Studies by electron-paramagnetic-resonance spectroscopy of the molybdenum centre of spinach (*Spinacia oleracea*) nitrate reductase

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The molybdenum centre of spinach (*Spinacia oleracea*) nitrate reductase has been investigated by e.p.r. spectroscopy of molybdenum(V) in reduced forms of the enzyme. The resting enzyme gives no signals attributable to Mo(V). However, on reduction with NADH, Mo(V) signals appeared at relatively short reaction times but decreased again on prolonged exposure to excess of the substrate as the enzyme was further reduced. On brief treatment of such samples with nitrate, Mo(V) signals reappeared but disappeared again on longer exposure to excess nitrate as the enzyme became fully reoxidized. Detailed investigation of the signals carried out in both $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ revealed the presence of two signal-giving species, referred to as 'signal A' and 'signal B', analogous to corresponding signals from nitrate reductase from *Escherichia coli* and from liver sulphite oxidase. Signal A has g_{av} 1.9767 and shows coupling to a single proton, exchangeable with the solvent, with $A(^1\text{H})_{\text{av}}$ 1.3 mT, whereas signal B shows no more than weak coupling to protons. Investigation of interconversion between the two species indicated that decreasing the pH from 8.0 to 6.7 had little effect, but that signal A was favoured by the presence of Cl^- . This suggests, by analogy with recent work on sulphite oxidase by Bray, Gutteridge, Lamy & Wilkinson [*Biochem. J.* (1983) 211, 227–236] that Cl^- is a ligand of molybdenum in the species giving signal A.

Nitrate reductases (EC 1.6.6.1–3; 1.7.99.4) purified from a range of different organisms are complex proteins of high molecular weight containing a number of different reducible centres. The types and number of centres in many of the enzymes has not been clearly established, although generally the reductases from eukaryotic species contain haem and flavin components, whereas iron–sulphur centres predominate in the enzymes from prokaryotes (see Hewitt & Notton, 1980; Jacob & Orme-Johnson, 1980). All of the enzymes, irrespective of their source, contain molybdenum. This is the site where nitrate binds and is reduced to nitrite (Vincent & Bray, 1978). Reducing substrates such as NADH interact via other components of the enzymes.

Detailed understanding of the enzymic mechanism of nitrate reduction at the molybdenum centre is limited. Oxygen-transfer mechanisms have been widely assumed (e.g. Garner *et al.*, 1974; Hewitt *et al.*, 1979) in the workings of molybdenum-

containing enzymes, including nitrate reductase. At the present time, however, the most direct evidence for such a mechanism comes from studies on xanthine oxidase (Gutteridge & Bray, 1980). The mechanisms of nitrate reduction by nitrate reductase and of sulphite oxidation by sulphite oxidase could well be similar to one another, despite the very different redox potentials of molybdenum in the two enzymes (see Bray, 1980). In support of this, e.p.r. studies (Bray, 1980) on the metal in these enzymes have certainly indicated close structural similarities between them (Vincent & Bray, 1978; Lamy *et al.*, 1980).

So far only one form of nitrate reductase, namely the dissimilatory enzyme from *Escherichia coli*, has been investigated systematically with the e.p.r. technique. Two major e.p.r. signals due to Mo(V) were distinguished, which were found to be interconverted in response to change of pH. A rhombic signal generated at low pH values, having g_{av} 1.9824, exhibited splitting due to a strongly coupled proton (Bray *et al.*, 1976; Vincent & Bray, 1978). With increasing pH, this was replaced by an unsplit signal with g_{av} 1.9762. Decreasing enzyme activity

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with increasing pH paralleled increasing proportions of the unsplit signal in the e.p.r. spectrum, and this suggested (Vincent & Bray, 1978) that the low-pH form of the enzyme was the one involved in catalysis.

In the case of sulphite oxidase it has long been known (Cohen *et al.*, 1971) that there is a similar interconversion between high-pH and low-pH forms of the enzyme, detected by e.p.r. However, recently, Bray *et al.* (1983) found that the low-pH form was favoured not only by low pH but also by the presence of high concentrations of Cl^- in the medium. They concluded that Cl^- was a ligand of molybdenum in this form of this enzyme.

Molybdenum e.p.r. signals have been reported for nitrate reductases from two eukaryotic organisms, *Neurospora crassa* (Jacob & Orme-Johnson, 1980) and *Chlorella vulgaris* (Solomonson, 1979), but the signals were not analysed in detail and no specific parameters were given. These signals are not markedly like those from the *E. coli* reductase. [Another nitrate reductase from a prokaryotic organism, that from *Pseudomonas aeruginosa*, has recently been investigated by G. N. George, R. C. Bray, C. Godfrey, C. Greenwood and A. Thomson (unpublished work) and shown to be very similar in its Mo(V) e.p.r. properties to the enzyme from *E. coli*.] We now report e.p.r. studies on nitrate reductase isolated and purified from spinach (*Spinacia oleracea*), and compare the well-defined molybdenum signals of this enzyme with those generated from the nitrate reductases from other sources and with the signals from other molybdenum-containing enzymes. A preliminary report of parts of this work has appeared (Notton & Hewitt, 1983); note, however, that two of the present authors (R. C. B. and S. G.) do not accept interpretations of the e.p.r. data there presented, which were based on the speculative schemes for nitrate reductase action and inactivation put forward by Hewitt *et al.* (1979).

Materials and methods

Nitrate reductase was isolated and purified from 7 kg of spinach leaves by using streptomycin sulphate and $(\text{NH}_4)_2\text{SO}_4$ fractionation, hydroxyapatite absorption, molecular-seive chromatography and affinity chromatography on Blue Sepharose (Pharmacia). All steps were basically as previously described (Notton *et al.*, 1977), except for the inclusion of $10\mu\text{M}$ -FAD during the purification and for elution of the enzyme from the Blue Sepharose with 5 mM-sodium phosphate buffer, pH 7.5, containing 1 mM-EDTA, $10\mu\text{M}$ -FAD and $50\mu\text{M}$ -NADH. The enzyme was precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 50% saturation, redissolved in 0.1 M-sodium Mops (4-morpholinepropanesulphonate) buffer, pH 7.0, containing $10\mu\text{M}$ -FAD and 1 mM-

EDTA, then dialysed against the same buffer. Gel electrophoresis at pH 9.5 indicated the product to be >90% homogeneous. It was assayed at 27°C and pH 7.5 by using the method of Wray & Filner (1970) and protein was calculated from A_{260}/A_{280} (Warburg & Christian, 1941). Though the specific activity was initially high, it fell during concentration and storage. The final solution had a specific activity of $2.59\mu\text{mol of NO}_2^- \text{ produced} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$, which is 11% of the best specific activity obtained for spinach nitrate reductase by Notton *et al.* (1977). The total yield of enzyme was 76 units.

E.p.r. measurements

The enzyme was dialysed against the required buffer. In some experiments EDTA (1 mM) and FAD (1 or $10\mu\text{M}$) were added to increase stability of the enzyme. However, the latter, particularly at the higher concentration, tended to give rise to free-radical e.p.r. signals, which interfered to some extent with observation of the Mo(V) e.p.r. signals. The enzyme was concentrated by using Minicon B concentrators (from Amicon Ltd., Stonehouse, Gloucestershire GL10 2BJ, U.K.) to a maximum concentration of about 300 units/ml and placed in 4 mm-internal-diameter e.p.r. tubes. Samples in $^2\text{H}_2\text{O}$ were dialysed against three changes of buffer made up in this solvent, thereby reducing the content of $^1\text{H}_2\text{O}$ to less than about 4%.

E.p.r. spectra were generated by aerobic addition of small quantities of NADH to the enzyme in the e.p.r. tubes. After being mixed the solutions were frozen manually within about 30 s. Where necessary, further additions were made to the enzyme from concentrated solutions after thawing the samples rapidly. E.p.r. spectra were recorded on a Varian E9 spectrometer linked to a computer and visual-display system (Bray *et al.*, 1978); difference spectra were obtained as described by these workers and by Bray & Gutteridge (1982). Spectra were recorded at 9.3 GHz at about 120 K at a microwave power of 20 mW and generally with a modulation amplitude of 0.25 mT. Simulation of e.p.r. spectra was carried out as described by Lowe (1978) or George (1983).

Results

Appearance and disappearance of Mo(V) e.p.r. signals from spinach nitrate reductase

E.p.r. signals from the molybdenum of the assimilatory nitrate reductases of higher plants have not been described before. Therefore it was important to establish that the metal could respond to reducing and to oxidizing substrates. Fig. 1 illustrates the changes that occur at the molybdenum centre as the result of progressive reduction and re-oxidation of samples of spinach nitrate reductase. The concentrated enzyme as prepared is fully oxidized and thus showed no e.p.r. signals due to Mo

Fig. 1. Effects of reduction by NADH and re-oxidation by nitrate on the e.p.r. spectrum of nitrate reductase. Different overall amplifications were used for the various spectra, as indicated on the Figure. Spectra in (a)–(e) were recorded after making successive additions to a single sample of nitrate reductase. Final concentrations, and the incubation time after each addition, were as follows: (a) no additions; (b) 0.5 mM-NADH, 30 s at 0°C; (c) 6 mM-NADH, 15 min at 25°C; (d) 4 mM-nitrate, 30 s at 0°C; (e) 10 mM-nitrate, 30 s at 0°C. The sample was in 100 mM-Mops adjusted to pH 7.0 with NaOH. The arrow here and in Figs. 2 and 3 corresponds to $g = 2.0037$.

(Fig. 1a). Addition of NADH as reductant produced an Mo signal as the metal was reduced from the non-detectable sexivalent state to the quinquevalent state (Fig. 1b). Increasing the concentration of NADH or the reaction time or both, as in Fig. 1(c), caused the signal to decline in intensity, as further reducing equivalents were taken up by the enzyme molecules and the molybdenum was further

reduced to the quadrivalent state. Figs. 1(d) and 1(e) show that the process is readily reversible and that on addition of the substrate, nitrate, the Mo(V) signals reappeared, disappearing ultimately when the Mo(VI) state was once again attained. Thus, the metal can cycle between the sexi- and quadri-valent states, with Mo(V) detectable in intermediate oxidation states of the enzyme molecules.

Form of the Mo(V) e.p.r. signals

The form of the Mo(V) signals is complex and was found to vary from one sample to another. Conditions affecting this variation will be considered below. The variation can be seen, for example, in Fig. 1. Thus the relative amplitudes of the two main derivative-type features of the spectrum are quite different in Fig. 1(b) and Fig. 1(d), with the feature at the lower field value relatively much enlarged in Fig. 1(d). With the aid of computer difference techniques (Bray *et al.*, 1978) such spectra could be resolved into two species which we shall call signal A and signal B. In fact, under specific conditions (see below), signal A could be obtained in relatively pure form (Figs. 2a₁ and 3a) without the use of difference techniques. This signal shows doublet features, typical of strong coupling to Mo of a single proton. This conclusion was confirmed by reducing the enzyme under appropriate conditions in ²H₂O (see Fig. 2a₂), which resulted in loss of the doublets. The same signal is shown again in Figs. 3(a) and 3(c), where our interpretation of the lineshape has been confirmed by computer simulation in ¹H₂O (Fig. 3b) and in ²H₂O (Fig. 3d), which gave good approximations to the experimental spectra. Parameters of signal A are given in Table 1.

In contrast, signal B was never obtained on its own and we therefore had to deduce its form by subtracting out signal A from spectra composed of mixtures of the two signals. This process is illustrated in Fig. 2, both for experiments in ¹H₂O (Fig. 2a₁ to Fig. 2c₁) and in ²H₂O (Fig. 2a₂ to Fig. 2c₂). The starting spectra are illustrated in Figs. 2(b₁) and 2(b₂) and the difference spectra, corresponding to signal B, in Figs. 2(c₁) and 2(c₂). The last named pair of spectra are somewhat different from one another, a conclusion which was confirmed by generating the difference spectra from other pairs of samples. Thus, for example, the g_1 feature of the difference spectrum in ²H₂O (Fig. 2c₂) is sharper than that in ¹H₂O (Fig. 2c₁). The position of the g_3 feature of signal B is not certain, but again there seemed to be substantial differences in this region of the spectrum in the two solvents. Although these observations are undoubtedly suggestive of a rather weak anisotropic coupling to an exchangeable proton in the species giving signal B, we have not attempted simulations, because of the possibility that other additional

Fig. 2. Effects of $^2\text{H}_2\text{O}$ and of Cl^- on the e.p.r. spectrum of nitrate reductase and the presence of two signal-giving species. The left-hand column, (a_1) – (c_1) , were obtained for enzyme in $^1\text{H}_2\text{O}$ and the right-hand column, (a_2) – (c_2) , were in $^2\text{H}_2\text{O}$. The spectra on the second line, (b_1) and (b_2) , were recorded on samples without added Cl^- and those on the first line, (a_1) and (a_2) , were obtained from samples prepared under comparable conditions but with the addition of 100 mM Cl^- . The third line, (c_1) and (c_2) , shows difference spectra obtained by subtracting the spectra on the first line from those on the second line. As is discussed in the text, (a_1) and (a_2) are believed to correspond to almost pure signal A, and (c_1) and (c_2) correspond to signal B. Spectra were generated by reduction with NADH in a buffer containing 25 mM-Pipes and 25 mM-Bicine adjusted to pH 6.7 with NaOH. The difference spectra were as follows: $(c_1) = [(b_1) - 0.48(a_1)]$ and $(c_2) = 1.6 \times [(b_2) - 0.58(a_2)]$.

species might be contributing to the spectra. Examination of signal B would of course be facilitated if conditions could be found under which it was obtained on its own, without signal A. Alternatively, spectra at 35 GHz might help to resolve this problem, which is particularly difficult because of the close similarity of signal B to the ^2H form of signal A.

Conditions affecting interconversion between signals A and B

We carried out a number of experiments to investigate factors that might have an influence on interconversion between signal A and signal B. Unexpectedly, by analogy with nitrate reductase from *E. coli* and with chicken liver sulphite oxidase, varying the pH value from pH 6.7 to 8.0, in samples to which Cl^- had not been added, did not influence the relative amounts of signals A and B. However, the total signal intensity was lower at the higher pH value. {This experiment was carried out in buffers consisting of 25 mM-Pipes (1,4-piperazinediethanesulphonic acid) and 25 mM-Bicine [*NN*-bis-(2-hydroxyethyl)glycine], adjusted with NaOH; pH values were checked with a microelectrode after the spectra had been recorded.}. In contrast, we found clear evidence that addition of Cl^- resulted in increasing proportions of signal A, and with this ion

at 100 mM, essentially complete conversion into this signal was obtained at both pH values. The spectra of Figs. 2(a_1) and 2(a_2) and Figs. 2(b_1) and 2(b_2), which illustrate effects of 100 mM Cl^- in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$, have already been referred to. The progressive conversion of signal B into signal A observed as additions of substrates were made to the enzyme (Fig. 1) has also been referred to, and was observed in other similar experiments. Such changes might, in part, be due to increasing contamination with Cl^- . On the other hand, change from signal B to signal A did not represent, for example, irreversible degradation of the enzyme. Thus the nitrate reductase could be recovered after e.p.r. experiments, freed from substrates by dialysis, and on re-exposure to NADH and then NO_3^- , gave once again the same pattern of signal changes.

Signal A (or signals of a form very similar to it) could also be obtained in relatively pure form from samples without added Cl^- but containing moderate concentrations of certain other anions such as nitrate (Fig. 3a), acetate, phosphate or fluoride.

Discussion

The Mo centre of spinach nitrate reductase responds to reducing and oxidizing substrates as expected for such a centre involved in catalysis and

as found for the nitrate reductases of *E. coli* and *N. crassa*. Since re-oxidation by nitrate of the enzyme, reduced by NADH, was rapid under all the conditions we tried, slow inactivation of the spinach enzyme by excess of the reducing substrate (cf. Hewitt *et al.*, 1979) was not a problem in the present work. Because of the probability that intra-molecular electron-transfer reactions are rapid be-

tween Mo and other redox active centres in the enzyme, experiments of the type we have performed cannot distinguish between one- and two-electron transfer from Mo to nitrate in the enzyme mechanism. Nevertheless, two-electron mechanisms are now widely assumed (Spence, 1980; Bray, 1980) for molybdenum-containing enzymes generally.

The similarities between nitrate reductases regarding reduction and re-oxidation of the metal are extended, in the case of the spinach and *E. coli* enzymes, to the generation of two different species of Mo(V), distinguishable by e.p.r. Though Jacob & Orme-Johnson (1980) presented no evidence suggesting two such species from the *N. crassa* enzyme, on the other hand the e.p.r. signal reported by Solomonson (1979) for the *Chlorella* enzyme (when allowance is made for the spectrum having been over-modulated) is quite similar to some of our spectra (see particularly, for example, our Fig. 2b₁), suggesting that in this enzyme, too, the Mo can exist in two Mo(V) states.

Before attempting to comment on the nature and possible significance of the two states of Mo(V) in the spinach enzyme, we may note the pattern which work on molybdenum centres in other enzymes has shown. The first stage inevitably consists of distinguishing different signal-giving species from one another. Two further stages may follow. One consists of deducing structural information from the e.p.r. parameters, particularly with the help of isotopic substitution. The other consists of establishing the relationship between the signal-giving species and the catalytic mechanism of the enzyme. Such work has reached its most advanced state on xanthine oxidase (see, for example, Bray & Gutteridge, 1982; George & Bray, 1983), though even for this enzyme much remains to be done. For sulphite oxidase, though recent e.p.r. work (Bray *et al.*, 1983) has provided evidence for a Cl⁻ ligand in the low-pH form of the enzyme, nevertheless the relationship of this form, and of the high-pH form, to the activity of the enzyme has still not been properly elucidated.

In these terms, e.p.r. work on nitrate reductase is still at an early stage. For the *E. coli* enzyme, the

Fig. 3. Simulation of signal A in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$. (a) Shows the experimental spectrum in $^1\text{H}_2\text{O}$ and was obtained by reducing, with NADH, a sample of the enzyme in 25 mM-imidazole adjusted to pH 6.5 with HNO_3 . (c) shows the experimental spectrum in $^2\text{H}_2\text{O}$ and is the spectrum of Fig. 2(a₂). The simulations in (b) and (d) respectively were obtained as described in the Materials and methods section, by using the parameters of Table 1, with appropriate replacement in (d) of coupling to ^1H by coupling to ^2H .

Table 1. E.p.r. parameters of Mo(V) signals from nitrate reductase and sulphite oxidase

The values given for spinach nitrate reductase signal A are those used in the simulation of Fig. 3. Data on other enzymes are taken from the literature, for comparison.

Enzyme	Signal	g-values				$A(^1\text{H})$ (mT)				Reference
		1	2	3	Av.	1	2	3	Av.	
Spinach nitrate reductase	A*	1.9957	1.9692	1.9652	1.9767	1.20	1.25	1.45	1.30	Present work
	B	1.995	1.967	—	—	—	—	—	—	Present work
<i>E. coli</i> nitrate reductase	Low pH	1.9989	1.9855	1.9628	1.9824	1.11	0.84	0.81	0.92	Vincent & Bray (1978)
	High pH	1.9870	1.9805	1.9612	1.9762	—	—	—	—	Vincent & Bray (1978)
Chicken liver sulphite oxidase	Low pH	2.0037	1.9720	1.9658	1.9805	0.85	0.80	1.30	0.98	Lamy <i>et al.</i> (1980)
	High pH	1.9872	1.9641	1.9531	1.9681	—	—	—	—	Lamy <i>et al.</i> (1980)

* Half-linewidths were respectively 0.30, 0.20 and 0.25 mT.

effect of anions on interconversion between high-pH and low-pH forms of the enzyme now clearly needs to be investigated. For the spinach enzyme, more work is required on interconversion between signal A and signal B, in solutions of controlled, low-Cl⁻ concentrations. Also effects of other anions on the e.p.r. spectrum need to be investigated in detail and related to their effects (cf. Hewitt, 1974) on activity of the enzyme. It is conceivable that our failure to observe a pH effect on the spectrum could be due to there being, by chance, more contamination with Cl⁻ ions in our high-pH than in our low-pH samples. However, this seems quite unlikely. The alternative possibility would be that interconversion between signal B and signal A involves anion uptake not accompanied by proton uptake, and presumably (noting the possibility mentioned earlier that signal B might involve a weakly coupled proton), accompanied by the proton's moving from a weakly coupled to a strongly coupled position.

Though Mo(V) species from essentially all Mo-containing enzymes show strong coupling of exchangeable protons to the metal, these protons are distinguished from one another by their coupling constants and by their exchange kinetics (Gutteridge *et al.*, 1978; J. P. G. Malthouse & R. C. Bray, unpublished work), and the species containing the protons are distinguished by effects of pH and of anions on conversion of the enzymes into these species (Malthouse *et al.*, 1980; Bray *et al.*, 1983; Vincent & Bray, 1978). For the species giving rise to signal A of spinach nitrate reductase, it is probable that there is a Cl⁻ ligand, which may be replaceable by other anions, apparently without a significant change of e.p.r. parameters. Presumably the proton is in the form of an MoOH group (cf. Yamase *et al.*, 1981).

With regard to e.p.r. parameters other than proton couplings, probably the most pertinent point in Table 1 is that, for the spinach enzyme alone, *g*-values of the two signals yielded by each enzyme scarcely differ from one another. (Note, however, that *g*₃ has not yet been determined for signal B.) This would tend to support the idea of no more than a minor structural change being involved in the interconversion of the species giving signals A and B. However, until some of the points mentioned above have been established, it seems futile to speculate further on the nature and mechanistic significance of the two signals. Nevertheless, our investigations clearly open the way for further potentially revealing e.p.r. studies on spinach nitrate reductase, using, for example, isotopic substitution and kinetic methods.

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