Purification and characterization of the assimilatory nitrate reductase of *Azotobacter vinelandii*

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1. A soluble reduced Methyl Viologen-dependent assimilatory nitrate reductase from *Azotobacter vinelandii* strain UW136 grown aerobically on nitrate was purified to homogeneity by the criteria of nitrate reductase activity staining, and coincidence of a Coomassie Blue-staining protein band on polyacrylamide gels run under non-denaturing conditions. The specific activity was 3 μmol of NO_2^- formed/min per mg of protein. 2. Gel filtration on Superose-12 and SDS/PAGE showed that the enzyme had an M_r of 105000 and was monomeric. The enzyme contained 1 Mo atom, 4 Fe atoms and 4 acid-labile sulphide atoms per molecule; no evidence for the presence of cytochrome or FAD was found. 3. Mo was present in a molybdenum cofactor, which on extraction was capable of activating apo-(*nit-1*) nitrate reductase present in crude extracts of *nit-1* mutants of *Neurospora crassa*. 4. As isolated, the enzyme had e.p.r. signals assigned to Mo(V) with g-values $g_1 = 2.023$; $g_2 = 1.998$; $g_3 = 1.993$ and with $g_{av.} = 2.004$ indicating an unusual environment of Mo in this enzyme. 5. Reduction with $S_2O_4^{2-}$ bleached the e.p.r. signals which, on reoxidation after the addition of NO_3^{2-} to initiate enzyme turnover, exhibited at short times Mo(V) signals similar to those of dissimilatory nitrate reductases, with $g_1 = 1.998$; $g_2 = 1.989$; $g_3 = 1.981$ and $g_{av.} = 1.989$. Prolonged incubation subsequently gave a mixture of both e.p.r. species. 6. Neither NADH nor NADPH was effective as an electron donor, but reduced Methyl Viologen (apparent K_m 988 μM) and reduced Bromophenol Blue (apparent K_m 158 μM) were effective. With these donors the apparent K_m values for nitrate were 70 μM and 217 μM respectively.

INTRODUCTION

The ability to use nitrate as a source of nitrogen for growth is widespread amongst plants, fungi and bacteria. Assimilatory nitrate reductase catalyses the first step in the metabolism of nitrate, the two-electron reduction of nitrate to nitrite. The enzymes from higher plants, fungi and green algae have been extensively characterized. The enzymes are complex, having several types of subunit, FAD, cytochrome *b* and a molybdenum-containing cofactor (Moco) as redox centres (for review see Solomonson and McCreery, 1986).

The dissimilatory nitrate reductases from bacteria, those enzymes involved in the use of nitrate as an alternative electron acceptor to O_2, have been characterized thoroughly. These respiratory enzymes are usually integral proteins of the cytoplasmic membrane, have a complex subunit composition and have Moco and Fe-S centres as prosthetic groups (Van't Riet et al., 1975; Carlson et al., 1982; Seki et al., 1987). In some organisms, depending on the methods used to solubilize the enzyme, cytochrome *b* is also present (DeMoss et al., 1981; Chaudhry and MacGregor, 1983a,b; Ishizuka et al., 1984; Craske and Ferguson, 1986). Unusually, in the case of some *Rhodobacter* species this type of enzyme is soluble and periplasmic (see McEwan et al., 1984).

In contrast, the assimilatory nitrate reductases of prokaryotes have not been extensively studied; in the limited range of organisms investigated these enzymes are usually particulate, e.g. *Anabaena cylindrica* (Hattori and Myers, 1967), *Plectonema boryanum* (Mikami and Ida, 1984) and *Ectothiorhodospira shaposknikovii* (Malofeeva et al., 1975). The solublized enzyme of *Plectonema boryanum* was found to be monomeric with an M_r of 85000 and contained 1 Mo atom, 4 Fe and 4 S^{2-} atoms. The first homogeneous preparation of a soluble bacterial (presumed) assimilatory nitrate reductase was obtained from the phototroph *Rhodobacter capsulans* strain AD2 (Alef and Klemme, 1979). This enzyme was a dimer of M_r 180000 contained 1 Mo atom and, unexpectedly for a prokaryotic assimilatory enzyme, had spectra characteristic of cytochrome *b*. However, it was subsequently shown (Alef et al., 1985) that this strain possesses two pathways of nitrate reduction and has a periplasmic dissimilatory enzyme which predominates in late stages of growth; consequently it is no longer clear that the enzyme purified by Alef and Klemme (1979) is the assimilatory enzyme.

The assimilatory nitrate reductase from *Azotobacter vinelandii* has not been studied in detail, but has been shown to be particulate and NAD(P)H-dependent. The activity could be solubilized by treatment with Triton X-100, and when solubilized, the enzyme was shown to be active with flavodoxin or ferredoxin as electron donor but not with NAD(P)H (Taniguchi and Ohmachi, 1960; Bothe and Häger, 1981). In contrast, the closely related organism *Azotobacter chroococcum* has, under some conditions, a soluble assimilatory nitrate reductase which has been partially purified and shown to have an M_r of 100000, but was not further characterized (Guerrero et al., 1973).

In the present paper we report the purification and characterization of a soluble form of the assimilatory nitrate reductase of *A. vinelandii* strain UW136 grown on nitrate. The enzyme is monomeric, with an M_r of 105000, contains 1 Mo atom in a Moco centre which exhibits e.p.r. signals assignable to Mo(V) in an unusual environment, and also contains 4 Fe and 4 S^{2-} atoms per molecule.

MATERIALS AND METHODS

Organism and growth conditions

The stain used was *A. vinelandii* UW136 (Bishop et al., 1977). Cultures were grown aerobically in a 200-litre pilot plant fermenter (New Brunswick Scientific) at a maintained dissolved

Abbreviation used: Moco, molybdenum cofactor.

oxygen concentration of 10% of air saturation in modified Burk's medium (Strandberg and Wilson, 1968) supplemented with nitrate (10 mM) as a nitrogen source.

Harvesting of cells and preparation of crude extract

The cells were harvested during the exponential growth phase, using an SA7 Westfalia multidisc stack centrifuge. Crude extracts were obtained by resuspending the cell paste (approx. 1.5 kg) in 1 litre of 50 mM Hepes/KOH buffer, pH 8, containing 0.1 g/l dithiothreitol, disrupting it in a precooled Manton–Gaulin homogenizer at 27 MPa and centrifuging at 33000 *g* at 10 °C for 120 min. The clear brown supernatant was frozen in liquid nitrogen.

Protein determination

Protein concentration was determined using the Biuret method (Gornall et al., 1949) for solutions of high protein concentration, or, for lower concentrations, the Folin phenol reagent was used (Lowry et al., 1951). BSA was used as a standard. The concentration of highly purified nitrate reductase was also measured from its absorbance at 280 nm using the relationship that $A_{280} = 1$ corresponds to 0.66 mg of protein/ml, determined by the Folin method.

Assay of nitrate reductase activity

The standard assay of nitrate reductase was carried at 30 °C, using reduced Methyl Viologen as the electron donor, by a modification of the method described by MacGregor (1978). After preliminary experiments designed to prolong the linearity of product formation with time, the assay was modified by the inclusion of cyanate (1 mM) (see below). Nitrite was estimated as described by Low and Evans (1964). Nitrate reductase specific activity is expressed as nmol of NO_2^- formed/min per mg of protein.

Distribution of nitrate reductase activity

The experiments were carried out as described by Bothe and Häger (1981). Frozen cells were thawed, resuspended in an equal volume of 50 mM Hepes buffer, pH 8.0, containing 0.1 g/l dithiothreitol and disrupted through a French press at 35.4 MPa (5000 ft-lb) at 5 °C. The resulting crude extract was centrifuged at 3000 *g* for 20 min, and the supernatant from this first step was centrifuged at 39000 *g* for 20 min and at 150000 *g* for 120 min in a Pegasus ultracentrifuge using a 6 × 5.5 ml swing-out rotor ($r_{av.}$ 8.18 cm) at 5 °C. Nitrate reductase activity was measured in the supernatants and the resuspended sedimented pellets.

Gel electrophoresis

SDS/PAGE was carried out as described by Weber and Osborn (1969). Native gel electrophoresis was carried out in 10% (w/v) polyacrylamide gels in Tris/HCl buffer, pH 8.8, by the method of Hedrick and Smith (1968). Nitrate reductase activity in the gel was detected essentially as described by Hucklesby and Hazeman (1973).

M_r and subunit composition

The native M_r of nitrate reductase was determined using an f.p.l.c. Superose-12 gel-filtration column (1 cm × 30 cm), equilibrated with 50 mM Tris/HCl buffer, pH 8.0, containing 0.1 M NaCl and calibrated with standard proteins of known M_r. Nitrate reductase (0.2 ml) was loaded on to the column and elution was detected by enzymic assay of fractions from the column; nitrate reductase activity correlated with the symmetrical elution peak detected by u.v. absorbance at 280 nm. Elution volumes (V_e) of standard proteins and nitrate reductase were measured and the void volume (V_0) was calculated from the elution volume of Blue Dextran (M_r 2000000). The M_r of nitrate reductase was determined from the graph of V_e/V_0 ratio against log M_r of the standards proteins.

The M_r of the polypeptides of nitrate reductase were estimated by SDS/PAGE using marker proteins of known M_r (Weber and Osborn, 1969).

Iron analysis

Protein samples were heated with 0.5 ml 1 M HCl to 80 °C for 10 min, and precipitated protein was removed by centrifuging (11000 *g*) for 2 min. The clear supernatants were used to determine iron as the bathophenanthroline complex as described by Eady et al. (1987).

Determination of molybdenum

Molybdenum was determined on wet-ashed samples of nitrate reductase (160 μg) by both the colorimetric toluenedithiol method as described by Eady et al. (1972) and differential pulse polarography of the 5-sulpho-7-nitro-8-hydroxyquinoline complex (Metrohm Application Bulletin No. 146e) using a Metrohm 646 VA processor and 647 VA stand. Good agreement between these methods was obtained. In the polarographic method, the digested enzyme (0.05 ml to 0.2 ml of the wet-ashed material) was added to 20 ml of distilled water followed by 2 ml of the complexing reagent (2 mM final concn.). After analysis of the digest, three sequential additions of a standard Mo solution (50 ng) to the vessel gave an internal calibration curve with a slope of 641 ng of Mo/μA.

Determination of selenium

Selenium was determined on wet-ashed samples of nitrate reductase (500 μg) by inverse voltammetry of a Cu(EDTA) complex using differential pulse polarography (Metrohm Application Bulletin No. 117e) and a Metrohm 646 VA processor and a 647 VA stand. After analysis of the digest, an internal calibration curve obtained from the sequential addition of 100 ng of a selenium standard gave a slope of 24.5 μg/μA. Since the selenium content of the nitrate reductase digest was below the limits of detection, a recovery experiment was performed which indicated that 10 ng of selenium added to the digest was detectable at 99.5% of the expected value; this corresponds to less than 0.05 g-atom of Se/mol of nitrate reductase.

Acid-labile sulphide

This was determined colorimetrically by the method of King and Morris (1967). A range of volumes of enzyme solution were assayed and the results extrapolated to infinite dilution.

U.v.–visible spectra

Spectra were recorded under Ar in a 1-cm light-path quartz cuvette capped with a rubber closure, using a Lambda 5 u.v.–visible spectrophotometer. Additions of solutions of dithionite

and nitrate were made using a long-needled Hamilton syringe (5 μl) through a rubber sealing cap.

E.p.r. measurements

E.p.r. spectra were recorded on a Bruker ESP 300 spectrometer fitted with a Bruker ERP 35M n.m.r. Gaussmeter and a Marconi Instruments 2440 microwave frequency counter. Integrated intensities were obtained by comparison with aqueous Cu(EDTA) with a *g*-value correction applied as described by Aasa and Vänngard (1975).

Samples of nitrate reductase in deuterated buffer were prepared using a P6DG desalting column (1 cm × 1 cm), equilibrated with the deuterated buffer. The ^2H content of samples prepared in this way was determined by ^1H n.m.r. spectroscopy. An equal volume of [^2H]acetone was added to the nitrate reductase sample in ^2H_2O-containing buffer and the ^1H n.m.r. spectrum measured. The increase in intensity of the ^1H_2O signal of the nitrate reductase sample was compared with that of the acetone. Subsequent additions (5, 10, 20 and 40 μl) of distilled water were made, and the increase in intensity of the ^1H_2O signals for each addition was recorded. A graph of the volume of water added against the increase in signal intensity allowed the amount of ^1H_2O present initially in the nitrate reductase solution to be calculated.

Reconstitution experiments of Mo cofactor with *nit-1* extract of *Neurospora crassa*

The preparation of Moco from nitrate reductase and the reconstitution of the apo-(nitrate reductase) present in crude extracts (of *nit-1* mutants) were carried out as described by Hawkes and Bray (1984) in an anaerobic glove box under N_2 containing less than 1 p.p.m. of O_2. Reconstituted NADPH-dependent nitrate reductase activity was measured under aerobic conditions using the method of Hawkes and Bray (1984). To establish the reproducibility of these methods in our hands, Moco was also extracted from purified xanthine oxidase and assayed using the reconsitution assay.

Isolation and fluorescence spectroscopy of form B of molybdopterin

Form B of molybdopterin was obtained by a method described by Ventom et al. (1988) and the fluorescence emission spectra were recorded on a Perkin–Elmer LS-3 spex fluorolog 2 fluorescence spectrometer, with an excitation wavelength of 370 nm.

RESULTS AND DISCUSSION

Distribution of nitrate reductase activity

Previous reports on the cellular location of the assimilatory nitrate reductase of *A. vinelandii* strain O (Taniguchi and Ohmachi, 1960; Bothe and Häger, 1981) indicated that it was particulate. Most of the activity sedimented after centrifugation for 20 min at 14000 *g*, and no activity was detectable in the supernatant after centrifugation at 110000 *g*. In our preparations, the resuspended pellet obtained by centrifugation of crude extract for 20 min at 39000 *g* contained only 20% of the starting units of nitrate reductase activity. Subsequently when the supernatant from this step was centrifuged at 150000 *g* for 120 min, 90% of the activity remained in the supernatant (results not presented).

The reasons for the differences in solubility compared with the earlier studies are not known. The imposition of a constant dissolved oxygen concentration throughout growth of cultures in the present study or small differences in the conditions under which the cells were disrupted may have been responsible. It has also been reported that repeated subculture of *A. chroococcum* in liquid media with nitrate as nitrogen source resulted in the normally soluble nitrate reductase activity being incorporated into the cytoplasmic membrane (Vila et al., 1977).

Properties of nitrate reductase activity in crude extracts

The specific activities of nitrate reductase in crude extracts of *A. vinelandii* were in the range of 1–2 nmol of NO_2^-- formed/min per mg of protein for different preparations. These activities were comparable with those reported by Bothe and Häger (1981) and are 5–10-fold lower than that observed in extracts of higher plants, fungi and algae. In assays where Methyl Viologen was omitted, the rate of nitrite formation was one-third of that observed under standard assay conditions. Preliminary experiments indicated that flavodoxin present in crude extracts was a potential electron donor capable of replacing Methyl Viologen. The activity of crude extracts of *A. vinelandii* increased approximately 2-fold on incubation at room temperature for 6 h, an increase that did not occur at 5 °C. Similar observations have been made with crude extracts of *A. chroococcum* (Guerrero et al., 1973), but the reasons for this increase in activity are not clear.

When nitrate reductase activity of crude extracts was measured, nitrite formation was proportional to time only for the first 2–3 min of the assay. After this time a dramatic decrease in activity was observed. Similar behaviour has been reported for nitrate reductase activity assayed in crude extracts of *A. chroococcum* (Guerrero et al., 1973), and these workers showed that addition of the competitive inhibitor cyanate (1 mM) extended the linear phase. In addition, when cyanate was added to assays in which the rate of nitrite formation was no longer linear, the initial rate was restored. These effects have been attributed to the ability of cyanate to prevent dithionite (present in the assay as an electron donor) from inactivating nitrate reductase. Similar observations have been made on extracts of *Plectonema boryanum* (Mikami and Ida, 1984), and cyanate stimulated the activity of nitrate reductase of *Rhodopseudomonas capsulatas* 2-fold (Alef and Klemme, 1979). When cyanate (1 mM) was included in our assay system, the reaction rate was constant for at least 15 min (up to 700 nmol of NO_2^- formed) and was therefore subsequently included in the assay mixture.

Purification of nitrate reductase

Unless stated to the contrary, purification was at room temperature in Tris/HCl buffer, pH 8.0, containing 0.1 g/l dithiothreitol. The elution pattern of coloured proteins was followed by using an LKB uvicord s monitoring system, fitted with a 365 nm filter. Gel-filtration, ion-exchange and f.p.l.c. materials were obtained from Pharmacia (U.K.). The purification procedure is summarized in Table 1.

Step 1: preparation of crude extracts

The crude extracts were prepared as in the Materials and methods section.

Step 2: DEAE-Sephacel chromatography of crude extract

Crude extract (approx. 3 l) was divided in three parts which were loaded on to three separate columns (15 cm × 5 cm) of

DEAE-Sephacal previously equilibrated with buffer. The columns were each washed with 200 ml of buffer which eluted membrane and particulate material. Adsorbed proteins were then eluted with a linear NaCl gradient 0–0.4 M NaCl in a total volume of 2 l. The nitrate reductase activity was eluted as a single band within an NaCl concentration range of 0.18–0.22 M NaCl.

Step 3: $(NH_4)_2SO_4$ precipitation

The active fractions from the three columns of step 2 were pooled, and solid $(NH_4)_2SO_4$ was added to 30% (w/v). The precipitated protein was collected by centrifugation at 15000 *g* (14000 rev./min) for 10 min at 10 °C. The pellet containing nitrate reductase activity was resuspended in a minimum volume of standard buffer and then centrifuged to remove any insoluble material.

Step 4: gel filtration in Sephacryl S-200

The concentrated nitrate reductase from step 3 was chromatographed in two batches on a column (45 cm × 8 cm) of Sephacryl S-200 equilibrated with buffer containing 50 mM NaCl. The protein separated into three major coloured bands, a fast running reddish–brown band, a central dark-brown band and a slower-running yellowish–brown band. Nitrate reductase activity was collected as a single peak from the middle dark-brown band. This material from both columns was combined.

Step 5: f.p.l.c. gradient elution on Q-Sepharose HP

The active fractions from the previous step were pooled, filtered using a ministart N filter (0.45 μm) (Sartorius GMbH) and loaded in two batches using a 50 ml superloop on to a column (2.6 cm × 10 cm) of Q-Sepharose Hp equilibrated with 20 mM Tris/HCl buffer, pH 8.0, containing 50 mM NaCl (buffer A). The column was then developed at a flow rate of 8 ml/min with a linear NaCl gradient, extending from 30% (90 mM NaCl) to 65% (195 mM NaCl) of buffer B (20 mM Tris/HCl, pH 8.0, containing 0.3 M NaCl) in a total volume of 800 ml. Nitrate reductase activity was eluted between 55% and 60% buffer B (165–180 mM NaCl) as a single peak. The elution of proteins was monitored at 280 nm.

Step 6: f.p.l.c. hydrophobic interaction chromatography on phenyl-Superose

The active fractions from step 5 were combined and solid $(NH_4)_2SO_4$ was added to give a final concentration of 1 M. The solution was centrifuged at 22000 *g* (10000 rev./min), to remove insoluble material and loaded on to a column (5 cm × 5 mm) of phenyl-Superose, equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 1 M $(NH_4)_2SO_4$. The column was developed at 0.5 ml/min with a linear descending gradient of 0.5 M $(NH_4)_2SO_4$ to 50 mM potassium phosphate buffer, pH 7.0, alone in a total volume of 24 ml. Nitrate reductase activity was eluted as the last peak off the column. A typical elution profile is shown in Figure 1 and the purification procedure is summarized in Table 1.

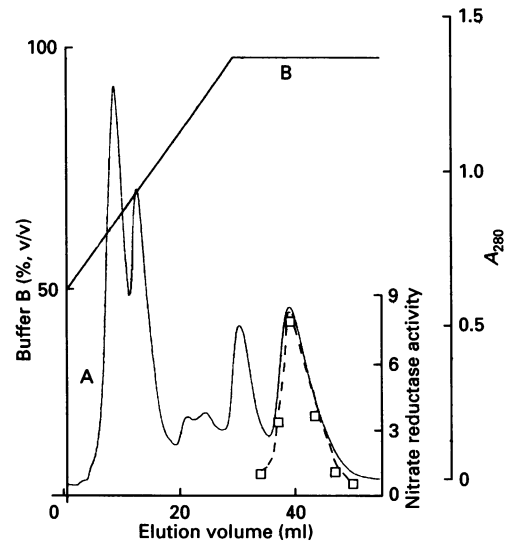

Figure 1 F.p.l.c. elution profile of nitrate reductase activity from a phenyl-Superose hydrophobic interaction column

The profile corresponds to step 6 of the purification procedure. A, eluted protein; □, nitrate reductase activity; B, the percentage of buffer B.

Assay conditions and electron donors for purified nitrate reductase

The optimum pH for nitrate reductase activity in the reduced Methyl Viologen assay used routinely was pH 8.0, and the apparent K_m values for nitrate and Methyl Viologen were 75 μM and 1 mM respectively. In the e.p.r. experiments of the enzyme during turnover, dithionite was used as the primary electron donor in the absence of Methyl Viologen. As has been reported for nitrate reductase from higher plants (Hoarau et al., 1986;

Table 1 Purification of nitrate reductase from *Azotobacter vinelandii* UW136

Step no.	Fraction	Volume (ml)	[Protein] (mg/ml)	Total activity (kunits)	Specific activity (nmol of NO_2^- formed/min per mg of protein)	Yield (%)
1	Supernatant from crude extract after centrifugation at 33000 *g*	3150	38.5	125	1	100
2	DEAE-Sephacel combined active fractions	500	19.6	310	32	248
3	$(NH_4)_2SO_4$, 0–30% (w/v) precipitate	130	54.6	280	39	224
4	Sephacyl S-200 combined active fractions	360	9.6	235	68	188
5	F.p.l.c.: Q-Sepharose active fractions	46	0.7	72	2236	57
6	F.p.l.c.: hydrophobic interaction chromatography on phenyl-Superose	6	0.2	37	30800	29

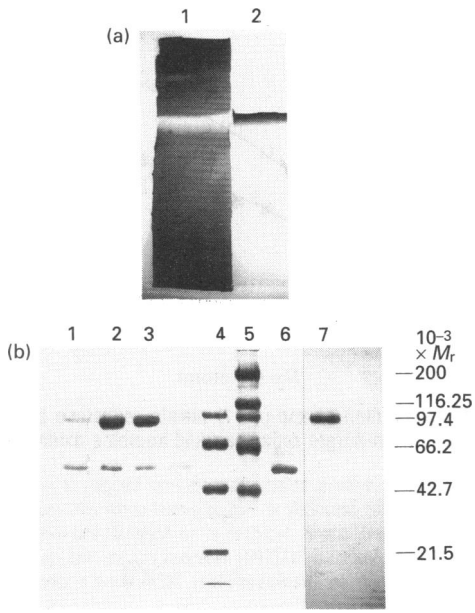

Figure 2 Gel electrophoresis of purified nitratre reductase

(**a**) Native gel electrophoresis: lane 1, nitrate reductase activity stain; lane 2, protein stained with Coomassie Blue. (**b**) SDS/PAGE of purified nitrate reductase fractions in a 10% acrylamide gel. Lanes 1–3, fractions obtained from an f.p.l.c. phenyl-Superose column (step 6); lane 6, nitrate reductase inactivated by storage at room temperature for 48 h; lane 7, material obtained when active enzyme from step 6 was subjected to f.p.l.c. gel filtration on Superose-12. Lanes 4 and 5 are M_r markers: myosin (200000); β-galactosidase (116250); phosphorylase *b* (97400); BSA (66200); ovalbumin (42700); soya bean trypsin inhibitor (21500).

Campbell, 1986), reduced Bromophenol Blue was also an effective electron donor, and under these conditions the apparent K_m values were 217 μM and 158 μM for nitrate and Bromophenol Blue respectively. NADH and NADPH were tested as potential electron donors to the purified enzyme and were found not to support nitrate reductase activity, as was observed with the partially purified solubilized enzyme (Bothe and Häger, 1981).

Stability

The purified enzyme was stable at room temperature for up to 24 h in 50 mM potassium phosphate buffer, pH 7.0, and for several months at −20 °C. The activity was lost on incubation at room temperature for 48 h, and loss of activity was associated with changes in the mobility of the protein on SDS/PAGE (see below).

M_r and subunit composition

Native gel electrophoresis of the purified enzyme from Step 6 produced a single protein band when stained with Coomassie Blue (Figure 2a, lane 2), which corresponded to a band of nitrate reductase activity when a separate section of the gel was immersed in an activity stain reaction mixture which bleached Tetrazolium Red in the presence of nitrate (Figure 2a, lane 1).

Electrophoresis of the purified enzyme from step 6 after denaturation at 100 °C in the presence of dithiothreitol in 10% polyacrylamide gels containing SDS showed a major component around M_r 100000, and an additional band of M_r approx. 50000, which was present in variable proportions across the elution peak (Figure 2b, lanes 1–3). Some preparations showed only the 50000-M_r component (Figure 2b, lane 6). Although no systematic studies were undertaken, this observation was made most frequently on protein preparations that had been stored at room temperature and had lost activity. When nitrate reductase from step 6 was subjected to gel filtration on an analytical f.p.l.c. column of Superose-12, an M_r of 105000 was determined (Figure 3), and when this material was analysed by SDS/PAGE only the 100000-M_r componment was detected (Figure 2b, lane 7). The removal of the 50000-M_r species by high-resolution gel filtration resulted in an increase in specific activity from 18000 to 35000 nmol of NO_2^-/min per mg of protein.

Our interpretation of these data is that the native M_r of the nitrate reductase corresponds to the larger component seen on SDS/PAGE and that the protein is therefore monomeric. The origin of the 50000-M_r component, which is a variable proportion of the total protein and can be removed by gel filtration, presumably arises as a consequence of cleavage of the larger polypeptide. Such cleavage could be spontaneous, or the result of limited proteolysis, forming two fragments of approximately equal size. Nitrate reductases from a number of sources have been shown to be susceptible to interdomain proteolytic cleavage and they also show changes in size and properties during the course of purification [see Howard and Solomonson (1982), Solomonson et al. (1986), Brown et al. (1981), MacGregor (1975) and Kubo et al. (1988)].

Owing to the limited amount of material available, and the losses consequent on further manipulation, most of the properties described here were obtained with the combined protein purified to step 6, i.e specific activity of 30000 nmol of NO_2^-/min per of protein, and with SDS protein profiles corresponding to lanes 2 and 3 of Figure 2(b).

Metal and acid-labile sulphide content

Analysis of the iron, molybdenum and acid-labile sulphide content of several preparations of nitrate reductase as described in the Materials and methods section gave average values of 0.89 ± 0.01 g-atom of Mo, 4.13 ± 0.19 g-atoms of Fe and 4.02 ± 0.07 mol of acid-labile sulphide per mol of enzyme, based on an M_r of 105000. Since these values were not significantly different for preparations ranging in specific activity from 10000 to 27000 nmol of NO_2^-/min per mg of protein, loss of activity was not necessarily associated with the loss of metals or acid-labile sulphide.

U.v.–visible absorption spectra

The purified enzyme was reddish-brown in colour and had a broad absorption spectrum in the visible region as shown in Figure 4. In addition to the absorbance maximum due to protein at 278 nm, there is a shoulder at 325 nm and broad bands centred around 412 nm and 540 nm. The absorption bands in the visible region decreased in intensity after the addition of dithionite (2 mM) and were restored to the original amplitude by addition of nitrate (5 mM). Features characteristic of cytochrome *b* were not observed in the spectra of the protein either as isolated or in the dithionite-reduced state. In addition, no haem was detectable when preparations were analysed using the pyridine haemochrome assay as described by Appleby and Morton, (1959).

Identification of molybdenum cofactor

Other than nitrogenase, all molybdoenzymes including nitrate reductases have a molybdenum cofactor in which molybdenum

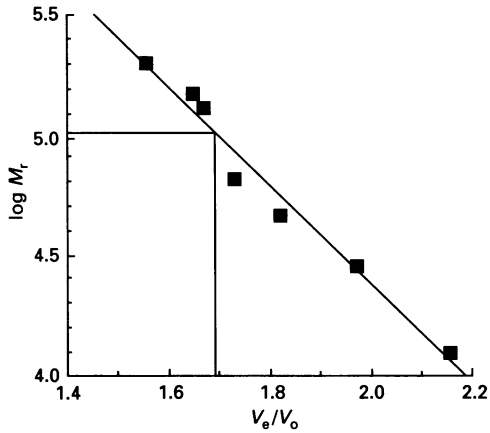

Figure 3 Determination of the native M_r of nitrate reductase by f.p.l.c. gel filtration on Superose-12

A column (1 cm × 30 cm) of Superose-12 was equilibrated with 50 mM Tris/HCl buffer, pH 8.0, containing 0.1 M NaCl. The column was operated at a flow rate of 0.5 ml/min, and 0.2 ml of nitrate reductase of standard proteins was loaded. M_r calibration standards were: cytochrome *c* (12400); carbonic anhydrase (28000); ovalbumin (47000); BSA (66000); lactate dehydrogenase (130000); alcohol dehydrogenase (150000); β-amylase (200000).

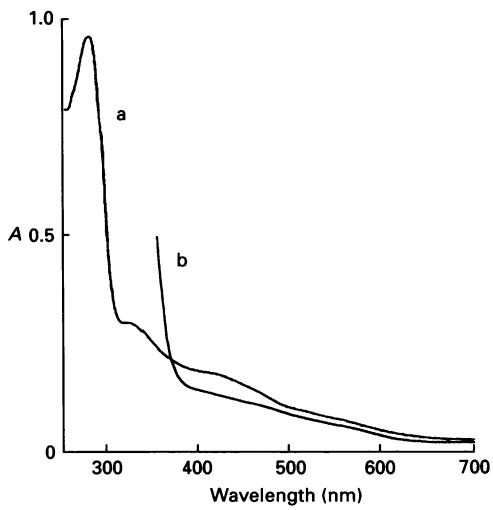

Figure 4 U.v.–visible absorption spectrum of nitrate reductase

Solutions of both oxidized enzyme as isolated (a) and enzyme reduced with an excess of $Na_2S_2O_4$ (b) were obtained in 50 mM phosphate buffer, pH 7.0, at a protein concentration of 0.6 mg/ml.

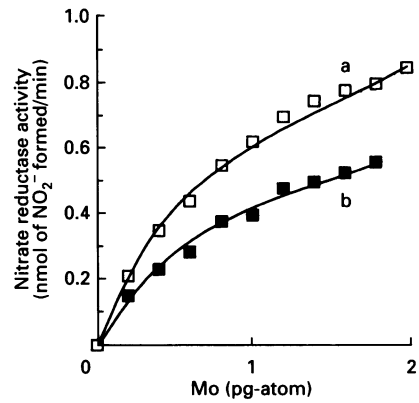

Figure 5 Reconstitution of apo-(*nit-1*) nitrate reductase by molybdenum cofactor isolated from nitrate reductase and xanthine oxidase

Increasing amounts of molybdenum cofactor liberated from samples of xanthine oxidase and nitrate reductase were added separately to a crude extract of the *nit-1* mutant of *Neurospora crassa* under anaerobic conditions as described in the Materials and methods section. After 24 h incubation, the reconstituted NAD(P)H-dependent nitrate reductase activity of the incubation mixtures were measured (Hawkes and Bray, 1984). Curve a, xanthine oxidase; curve b, nitrate reductase of *A. vinelandii*.

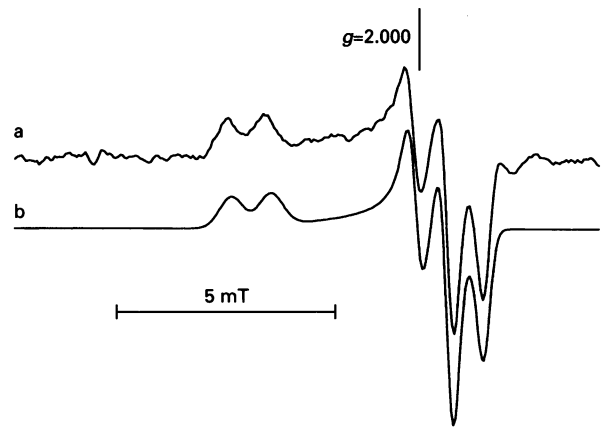

Figure 6 Mo(V) e.p.r. spectra of nitrate reductase from *A. vinelandii*

The spectra are: a, nitrate reductase (7.6 μM) as isolated in 50 mM phosphate buffer, pH 7.0. (e.p.r. running conditions: temperature 120 K; microwave frequency, 9.34 GHz; power, 2.01 mW; modulation intensity, 0.2 mT at 100 KHz; protein concentration, 7.6 μM); b, computer simulation using the parameters $g_1 = 2.02300$, $g_2 = 1.99810$, $g_3 = 1.99310$ and half-line-widths of 2.7, 1.5 and 1.5 mT.

is associated with pterin (molybdopterin). Molybdopterin can be extracted from these enzymes under oxidizing conditions in two derivative forms A and B, which at alkaline pH have characteristic fluorescence spectra (Johnson et al., 1984).

When the assimilatory nitrate reductase of *A. vinelandii* was denatured under conditions which release oxidized Moco (see the Materials and methods section), material with fluorescence characteristics of molybdopterin was released. In alkaline solution, fluorescence excitation at 370 nm gave a fluorescence emission spectrum with a maximum at 482 nm characteristic of the B form of Mo cofactor. The addition of an equivalent concentration of FAD (1 μM) to the solution resulted in a general increase in fluorescence between 500 and 600 nm and shifted the emission maximum to 510 nm. This latter spectrum is very similar to that observed when Mo cofactor was extracted together with FAD from purified xanthine oxidase, and indicates that no significant amount of flavin was extracted from nitrate reductase under these conditions.

Mo cofactor released from nitrate reductase under anaerobic conditions was tested for its activity in the apo-(*nit-1*) nitrate reductase assay. The conditions used, developed by Hawkes and Bray (1984), provide a quantative basis for the assay of Mo cofactor. For comparison, Mo cofactor extracted from purified xanthine oxidase was also assayed under the same conditions. Figure 5 shows that a typical saturation curve was obtained when increasing amounts of Mo cofactor isolated from both nitrate reductase and xanthine oxidase were incubated with crude extracts of the *nit-1* mutant, and then assayed for NADPH-dependent nitrate reductase activity.

Using partially purified apo-(*nit-1*) nitrate reductase, in similar titrations, and by extrapolating the experimental data to infinite concentration, a theoretical maximum of 26 ± 6 μmol of NO_2^- formed/min per ng-atom of Mo was derived by Hawkes and Bray (1984). In our hands, using a crude extract of the *nit-1* mutant, a value of 11 μmol of NO_2^- formed/min per ng-atom of Mo was obtained for xanthine oxidase, corresponding to 44% efficiency of extraction and transfer to the apoenzyme. For nitrate reductase, the Mo cofactor activity was 9 units, equivalent to 36% efficiency of extraction and transfer. These data show that the assimilatory nitrate reductase of *A. vinelandii* contains Mo cofactor capable of activating apo-(*nit-1*) nitrate reductase.

It has recently been established that the pterin cofactor of molybdoenzymes isolated from a number of bacteria is different from that of molybdoenzymes from eukaryotic sources (Krüger and Meyer, 1987; Rajagopalan, 1991; Rajagopalan and Johnson, 1992). Of interest in the present context is the finding that the dissimilatory nitrate reductases from *Escherichia coli*, *Pseudomonas carboxydoflava* and *Rhodobacter sphaeroides* have modified forms of Mo cofactor which contain a molybdopterin guanosine dinucleotide, and that the assimilatory enzymes from corn and *Chlorella vulgaris* have unmodified molybdopterin (see Rajagopalan and Johnson, 1992). Since both forms of Mo cofactor are active in the *nit-1* cofactor assay, our data do not allow us to distinguish between the presence of molybdopterin or some variant in the nitrate reductase of *A. vinelandii*; however, our e.p.r. data presented below indicate that the Mo in this enzyme has unusual ligation.

E.p.r. spectroscopy

E.p.r. spectroscopy of molybdoenzymes in the Mo(V)-oxidation state has been used extensively to study the ligation of the metal in molybdenum cofactor centres and in mechanistic studies on these types of enzyme (Bray, 1988). However, of the 25 nitrate reductases that have been purified, only a few have been studied by e.p.r. spectroscopy [*E. coli*, Vincent and Bray (1978) and George et al. (1989); *Pseudomonas aeruginosa*, Godfrey et al. (1987); *Paracoccus denitrificans*, Turner et al. (1988); spinach, Gutteridge et al. (1983) and Kay et al. (1989); *Chlorella vulgaris*, Kay and Barber (1989); *Candida nitratophila*, Kay et al. (1990)]. In these enzymes the e.p.r. properties of the molybdenum centres are very similar, and capable of undergoing a transition between two different states, that is both pH- and anion-dependent. The similarities in the e.p.r. properties of these enzymes established that the ligand environment of molybdenum in their active centres is identical. The e.p.r. spectra are superficially rather alike, with $g_{av.}$ in the range 1.96–1.98, and show splitting due to relatively strong hyperfine coupling of molybdenum to one or more exchangeable protons associated with the -OH or -SH ligands to the molybdenum.

E.p.r. data for the assimilatory nitrate reductase is restricted to the enzymes from spinach (Gutteridge et al., 1983; Kay et al., 1989), *Chlorella* (Kay et al., 1988) and the yeast *Candida nitratophila* (Kay et al., 1990). These proteins when partially reduced have very similar Mo(V) e.p.r. parameters assignable to a single species with a $g_{av.}$ value of 1.977; the signals have near-axial symmetry and show superhyperfine splitting due to interaction with a single, $s = 1/2$, nucleus.

The e.p.r. spectrum of purified nitrate reductase of *A. vinelandii* as isolated also exhibited Mo(V) signals with close to axial symmetry showing hyperfine splitting due to interaction with a single, $I = 1/2$, nucleus (Figure 6, spectrum a). The integrated spin intensity was 10 ± 2% electrons per mol of enzyme. Computer simulation of the experimental spectrum gave g values of $g_1 = 2.0230$, $g_2 = 1.9981$ and $g_3 = 1.9931$ ($g_{av.} = 2.004$) (Figure 6, spectrum b). With a $g_{av.}$ value above 2.002, the nitrate reductase of *A. vinelandii* shows an environment of the molybdenum that is different from other molybdoenzymes that have been subjected to e.p.r. studies. In addition, the e.p.r. spectrum was essentially unchanged after incubation for 45 min in phosphate buffer containing ^2H_2O (99.5%) at 23 °C (Figure 7, spectra a and b), unlike other molybdoenzymes where proton exchange results in the disappearance of doublet features observed in ^1H_2O. This difference was not due to the $I = 1/2$ nucleus being selenium (which could potentially replace sulphur as a ligand to Mo), since analysis of the enzyme showed no detectable selenium in the preparations used in these experiments (see the Materials and methods section). If the splitting is due to a proton, then it must exchange very slowly. Without further supplies of protein we are unable to eliminate the possibility that this nucleus is ^{31}P, but we consider this unlikely since other molybdoproteins do not show doublet features in phosphate buffers containing ^2H_2O [see for example Bray et al. (1976)].

The Mo(V) e.p.r. spectrum was bleached by the addition of $S_2O_4^{2-}$ (2 mM) (Figure 8b). No e.p.r. signal attributable to the presence of a reduced Fe-S centre was observed at 10 K,

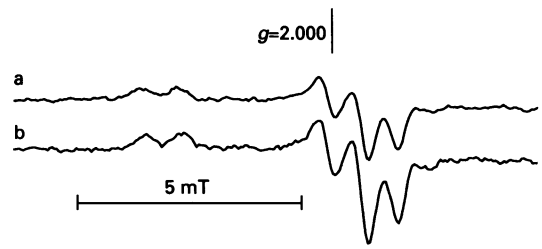

Figure 7 E.p.r. spectrum of Mo(V) in nitrate reductase in ^2H_2O

Curve a, enzyme (7.6 μM) as isolated in 50 mM phosphate buffer, pH 7.0; curve b, enzyme (11 μM) in 50 mM phosphate buffer, pH 7.0, containing 99.5% ^2H_2O. Samples were prepared and the deuterium content of the buffer was measured as described in the Materials and methods section. No corrections for the effect of ^2H_2O on pH measurements using a glass electrode were made. E.p.r. running conditions were as in Figure 6.

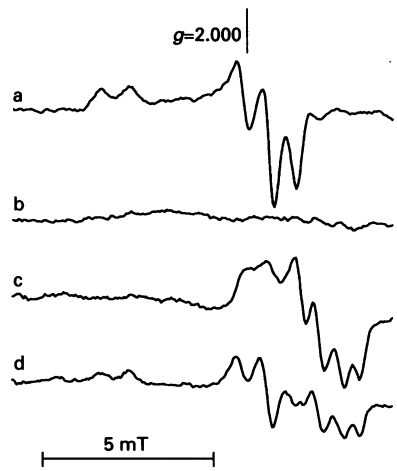

Figure 8 E.p.r. spectra of nitrate reductase reduced with $S_2O_4^{2-}$ and during enzyme turnover

Curve a, enzyme (7.6 μM) as isolated; b, reduced with $S_2O_4^{2-}$ (2 mM); c, sample b frozen 30s after the addition of nitrate (5 mM) to initiate enzyme turnover; d, sample frozen 90 min after addition of nitrate (5 mM). E.p.r. running conditions were as in Figure 6.

presumably because of the relatively low concentration of nitrate reductase used in these experiments.

Samples of enzyme containing $Na_2S_2O_4$ (2 mM), which were frozen 30 s after the addition of NO_3^- (5 mM) to initiate enzyme turnover, developed a Mo(V) e.p.r. spectrum with g values within the range observed with other Moco-containing enzymes ($g_1 = 1.998$; $g_2 = 1.989$; $g_3 = 1.981$; $g_{av.} = 1.989$) (see Figure 8, spectrum c) with a spin intensity of $7 \pm 2\%$ electrons per mol of protein. On prolonged incubation (90 min) under these conditions of limiting reductant, the spectrum assignable to Mo(V) was a mixed species of the e.p.r. spectra seen during turnover at short times, and that of the protein as isolated (compare Figure 8 spectrum 9 with Figure 8 spectra c, d). These data are consistent with the reversible interconversion of the unusual environment of Mo in the protein, as isolated, into a ligand environment during turnover, more typical of other molybdoproteins. In such a kinetically complex system with multiple potential redox states, it is not unexpected that only a small proportion of the enzyme (about 7%) contains the e.p.r.-detectable species Mo(V) during turnover.

The properties of the assimilatory nitrate reductase of *A. vinelandii* described here are similar to the assimilatory enzyme of the cyanobacterium *Plectonema boryanum* (Mikami and Ida, 1984) in that they are both monomeric (M_r 85000–105000) and contain Moco and Fe-S but do not have FAD or cytochrome components, as found in the assimilatory enzyme of higher plants. The dissimilatory enzyme from *Clostridium perfringens* has similar properties (Seki *et al.*, 1987). However, the unusual e.p.r. properties we report cannot be compared with these enzymes since no data for these prokaryotic enzymes are available.

We thank Mr. Colin Macdonald for performing the n.m.r. experiments, Professor Bob Bray and Dr. Richard Hughes for advice and material for the Moco assays. We also thank Professor Barry Smith for constructive comments on the manuscript and gratefully acknowledge financial assistance to R. G. from the British Council.

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Received 29 May 1992/30 July 1992; accepted 4 August 1992