biochem. Soc. Trans.* **1**, 1067–1072
Bray, R. C., Vincent, S. P., Lowe, D. J., Clegg, R. A. & Garland, P. B. (1976) *Biochem. J.* **155**, 201–203
Bray, R. C., Barber, M. J. & Lowe, D. T. (1978) *Biochem. J.* **171**, 653–658
Clegg, R. A. (1976) *Biochem. J.* **153**, 533–541
Cohen, H. J., Fridovich, I. & Rajagopalan, K. V. (1971) *J. Biol. Chem.* **246**, 374–382
Cotton, F. A. (1973) in *The Chemistry and Uses of Molybdenum* (Mitchell, P. C. H., ed.), pp. 6–10, Climax Molybdenum Co., London
Dervartanian, D. V. & Forget, P. (1975) *Biochim. Biophys. Acta* **379**, 76–80
Durant, R., Garner, C. D., Hyde, M. R., Mabbs, F. E., Parsons, J. R. & Richens, D. (1977) in *Chemistry and Uses of Molybdenum* (Mitchell, P. C. H. & Seaman, A., eds.), pp. 234–236, Climax Molybdenum Co., London
Eady, R. & Postgate, J. R. (1974) *Nature (London)* **249**, 805–810
Enoch, H. G. & Lester, R. L. (1974) *Biochem. Biophys. Res. Commun.* **61**, 1234–1240
Enoch, H. G. & Lester, R. L. (1975) *J. Biol. Chem.* **250**, 6693–6705
Forget, P. (1974) *Eur. J. Biochem.* **42**, 325–332
Forget, P. & Dervartanian, D. V. (1972) *Biochim. Biophys. Acta* **256**, 600–606
Garland, P. B. (1975) *Biochem. J.* **152**, 547–559
Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S. & Singh, R. M. M. (1966) *Biochemistry* **5**, 467–477
Hart, L. I., McGartoll, M. A., Chapman, H. R. & Bray, R. C. (1970) *Biochem. J.* **116**, 851–864
Kemp, M. B., Haddock, B. A. & Garland, P. B. (1975) *Biochem. J.* **148**, 329–334
Kessler, D. L., Johnson, J. L., Cohen, H. J. & Rajagopalan, K. U. (1974) *Biochim. Biophys. Acta* **334**, 86–96
Kon, H. (1968) *J. Biol. Chem.* **243**, 4350–4357
Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
Lowe, D. J. (1978) *Biochem. J.* **171**, 649–651
Lowe, D. J., Lynden-Bell, R. M. & Bray, R. C. (1972) *Biochem. J.* **130**, 239–249
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Lund, K. & DeMoss, J. A. (1976) *J. Biol. Chem.* **251**, 2207–2216
MacGregor, C. H., Schnaitman, C. A., Normansell, D. E. & Hodgkins, M. G. (1974) *J. Biol. Chem.* **249**, 5321–5327

Marov, N. I., Belgaeva, U. K., Dubrov, Y. N. & Ermakov, A. N. (1972) *Russ. J. Inorg. Chem.* **17**, 1561–1565

Olson, J. S., Ballou, D. P., Palmer, G. & Massey, V. (1974) *J. Biol. Chem.* **249**, 4363–4382

Ruiz-Herrera, J. & DeMoss, J. A. (1969) *J. Bacteriol.* **99**, 720–729

Stiefel, E. I., Newton, W. E. & Pariyadath, N. (1977) in *Chemistry and Uses of Molybdenum* (Mitchell, P. C. H. & Seaman, A., eds.), pp. 265–270, Climax Molybdenum Co., London

Stouthamer, A. H. (1976) *Adv. Microbial Physiol.* **14**, 315–375

Taniguchi, S. & Itagaki, E. (1960) *Biochim. Biophys. Acta* **44**, 263–279

Van't Riet, Van Ee, J. H., Wever, R., Van Gelder, B. F. & Planta, R. J. (1975) *Biochim. Biophys. Acta* **405**, 306–317

Williams-Smith, D. L., Bray, R. C., Barber, M. J., Tsopanakis, A. D. & Vincent, S. P. (1977) *Biochem. J.* **167**, 593–600