Electron-paramagnetic-resonance spectroscopy studies on the dissimilatory nitrate reductase from *Pseudomonas aeruginosa*

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1. Preparations of nitrate reductase in the resting state from *Pseudomonas aeruginosa* exhibit an Mo(V) e.p.r. signal. Progressive reduction of the enzyme results at first in the intensification and then in the disappearance of the signal. 2. Three different species of Mo(V) were detected by e.p.r. These are the high-pH species ($g_1 = 1.9871$; $g_2 = 1.9795$; $g_3 = 1.9632$) and nitrate and nitrite complexes of a low-pH species (respectively $g_1 = 2.0004$; $g_2 = 1.9858$; $g_3 = 1.9670$; and $g_1 = 1.9975$; $g_2 = 1.9848$; $g_3 = 1.9652$). These signals are closely analogous to those for the enzyme from *Escherichia coli* described by Vincent & Bray [(1978) *Biochem. J.* **171**, 639–647]. 3. Signals typical of iron–sulphur clusters were also detected. In the oxidized enzyme these are believed to arise from a [3Fe-4S] cluster ($g = 2.01$) and in the reduced enzyme from an unusual low-potential [4Fe-4S]⁺ cluster ($g_1 = 2.054$; $g_2 = 1.952$; $g_3 = 1.878$). 4. The iron–sulphur centres were also studied in a ‘high-catalytic-activity’ form of the enzyme. Reduction with Na₂S₂O₄ resulted in the formation of a complex signal with g values at 2.054, 1.952, 1.928, 1.903 and 1.878. The signal could be deconvoluted by reductive titration of the enzyme into two species ($g_1 = 2.054$; $g_2 = 1.952$; $g_3 = 1.878$; and $g_1 = 2.036$; $g_2 = 1.928$; $g_3 = 1.903$). 5. The degradation of a [4Fe-4S] into a [3Fe-4S] cluster in the enzyme is suggested by these studies, the process being dependent on the method used to purify the enzyme. 6. The addition of nitrate to the reduced enzyme results in the oxidation of Mo(IV) to Mo(V) and of all the iron–sulphur centres.

Nitrate reductases have been isolated from bacteria, plants and fungi, and the enzymes studied from these various sources all contain molybdenum. In addition, cytochrome and flavin are generally present in the enzyme from eukaryotic sources, whereas iron–sulphur clusters usually predominate in the bacterial nitrate reductases (Hewitt & Notton, 1980). All of these sites are believed to participate in the transfer of electrons to nitrate, which is bound and reduced to nitrite at the molybdenum centre (Vincent & Bray, 1978). Understanding of the mechanism of nitrate reduction is at present limited. Although a two-electron transfer mechanism involving a Mo(IV)–Mo(VI) couple is currently favoured (Hewitt & Notton, 1980), experimentally obtained e.p.r. data have not progressed much beyond characterizing the various signal-giving species.

Mo(V) was first detected by e.p.r. in the enzyme

from *Pseudomonas aeruginosa* (Fewson & Nicholas, 1961), but the most extensive investigation of this species has been performed on the enzyme from *Escherichia coli*. Two major signals arising from a high-pH and a low-pH species of Mo(V) have been identified in the enzyme from this source (Vincent & Bray, 1978). The low-pH species was reported by these workers to possess a strongly coupled proton, and more recent work (Morpeth *et al.*, 1983; G. N. George, R. C. Bray, F. F. Morpeth & D. Boxer, unpublished work) has shown that there is also an anionic ligand, such as Cl[−], NO₃[−] or NO₂[−]. Information about the Mo(V) species in the enzyme from eukaryotic sources is rather more limited, although two states of Mo(V) have been identified in the spinach (Gutteridge *et al.*, 1983) and in the *Chlorella* (Solomonson *et al.*, 1984) enzymes.

A variety of e.p.r. signals apparently arising

from iron-sulphur clusters have been recorded in nitrate reductases extracted from a variety of bacterial sources. The enzyme from *Micrococcus denitrificans* exhibits a signal at $g = 2.01$, as isolated, that is lost on reduction with dithionite to be replaced by a rhombic set of signals at $g = 2.06$, 1.95 and 1.88. A second set of rhombic signals of similar g -values is also apparent (Forget & DerVartanian, 1972). Similar observations have been made for the *E. coli* enzyme (DerVartanian & Forget, 1975; Vincent & Bray, 1978), and the redox potentials of some of the signals have been determined by e.p.r. spectroscopy (Vincent, 1979). E.p.r. signals attributable to iron-sulphur centres have been reported in membrane vesicles of nitrate reductase from *Klebsiella aerogenes* (Bosma *et al.*, 1978) and *E. coli* (Blum & Poole, 1982). The definitive assignment of these signals to cluster type has not been achieved. Integration of the spin intensities invariably leads to an estimation of cluster concentrations well below the expected stoichiometric level of at least one per molecule of enzyme. One of us has recently reported an improved purification of the nitrate reductase from *Ps. aeruginosa* (Godfrey, 1983), and we now report an e.p.r. study of the molybdenum and iron-sulphur centres in this enzyme. This leads to the conclusion that the centres are very similar to those in the enzyme from *E. coli*. However, two forms of the enzyme, which differ in their specific activity, being 10 and 50 units/mg of protein, and which are isolated from bacteria grown either in the absence or in the presence of azide, have been examined spectroscopically. Furthermore, a comparison has been made between preparations of the enzyme prepared either by detergent extraction from or by heat treatment of membrane fragments to release enzyme.

Materials and methods

Nitrate reductase from *Ps. aeruginosa* was purified and characterized as previously described (Godfrey, 1983). The purification involved $(\text{NH}_4)_2\text{SO}_4$ fractionation, solubilization by detergent or heat treatment, and chromatography on phenyl-Sepharose (or DEAE-cellulose) and Sepharose 6B-CL (both from Sigma Chemical Co., Poole, Dorset, U.K.). The purified enzyme had a specific activity of 10 units/mg of protein, an M_r of 220 000 and contained 0.45 mol of molybdenum, 9.77 mol of non-haem iron and 12.5 mol of labile sulphide per mol of enzyme. A second form of the enzyme was isolated by the same method from *Ps. aeruginosa* grown in the presence of 0.2 mM- NaN_3 . It had a specific activity of 50 units/mg of protein, contained 0.4 mol of molybdenum/mol and is referred to as the 'high-activity' enzyme. One unit

of activity is 1 μmol of nitrate reduced/min at 30°C.

E.p.r. spectroscopy

The sample was concentrated to 24–60 mg/ml in the appropriate buffer. Where necessary, solutions and samples were made anaerobic by degassing and replacing the atmosphere with either N_2 or Ar (British Oxygen Co., London S.W.19, U.K.). $\text{Na}_2\text{S}_2\text{O}_4$ was added anaerobically to the sample in an e.p.r. tube from a stock solution, usually either 0.1 or 1.0 M, made up in the sample buffer. Samples were next incubated for 5 min at 0°C and then rapidly frozen.

Mo(V) e.p.r. spectra were recorded on a Varian E9 spectrometer linked to a computer and visual-display unit (Bray *et al.*, 1978). Iron-sulphur e.p.r. spectra were recorded on a Varian E3 spectrometer fitted with an Oxford Instruments ESR-9 continuous-flow cryostat and DTC2 temperature controller, enabling the sample temperature to be varied over the range 4.2–300 K.

Quantification of e.p.r. signals was performed by double integration, and intensities were corrected for g -value-dependence on transition probability by the method of Aasa & Vänngård (1975). Cupric EDTA and metmyoglobin cyanide were used as standards during the integration of Mo(V) and iron-sulphur signals respectively.

Simulation of e.p.r. spectra was carried out as devised by George (1983, and unpublished work) on the University of Sussex Digital Equipment Co. VAX 11/780 computer.

Buffers

The following buffers were used (supplied by Sigma Chemical Co.): Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and Mes (4-morpholine-ethanesulphonic acid) both adjusted to the appropriate pH with NaOH. Carbonate buffer was prepared by mixing solutions of Na_2CO_3 and NaHCO_3 to the required pH.

Results

Mo(V) e.p.r. signals

Ps. aeruginosa nitrate reductase, as prepared, exhibited an e.p.r. signal at 123 K that we ascribe to Mo(V). The signal was observed to double in intensity on partial reduction with $\text{Na}_2\text{S}_2\text{O}_4$, as the non-e.p.r.-detectable Mo(VI) species was reduced to Mo(V). Subsequent reduction of Mo(V) to Mo(IV) by further additions of dithionite was accompanied by a decline in the intensity of the signal and finally in its disappearance (Fig. 1). The addition of nitrate to the reduced enzyme restored the Mo(V) signal. The maximum spin intensity

Fig. 1. Plot of the spin intensity of the Mo(V) resonance of *Ps. aeruginosa* nitrate reductase against the corresponding $\text{Na}_2\text{S}_2\text{O}_4$ concentration used to reduce the enzyme. $\text{Na}_2\text{S}_2\text{O}_4$ concentrations were 0.1, 0.15, 0.2 and 0.3 mM. Reduction was performed anaerobically for 5 min at 4°C on a sample in 20 mM-potassium phosphate buffer, pH 7.4. The sample concentration was 60 mg of protein/ml. Conditions of running e.p.r. spectra were: temperature 123 K; microwave power 30 mW; microwave frequency 9.363 GHz; modulation amplitude 0.25 mT.

recovered on reduction was 0.041 mol of molybdenum/mol of enzyme, corresponding to 9.1% of the chemically determined molybdenum.

Three different Mo(V) e.p.r. signals were observed and identified in the enzyme. Using the terminology of Vincent & Bray (1978), we refer to these as the high-pH signal (Fig. 2a) and the low-pH nitrate (Fig. 2c) and nitrite (Fig. 2e) signals. Although all three are rhombic in form, the spectra of the two low-pH species differ slightly from one another and exhibit well-resolved hyperfine structure due to strong coupling to a single proton. The e.p.r. parameters of these signals obtained with the help of computer simulations are given in Table 1.

The high-pH and low-pH signals, recorded at pH 10 and 5.5 respectively, were interconvertible by adjusting the pH to an appropriate value. This phenomenon was first characterized for the *E. coli* enzyme by Vincent & Bray (1978).

In the low-pH species, the form of the spectrum depends on the anions present in the sample buffer. Thus two different low-pH Mo(V) spectra were observed in the presence of nitrate and of nitrite. These, we believe, correspond to specific complexes of these anions with the molybdenum centre. The computer-simulated e.p.r. spectra shown in Figs. 2(d) and 2(f) confirm that these signals both arise from single chemical species.

A third low-pH signal, recorded in the absence of nitrate and nitrite, was also observed (results not

Fig. 2. Mo(V) signals of resting oxidised *Ps. aeruginosa* nitrate reductase

(a) High-pH form; (c) low-pH nitrate form; (e) low-pH nitrite form. (b), (d) and (f) are simulations of (a), (c) and (e) respectively. The buffers used were (a) 50 mM-carbonate/bicarbonate buffer, pH 10; (c) and (e) 50 mM-Mes buffer, pH 5.5. Sample concentration in each case was 60 mg of protein/ml. (c) contained 1 M- NaNO_3 , and (e) 1 M- NaNO_2 . Conditions of e.p.r. running were: temperature 123 K; microwave power 30 mW; microwave frequency about 9.4 GHz; modulation amplitude 0.32 mT. Simulations were carried out by using the parameters shown in Table 1, as described in the Materials and methods section. The arrows correspond to $g = 2.0037$.

shown). However, by analogy with the *E. coli* enzyme (Morpeth *et al.*, 1983; G. N. George, R. C. Bray, F. F. Morpeth & D. Boxer, unpublished work) there must presumably be a molybdenum-bound anion present. This may be phosphate or chloride from a contamination in the buffer. We have observed similar e.p.r. signals from Mo(V) in all four types of enzyme, both high-activity and low-activity and both detergent-extracted and heat-treated, but no differences among the spectra of these forms were apparent.

Iron-sulphur e.p.r. signals

In the oxidized heat-treated enzyme a $g = 2.01$ signal was observed (Fig. 3b). However, the intensity of this signal was variable, depending on

Table 1. *Parameters of e.p.r. signals from nitrate reductase*

The relevant spectra are shown in Figs. 2(a), 2(c) and 2(e) respectively. The haem-NO spectra are not shown. Δ is half-linewidth in mT.

Signal	$g_1 (\Delta_1)$	$g_2 (\Delta_2)$	$g_3 (\Delta_3)$	Proton hyperfine (MHz)		
				A ₁	A ₂	A ₃
High-pH Mo(V)	1.9871 (0.4)	1.9795 (0.3)	1.9632 (0.35)	Not determined		
Low-pH Mo(V) nitrate	2.0004 (0.27)	1.9858 (0.23)	1.9670 (0.25)	40.1	28.6	27.5
Low-pH Mo(V) nitrite	1.9975 (0.29)	1.9848 (0.24)	1.9652 (0.28)	39.1	27.8	27.5
				¹⁴ N coupling (MHz)		
Haem-NO	2.0800 (1.4)	2.0340 (1.6)	2.0115 (0.4)	85	54	46

Fig. 3. *E.p.r. spectra of (a) the detergent-solubilized high-activity form and (b) the heat-treated form of Ps. aeruginosa nitrate reductase*
Both samples were in 50 mM-Hepes buffer, pH 7.4, at a concentration of 100 μ M. Conditions of running spectra were: temperature 10 K; microwave power 20 mW; modulation amplitude 0.63 mT; microwave frequency 9.225 GHz; receiver gains (a) 1.5×10^5 and (b) 1.5×10^4 .

the method used to solubilize the enzyme. In the detergent-solubilized nitrate reductase the 'g = 2.01' signal was either absent or very weak. The anaerobic addition of nitrate (10 mM) to the enzyme prepared by both of these methods did not result in an increase in the intensity of the g = 2.01 signal, suggesting that the clusters in the enzyme are already oxidized.

Fig. 4. *E.p.r. spectra of Ps. aeruginosa nitrate reductase (detergent-solubilized low-activity form) reduced with (a) 50 mM-Na₂S₂O₄ and (b) 100 mM-Na₂S₂O₄*
The reductions were performed anaerobically at 4°C for 5 min. The sample concentration was 100 μ M; sample buffer was 50 mM-Hepes, pH 8.0. E.p.r. running conditions were: temperature 10 K; microwave power 20 mW; microwave frequency 9.235 GHz; modulation amplitude 0.63 mT.

The high-activity enzyme also exhibited a variable g = 2.01-type signal, as described above. The spectrum of the detergent-solubilized enzyme (Fig. 3a) additionally had a Mo(V) contribution at 5 K that, in contrast with the g = 2.01 signal, was still present at 77 K.
Partial reduction of both the detergent-solubilized and heat-treated enzyme generates a spectrum of the type shown in Fig. 4(a), with g values at 2.054, 1.952 and 1.878. On complete reduction with Na₂S₂O₄ the g = 2.01 feature disappears, leaving a rhombic signal (Fig. 4b). Partial reduc-

Fig. 5. E.p.r. spectra of *Ps. aeruginosa* nitrate reductase (heat-treated high-activity form) (a) as isolated, (b) with 3 mM- $\text{Na}_2\text{S}_2\text{O}_4$ and (c) with 4 mM- $\text{Na}_2\text{S}_2\text{O}_4$.

Reduction was performed anaerobically at 4°C with an incubation time of 5 min. The sample concentration was 100 μM ; sample buffer was 50 mM-Hepes, pH 7.4. Conditions of recording spectra were: temperature 10 K; microwave power 20 mW; microwave frequency 9.22 GHz; modulation amplitude 0.63 mT.

tion of the high-activity enzyme resulted at first in the disappearance of the $g = 2.01$ signal (Fig. 5) and then in the appearance of a rhombic signal with g values at 2.054, 1.952 and 1.878, but with an additional contribution at $g = 2.036$ (Fig. 6a). Complete reduction led to the appearance of further structure in the spectrum, with g values at 2.054, 2.036, 1.952, 1.928, 1.903 and 1.878 (Fig. 6b). These signals, we believe, are due to contributions from two different types of low-potential clusters, with g values at 2.054, 1.952 and 1.878 and at 2.036, 1.928 and 1.903 respectively. No Mo(V) e.p.r. signals were detectable at 80 K in these experiments, confirming that all the low-temperature signals arise from iron-sulphur clusters. Similar complex signals have been observed in the enzymes from *M. denitrificans* (Forget & DerVartanian, 1972) and *E. coli* (Vincent & Bray, 1978; DerVartanian & Forget, 1975).

The addition of NaNO_3 (100 mM) to the fully reduced enzyme, whether heat-treated or detergent-solubilized, high-activity or low-activity forms, resulted in the appearance of a $g = 2.01$ signal and in the disappearance of the rhombic signals. This cor-

Fig. 6. E.p.r. spectra of the detergent-solubilized high-activity form of *Ps. aeruginosa* reductase reduced with (a) 2.5 mM- $\text{Na}_2\text{S}_2\text{O}_4$ and (b) an excess of $\text{Na}_2\text{S}_2\text{O}_4$.

Reduction was performed anaerobically at 4°C for 5 min. The sample concentration was 120 μM ; sample buffer was 50 mM-Hepes, pH 7.4. E.p.r. running conditions were: temperature 5 K; microwave power 20 mW; microwave frequency 9.220 GHz; modulation amplitude 0.63 mT. The arrows correspond to $g = 2.0037$.

responds to the oxidation of iron-sulphur centres in the enzyme.

Quantification of the fully developed $g = 2.01$ signal in the oxidized enzyme and of the rhombic signal in the reduced enzyme gave values of 0.212 and 0.22 spin/molecule of enzyme respectively.

Detection of a haem-NO species in the enzyme

In a sample of the enzyme that contained cytochrome a three-line e.p.r. spectrum with g -values of 2.0800, 2.0340 and 2.0115 (Table 1) appeared on reduction with $\text{Na}_2\text{S}_2\text{O}_4$ and persisted after oxidation with nitrate. It is likely to arise from a haemoprotein-NO complex, and appears to correspond to a single chemical species, as demonstrated by simulation of the spectrum (not shown). Similar signals are known in other systems (see, e.g., Kon, 1968) and have been detected in oxidized preparations of the *E. coli* nitrate reductase (Vincent & Bray, 1978).

Discussion

The properties of Mo(V) in the *Ps. aeruginosa* enzyme as determined by e.p.r. spectroscopy are very similar to those of the *E. coli* enzyme. Both exhibit an apparently unsplit high-pH signal and a protonated low-pH form of Mo(V) signal. Interconversion of the two forms may be achieved by adjusting the pH, although anion concentration is also important (Morpeth *et al.*, 1983). The pK_a of the interconversion for the *E. coli* enzyme was determined originally to be 8.2 (Vincent & Bray, 1978), but later work showed that in chloride-free buffer the apparent pK_a value was considerably lower (G. N. George, R. C. Bray, F. F. Morpeth & D. Boxer, unpublished work). Similarly, at low anion concentration at pH 7.4, the *Ps. aeruginosa* enzyme exhibits a high-pH signal similar to that shown in Fig. 2(a).

The poor fit of the simulation (Fig. 2b) of the high-pH signal might indicate the presence of more than one species. Alternatively, by analogy with the *E. coli* enzyme (Morpeth *et al.*, 1983; G. N. George, R. C. Bray, F. F. Morpeth & D. Boxer, unpublished work), there might be unresolved proton hyperfine structure, resulting in a more complex lineshape. Further experiments with enzyme in deuterated water or at 35 GHz would be necessary to determine unambiguously the nature of the signal.

By analogy with the *E. coli* enzyme (Morpeth *et al.*, 1983), we assume that the nitrate complex of *Ps. aeruginosa* nitrate reductase corresponds to a species of Mo(V) with a nitrate ligand and a strongly coupled proton. This is consistent with nitrate being bound and reduced at the molybdenum centre. In this respect, the enzyme is similar to other molybdenum-containing enzymes, which are believed to bind and react with their substrates at the molybdenum site (Bray, 1980). Overall, the close resemblance of the Mo(V) e.p.r. signals of *Ps. aeruginosa* nitrate reductase with those of the *E. coli* enzyme indicates that the ligands and geometry of the molybdenum site are similar in the two enzymes.

The results given in Figs. 3–6 show clearly that, in common with the dissimilatory bacterial nitrate reductases from *E. coli* (DerVartanian & Forget, 1975; Vincent & Bray, 1978) and *M. denitrificans* (Forget & DerVartanian, 1972), at least two types of iron–sulphur clusters can be detected in the enzyme from *Ps. aeruginosa*. They have, respectively, a signal at $g = 2.01$ in the oxidized state and a rhombic set of signals in the reduced state with average g -values just below 2.0. Our data do not show directly that these two types of e.p.r. signal belong to different classes of iron–sulphur cluster, but we argue below, by comparison with e.p.r. data

on better-defined bacterial ferredoxins, that they can be so assigned.

The e.p.r. signal at $g = 2.01$ has a very low intensity in some detergent-solubilized enzyme, is always present in heat-treated enzyme and has a variable intensity. NO_3^- ion re-oxidizes this centre after it has been reduced by dithionite. We conclude that the variability in signal intensity results from a variation in the concentration of this cluster in the enzyme rather than from a percentage of the clusters being reduced. The $g = 2.01$ signal has similar characteristics to that seen in the ferredoxin II from *Desulfovibrio gigas* (Huynh *et al.*, 1980). The shape is the same and the electronic relaxation properties are broadly similar, the signal disappearing between 30 and 40 K. There is little doubt now that the cluster that gives rise to the signal in *D. gigas* and in a range of other ferredoxins, such as *Thermus thermophilus* ferredoxin (Hille *et al.*, 1983), *Azotobacter vinelandii* ferredoxin I (Johnson *et al.*, 1983) and ferricyanide-damaged ferredoxin from *Clostridium pasteurianum* (Thomson *et al.*, 1981), is identical with the three-iron cluster in aconitase (Beinert & Thomson, 1983). There is evidence, including extended-X-ray-absorption-fine-structure data that give an iron–iron bond distance of 0.27 nm (2.7 Å) for *D. gigas* ferredoxin II (Antonio *et al.*, 1982) and for aconitase (Beinert *et al.*, 1983), and analytical data on aconitase, that all point to the structure of the three-iron cluster as [3Fe-4S], a [4Fe-4S] centre with one iron atom missing from a corner. The number of cysteine ligands, whether three, four or even five, is unknown. However, the basic core structure is very different from that proposed by Ghosh *et al.* (1980) to be the structure of the [3Fe-3S] cluster in his crystals of *A. vinelandii* ferredoxin. There is interconvertibility of clusters in aconitase and in the ferredoxins I and II from *D. gigas* (Moura *et al.*, 1982). A [4Fe-4S] cluster can lose iron to form a [3Fe-4S] core cluster on oxidative damage by ferricyanide (Thomson *et al.*, 1981), or in the absence of substrate in the case of aconitase (Emptage *et al.*, 1983). At present there is no proven enzyme function for a [3Fe-4S] cluster.

We therefore consider it most likely that the $g = 2.01$ signal in the dissimilatory nitrate reductases of bacteria belongs to [3Fe-4S] clusters that arise as a result of iron loss from a [4Fe-4S] core cluster. The variability of the signal intensity lends some support to this view. There is evidence from the low-temperature magnetic-c.d. spectra of the enzymes from *Ps. aeruginosa* (C. Godfrey & A. J. Thomson, unpublished work) and from *E. coli* (M. K. Johnson, M. C. W. Adams, L. E. Mortensen, C. Godfrey, C. Greenwood & A. J. Thomson, unpublished work) that the cluster giving rise to the e.p.r. signal $g = 2.01$ is a [3Fe-4S] cluster very similar

spectroscopically to that in *D. gigas* ferredoxin II. Vincent (1979) determined a mid-point potential for the $g = 2.01$ signal in *E. coli* nitrate reductase of $+80 \pm 20$ mV, compared with a value of -130 mV for *D. gigas* ferredoxin II (Cammack *et al.*, 1977).

The e.p.r. signals that appear on reduction with dithionite both of the low-activity detergent-solubilized and heat-treated enzyme show a rhombic set of g -values 2.054, 1.952 and 1.878. The signals appear to belong to a magnetically isolated centre, since there are no additional peaks expected if there were interaction between two or more reduced iron-sulphur clusters. Their assignment to a cluster $[4\text{Fe-4S}]^+$ is not unambiguous. The signals belong to a rapidly relaxing paramagnetic centre, since they cannot be observed above approx. 30 K. This makes it unlikely that they arise from a $[2\text{Fe-2S}]^+$ cluster, although the g -values match with that of plant-type ferredoxin, such as spinach, namely $g = 2.05$, 1.96 and 1.89 (Fritz *et al.*, 1971). The more likely assignment is to an isolated $[4\text{Fe-4S}]^+$ centre. The g -values of a typical ferredoxin containing a single centre from, for example, *Bacillus stearothermophilus* are 2.06, 1.92 and 1.88 (Mullinger *et al.*, 1975).

The iron-sulphur e.p.r. signals from the high-activity detergent-extracted enzyme are considerably more complex (Fig. 6) than those of the low-activity detergent-solubilized and the heat-treated enzyme. On partial reduction with dithionite a set of rhombic g -values, at 2.054, 1.952 and 1.878, dominate the spectrum and clearly arise from a cluster identical with that in the low-activity form of the enzyme. Further reduction, however, brings about the development of new features at $g = 2.045$ and 2.036 plus some poorly resolved peaks between $g = 1.952$ and 1.878. Since the rhombic trio $g = 2.054$, 1.952 and 1.878 remain unaffected by the presence of the new peaks, it is unlikely that these new features are a result of spin-coupling between centres involving the rhombic trio signals. A similar set of signals have been reported from the enzyme from *M. denitrificans* (Forget & DerVartanian, 1972) and assigned to two types of iron-sulphur centres. We can conclude little further about the nature of the iron-sulphur clusters in this protein. They pose many puzzles. The e.p.r. signals integrate to a very low spin concentration. The redox potentials of the e.p.r.-detectable clusters in the dithionite-reduced state are low, much lower than would appear to be required for transferring electrons to the molybdenum centre. Clearly, other spectroscopic techniques are now required in order to assist in the understanding of the structural complexity of the iron-sulphur centres in dissimilatory nitrate reductase.

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References

- Aasa, R. & Vänngård, T. (1975) *J. Magn. Reson.* **19**, 308–315
- Antonio, M. R., Averill, B. A., Moura, I., Moura, J. J. G., Orme-Johnson, W. H., Teo, B.-K. & Xavier, A. V. (1982) *J. Biol. Chem.* **257**, 6646–6649
- Beinert, H. & Thomson, A. J. (1983) *Arch. Biochem. Biophys.* **222**, 333–361
- Beinert, H., Emptage, M. H., Dreyer, J.-L., Scott, R. A., Hanh, J. E., Hodgson, K. O. & Thomson, A. J. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 393–396
- Blum, H. & Poole, R. J. (1982) *Biochem. Biophys. Res. Commun.* **107**, 903–909
- Bosma, H. J., Wever, R. & Van't Riet, J. (1978) *FEBS Lett.* **90**, 107–111
- Bray, R. C. (1980) *Adv. Enzymol. Relat. Areas Mol. Biol.* **51**, 107–165
- Bray, R. C., Barber, M. J. & Lowe, D. J. (1978) *Biochem. J.* **171**, 653–658
- Cammack, R., Rao, K. K., Hall, D. O., Moura, J. J. G., Xavier, A. V., Bruschi, M., LeGall, J., Deville, A. & Gayda, J. P. (1977) *Biochim. Biophys. Acta* **490**, 311–321
- DerVartanian, D. V. & Forget, P. (1975) *Biochim. Biophys. Acta* **379**, 74–80
- Emptage, M. H., Dreyer, J.-L., Kennedy, M. C. & Beinert, H. (1983) *J. Biol. Chem.* **258**, 11106–11111
- Fewson, C. A. & Nicholas, D. J. D. (1961) *Biochim. Biophys. Acta* **49**, 335–349
- Forget, P. & DerVartanian, D. V. (1972) *Biochim. Biophys. Acta* **256**, 600–606
- Fritz, J., Anderson, R., Fee, J., Palmer, G., Sands, R. H., Orme-Johnson, W. H., Beinert, H., Tsibris, J. C. M. & Gunsalus, I. C. (1971) *Biochim. Biophys. Acta* **253**, 110–133
- George, G. N. (1983) D.Phil. Thesis, University of Sussex
- Ghosh, D., O'Donnell, D., Patthabi, B. & Robbins, A. H. (1980) *J. Biol. Chem.* **255**, 1797–1800
- Godfrey, C. (1983) Ph.D. Thesis, University of East Anglia
- Gutteridge, S., Bray, R. C., Notton, B. A., Fido, R. J. & Hewitt, E. J. (1983) *Biochem. J.* **213**, 137–142
- Hewitt, E. J. & Notton, B. A. (1980) in *Molybdenum and Molybdenum-Containing Enzymes* (Coughlan, M., ed.), pp. 275–325, Pergamon Press, Oxford
- Hille, R., Yoshida, T., Tarr, G. E., Williams, C. H., Ludwig, M. L., Fee, J. A., Kent, T. A., Huynh, B. H. & Münck, E. (1983) *J. Biol. Chem.* **258**, 13008–13013
- Huynh, B. H., Moura, J. J. G., Moura, I., Kent, T. A., LeGall, J., Xavier, A. V. & Münck, E. (1980) *J. Biol. Chem.* **255**, 3242–3244
- Johnson, M. K., Czernuszewicz, R. S., Spiro, T. G., Fee, J. A. & Sweeney, W. V. (1983) *J. Am. Chem. Soc.* **105**, 6671–6678
- Kon, H. (1968) *J. Biol. Chem.* **243**, 4350–4357

- Morpeth, F. F., Boxer, D. H., Bray, R. C. & George, G. N. (1983) in *Recent Developments in the Chemistry of Chromium, Molybdenum and Tungsten* (Dilworth, J. R. & Lippert, M. F., eds.), p. P10, Royal Society of Chemistry, London
- Moura, J. J. G., Moura, I., Kent, T. A., Lipscomb, J. D., Huynh, B. H., LeGall, J., Xavier, A. V. & Münck, E. (1982) *J. Biol. Chem.* **257**, 6259–6267
- Mullinger, R. N., Cammack, R., Rao, K. K., Hall, D. O., Dickson, D. P. E., Johnson, C. E., Rush, J. D. & Simopoulos, A. (1975) *Biochem. J.* **151**, 75–83
- Solomonson, L. P., Barber, M. J., Howard, W. D., Johnson, J. L. & Rajagopalan, K. V. (1984) *J. Biol. Chem.* **259**, 849–853
- Thomson, A. J., Robinson, A. E., Johnson, M. J., Cammack, R., Rao, K. K. & Hall, D. O. (1981) *Biochim. Biophys. Acta* **637**, 423–432
- Vincent, S. P. (1979) *Biochem. J.* **177**, 751–759
- Vincent, S. P. & Bray, R. C. (1978) *Biochem. J.* **171**, 639–647