Investigation by electron paramagnetic resonance spectroscopy of the molybdenum centre of respiratory nitrate reductase from *Paracoccus denitrificans*

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The molybdenum centre of respiratory nitrate reductase from *Paracoccus denitrificans* has been investigated by e.p.r. spectroscopy of Mo(V). In common with the centres of the analogous enzymes from *Escherichia coli* and *Pseudomonas aeruginosa*, it undergoes a pH- and anion-dependent transition between two different e.p.r. signal-giving species. Comparison of the relevant e.p.r. parameters extracted with the help of computer simulations reveals ligation of the metal in the active centres of the three enzymes to be identical.

INTRODUCTION

E.p.r. spectroscopy of molybdoenzymes in the Mo(V) oxidation state is capable (Bray, 1988) of yielding very substantial information on the ligation of the metal in their active centres and on the mechanism of their catalytic reactions. Work (e.g. Dowerah *et al.*, 1987) on relevant low-molecular-mass molybdenum model compounds is now playing an important role. Nevertheless, of some 20 or 30 nitrate reductases that have been purified (Adams & Mortenson, 1985), only a few have so far been studied by e.p.r. The one most extensively investigated is the respiratory enzyme from anaerobically grown *Escherichia coli*. For this, the Mo(V) e.p.r. spectrum undergoes a well-defined transition, that is both pH- and anion-dependent, between two different forms of the enzyme. These forms have been carefully characterized and their e.p.r. parameters have been extracted with the help of computer simulation (Vincent & Bray, 1978; George *et al.*, 1985). The e.p.r. data established that the nitrate reductase species giving the low-pH spectrum clearly bears both a hydroxy and an anion ligand of molybdenum (Bray, 1986). On the other hand, the structure of the species giving the high-pH spectrum is rather less certain. Also, the exact relation of the two signal-giving species to the catalytic reaction has as yet not been fully defined. The respiratory nitrate reductase from the denitrifying bacterium *Pseudomonas aeruginosa* has also been studied in detail by e.p.r. by Godfrey *et al.* (1984), with the finding that its molybdenum centre is extremely similar to that of the *E. coli* enzyme. Similarly, the molybdenum centre of hepatic sulphite oxidase undergoes (Bray *et al.*, 1983) a high-pH/low-pH transition analogous to that in these nitrate reductases. On the other hand, e.p.r. investigations (Gutteridge *et al.*, 1983; Barber & Solomonson, 1986) of molybdenum in assimilatory nitrate reductases of eukaryotic organisms are at a relatively unadvanced state. Though the situation may be more complicated in the enzymes from these organisms, there are indications that a comparable high-pH/low-pH transition is involved. However, only in one case for e.p.r. data from a eukaryotic nitrate reductase, that for the low-pH form of

the spinach enzyme (Gutteridge *et al.*, 1983), has the spectrum been of sufficient quality that a simulation has been attempted.

It is clearly desirable that more nitrate reductases should be investigated by e.p.r. Recent availability of homogeneous preparations (Craske & Ferguson, 1986) of the complete respiratory nitrate reductase from *Paracoccus denitrificans* (formerly *Micrococcus denitrificans*) encouraged us to examine the spectra of this enzyme. It was particularly important to do so, since an early study by Forget & Dervartanian (1972) had failed to reveal Mo(V) e.p.r. spectra bearing any obvious relationship to those discussed above. Further, the recent discovery of periplasmic respiratory nitrate reductases (Ferguson *et al.*, 1987) illustrates that there is no guarantee that nitrate reductases from different bacteria will be identical.

MATERIALS AND METHODS

Nitrate reductase was isolated from *P. denitrificans* as described by Craske & Ferguson (1986), and concentrated by ultrafiltration. E.p.r. spectroscopy and computer simulation of e.p.r. spectra were carried out as described previously (Bray & George, 1985; George & Bray, 1988). Running conditions were: temperature -120°C ; microwave power 30 mW; modulation amplitude 0.25 mT. Mo(V) e.p.r. signals were quantified by double integration, with Cu^{2+} -EDTA as standard; the enzyme was assumed to contain 1 mol of Mo/230 000 g of protein.

RESULTS AND DISCUSSION

The untreated enzyme gave no detectable e.p.r. signals from Mo(V). However, on cautious anaerobic addition of dithionite solution to a sample in 50 mM- Na^{+} /Bicine buffer, pH 8.2, the spectrum of Fig. 1(a) was obtained. Lowering the pH of the sample to approx. 6.3 by the anaerobic addition of Na^{+} /Mops buffer (final concn. 100 mM) in the presence of NaNO_3 (10 mM) caused the spectrum to change to that of Fig. 1(c). Computer

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

(a) and (c) are experimental spectra and (b) and (d) are computer simulations. The high-pH spectrum (a) was obtained by reduction of a sample of the enzyme in 50 mM- Na^+ /Bicine buffer, pH 8.2. The low-pH spectrum (c) was obtained by anaerobic addition, to the sample of (a), of Na^+ /Mops buffer (final concentration, 100 mM) and NaNO_3 (10 mM) giving a final pH of approx. 6.3. Parameters used in the simulation were: (b), $g_{1,2,3}$ 1.9869, 1.9798, 1.9623; half linewidths 0.39, 0.37, 0.40 mT; (d) $g_{1,2,3}$ 2.0003, 1.9858, 1.9659; $A(^1\text{H})_{1,2,3}$ 1.46, 1.11, 1.09 mT; half linewidths 0.27, 0.22, 0.25 mT.

simulations of the experimental spectra, using the parameters given in the caption, are illustrated in Figs. 1(b) and (d).

Though the spectra are relatively weak, corresponding only to about 5% of the molybdenum present in the sample, their forms are clearly defined, and the fit of the simulated spectra to the experimental ones is good. The similarity of the spectra to the corresponding ones from the nitrate reductases from *E. coli* and *Ps. aeruginosa* is striking. Thus, Fig. 1(a) is the spectrum of the pure high-pH form of the enzyme and Fig. 1(c) that of the pure low-pH nitrate complex. The *E. coli* enzyme yields two low-pH nitrate spectra, designated type 1 and 2 (George *et al.*, 1985). That of Fig. 1(c) is of the type 1 form.

Comparison of the parameters of the high-pH and low-pH signals with those in the literature (George *et al.*,

1985; Godfrey *et al.*, 1984) indicates these to be the same for the three enzymes, almost to the limits of the experimental errors (usually quoted in work from this laboratory as ± 0.0003 for g -values). Thus, there are no g -value differences > 0.0021 amongst the three enzymes and in one case (g_2 of the low-pH spectrum) they show g -values identical to the fourth decimal place. The low-pH spectrum from the *P. denitrificans* shows coupling to a single proton with $A(^1\text{H})_{\text{av.}}$ 1.22 mT. For the high-pH spectrum, the rather large linewidths that had to be used for the simulation (Fig. 1b) are consistent with the presence (cf. George *et al.*, 1985) of unresolved hyperfine structure from a weakly coupled proton.

Our work establishes that the ligand environment of molybdenum in the active centre of nitrate reductase from *P. denitrificans* is identical to that in the enzymes from *E. coli* and *P. aeruginosa*. Thus there are now two examples of this centre in respiratory nitrate reductases from denitrifying bacteria, but whether a comparable centre is common to all nitrate reductases, including those from periplasm, remains to be seen. The similarity between the *E. coli* and *P. denitrificans* nitrate reductases contrasts with the differences between the other electron transport proteins of these organisms (Craske & Ferguson, 1986).

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