E.p.r.-spectroscopic studies on the molybdenum–iron site of nitrogenase from *Clostridium pasteurianum*

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The e.p.r. spectroscopy of the nitrogenase molybdenum–iron protein from *Clostridium pasteurianum* was re-investigated. The sharpness of the $\Delta M_s = \pm 3$ g_z peak from the $\pm \frac{3}{2}$ Kramer's doublet enables the observation and quantification of incompletely resolved hyperfine splittings from the stable magnetic nuclei ^{95}Mo and ^{57}Fe in samples enriched in these isotopes. No couplings to ^1H or ^{17}O could be discerned by examination of spectra from samples exchanged into $^2\text{H}_2\text{O}$ and H_2^{17}O respectively. Simulation of the spectrum from ^{95}Mo -enriched samples yields a hyperfine coupling of 2.9 MHz, and indicates that the earlier electron–nuclear-double-resonance-derived estimate of 8.1 ± 0.2 MHz is substantially in error.

INTRODUCTION

The central chemical reaction of the biological nitrogen cycle is the six-electron reduction of N_2 to NH_3 by the enzyme complex known as nitrogenase. Many bacteria are capable of this reaction, and, despite their taxonomic diversity (e.g. see Woese, 1987), their nitrogenase systems appear to be remarkably similar (Postgate, 1982). There are two protein components of nitrogenase. The smaller iron protein contains a single $[4\text{Fe-4S}]$ cluster, and supplies electrons in an ATP-dependent process to the larger protein, which in turn catalyses N_2 reduction. In most cases the latter is a molybdenum–iron protein, although alternative systems exist in which vanadium, and possibly iron, substitute for molybdenum (Robson *et al.*, 1986; Chisnell *et al.*, 1988). The molybdenum–iron protein from *Clostridium pasteurianum* has an $\alpha_2\beta_2$ subunit structure and contains two different types of redox-active metal sulphide centres, called P and M clusters. The M clusters are thought to be the active site of N_2 reduction, and are known to be molybdenum–iron–sulphur clusters with stoichiometry $\text{MoFe}_{(6-7)}\text{S}_{(7-9)}$ (Stiefel & Cramer, 1985) that also contain homocitrate (Hoover *et al.*, 1989). In the resting, reduced, form the iron–molybdenum site possesses an $S = \frac{3}{2}$ ground state, with a zero-field splitting of about 5 cm^{-1} (Palmer *et al.*, 1972; Münck *et al.*, 1975; Venters *et al.*, 1986); it gives a well-defined e.p.r. signal from the $\pm \frac{1}{2}$ Kramer's doublet, which has been the subject of much study, including sophisticated magnetic-resonance techniques such as e.n.d.o.r. (Venters *et al.*, 1986; McLean *et al.*, 1987; True *et al.*, 1988) and electron-spin-echo (Thomann *et al.*, 1987) spectroscopies. In the present paper we report a study of an e.p.r. signal from the excited-state $\pm \frac{3}{2}$ Kramer's doublet, which, because of its much narrower linewidth, enables direct observation of hyperfine interactions from ^{95}Mo and ^{57}Fe .

MATERIALS AND METHODS

C. pasteurianum was grown (Carnahan *et al.*, 1960; Mortenson *et al.*, 1963) in a 400-litre fermenter (New

Brunswick Scientific Co., Edison, NJ, U.S.A.), and the molybdenum–iron protein was isolated by a modification of the procedure of Zumft & Mortenson (1973). ^{95}Mo and ^{96}Mo (94.9% and 95.9%-enriched) were obtained from Isotec (Miamisburg, OH, U.S.A.). The proportions of $I = \frac{5}{2}$ Mo in the enzyme samples were 5% and 95% for ^{96}Mo and ^{95}Mo samples respectively [determined from e.p.r. of the Mo(V)–tris(toluenedithiol) complexes (Stiefel *et al.*, 1966) prepared from wet-ashed protein (Clark & Axley, 1955)]. H_2^{17}O at 48.6% enrichment was obtained from Monsanto Research Corp. (Miamisburg, OH, U.S.A.). Samples in enriched water were prepared by dilution of highly concentrated protein. ^{57}Fe -enriched protein was purified from cells grown as described by Rusnak *et al.* (1987). Enzyme activities were 1800 ± 200 nmol of $\text{C}_2\text{H}_2/\text{min}$ per mg of protein (Zumft & Mortenson, 1973).

E.p.r. spectra of enzyme samples (40–60 mg of protein/ml in 50 mM-Tris/HCl buffer, pH 7.5) were recorded as previously described (George *et al.*, 1988), with typical instrument settings of 0.25 mT modulation amplitude, 10 mW of applied microwave power and a sample temperature of 16 K. Powder lineshape simulations were calculated on a Digital Equipment Corp. VAX 11-8350 computer, using the $S' = \frac{1}{2}$ representation (e.g. see Abraham & Bleaney, 1970; Pilbrow, 1978) of the $S = \frac{3}{2}$ system with g' values obtained from diagonalization of the $S = \frac{3}{2}$ spin Hamiltonian matrix. The spin Hamiltonian included electronic Zeeman and zero-field splitting terms; nuclear hyperfine couplings were added as a first-order perturbation.

RESULTS AND DISCUSSION

The well-known first-derivative e.p.r. spectrum of the *C. pasteurianum* molybdenum–iron protein is shown in Fig. 1. The clearly defined rhombic e.p.r. signal, with g' values of 4.27, 3.79 and 2.015, derives from the $\pm \frac{1}{2}$ Kramer's doublet of the $S = \frac{3}{2}$ system. The true g -values are probably close to 2.0 (e.g. see Münck *et al.*, 1975), and the observed g' anisotropy derives chiefly from the

Fig. 1. (a) E.p.r. signal of *C. pasteurianum* molybdenum-iron protein at 9.234 GHz and (b) energy-level diagram for the nitrogenase molybdenum-iron protein

(a) E.p.r. signal of *C. pasteurianum* molybdenum-iron protein at 9.234 GHz. The expanded region shows the $\Delta M_s = \pm 3$ g'_z signal from the $\pm \frac{3}{2}$ Kramers' doublet. (b) Energy-level diagram for the nitrogenase molybdenum-iron protein, calculated by diagonalizing the $S = \frac{3}{2}$ spin Hamiltonian, by using the parameters discussed in the text, and with the magnetic field oriented along D (i.e. g'_z). The $\Delta M_s = \pm 1$ ($g' = 2.015$) and $\Delta M_s = \pm 3$ ($g' = 6.06$) transitions, within the lower and the upper Kramers' doublets respectively, are indicated.

zero-field splitting caused by interactions among the unpaired electrons, and described by the traceless tensor $\mathbf{D}$ with principal components D_x , D_y and D_z . The zero-field splitting can be conveniently described by two parameters: a magnitude D ($D = 3D_z/2$) and a 'rhombicity' λ [$\lambda = (D_x - D_y)/3D_z$]. According to the convention of Blumberg (1967), which stipulates $0 \leq \lambda \leq \frac{1}{3}$, the 4.27, 3.79 and 2.015 features represent the y , x and z axes of $\mathbf{D}$ respectively, and are thus labelled g'_y , g'_x and g'_z .

As reported by Münck *et al.* (1975), the nominally forbidden $\Delta M_s = \pm 3$ features from transitions within the $\pm \frac{3}{2}$ Kramers' doublet are not readily apparent in the spectrum. Nevertheless, the $\Delta M_s = \pm 3$ g'_z feature becomes clearly visible at about $g' = 6.06$ at higher amplification (Fig. 1). At low temperatures (i.e. close to 4 K) the $g' = 6.06$ signal diminishes (result not shown). This is expected for a $\Delta M_s = \pm 3$ transition, as low temperatures will thermally depopulate the excited Kramers' doublet. Using the measured g' values (at 9.2316 GHz) as a guide and assuming an axial g -tensor and $D = 5.1 \text{ cm}^{-1}$ (Venters *et al.*, 1986), we estimate a rhombicity λ of 0.0401 and g_x , g_y and g_z values of 2.0167, 2.0167 and 2.0245 respectively. Diagonalization of the Hamiltonian matrix yields $\Delta M_s = \pm 1$ g'_z , g'_y and g'_x values of 3.786, 4.270 and 2.0147 respectively, which agree well with the experimental values, and $\Delta M_s = \pm 3$ g'_z , g'_y and g'_x values 0.3378, 0.1599 and 6.0638 for the $\Delta M_s = \pm 3$ signal. For the $\Delta M_s = \pm 3$ signal, only g'_z is within the range of our spectrometer. Other transitions within the lowest two levels are predicted at extremely high fields with $g' \approx 0.06$ (at 9.27 GHz).

The sharpness of the $\Delta M_s = \pm 3$ g'_z feature prompted us to look for nuclear hyperfine couplings to the stable magnetic isotopes ^{95}Mo and ^{57}Fe in protein derived from organisms grown on isotopically enriched media, and to ^{17}O and ^1H from atoms exchangeable with solvent water.

Isotopic enrichment with ^{95}Mo and ^{96}Mo

The stable isotopes ^{95}Mo and ^{96}Mo possess nuclear spins $I = \frac{5}{2}$ and $I = 0$ respectively, so that although the presence of the $I = 0$ ^{96}Mo nucleus should cause no hyperfine splitting of the e.p.r. signal, hyperfine coupling to a single ^{95}Mo nucleus would be expected to give rise to six hyperfine lines. Palmer *et al.* (1972) examined the e.p.r. of the $\pm \frac{1}{2}$ signal from the molybdenum-iron protein of *C. pasteurianum* and observed that the spectra from ^{95}Mo -enriched and unenriched samples were essentially indistinguishable, indicating at most a quite small hyperfine coupling. Similar results were obtained with the molybdenum-iron protein from *Klebsiella pneumoniae* by Smith *et al.* (1973). Fig. 2 shows the $\Delta M_s = \pm 3$ g'_z feature for samples enriched with ^{95}Mo and ^{96}Mo . The distinct broadening of the peak of the ^{95}Mo -enriched sample, relative to the ^{96}Mo -enriched sample, can be simulated by using the linewidth obtained from simulating the ^{96}Mo -enriched sample plus a hyperfine coupling of 2.9 MHz (note that we cannot determine the sign of the coupling). This value differs quite markedly from the value of $8.1 \pm 0.2 \text{ MHz}$ estimated by Hoffman, Orme-Johnson and co-workers (Venters *et al.*, 1986) from e.n.d.o.r. measurements. A simulation with their larger hyperfine coupling is shown in Fig. 2. We conclude that their analysis is in error, and suggest a simple explanation for the disparate conclusions. The ^{95}Mo e.n.d.o.r. spectrum at g'_z observed by Venters *et al.* (1986) is broad, with only a single resolved peak. E.n.d.o.r. spectra for $I > \frac{1}{2}$ are sometimes quite complex, and are often incompletely resolved owing to the presence of overlapping transitions. This is especially true if a significant nuclear electric quadrupole coupling is present; for $I = \frac{5}{2}$ a minimum of ten e.n.d.o.r. transitions are expected, plus additional satellite transitions made probable by the nuclear quadrupole coupling. To a first-order perturb-

Fig. 2. Effects of ^{95}Mo and ^{96}Mo enrichment upon the $\Delta M_s = \pm 3 g'_z$ signal

The continuous lines are the signals from ^{95}Mo - (upper trace) and ^{96}Mo - (lower trace) enriched enzyme. The dashed lines show the least-squares best-fit simulations of the spectra. The ^{95}Mo spectrum was fitted by using the half-linewidth obtained from fitting the ^{96}Mo spectrum (0.3 mT), plus a hyperfine coupling to a single $I = \frac{5}{2}$ nucleus (2.9 MHz). The dotted line is a simulation made by using the ^{95}Mo hyperfine coupling estimated by Venters *et al.* (1986).

ation approximation, the Larmor doublet associated with the $M_1 = \pm \frac{1}{2}$ levels is unaffected by the nuclear quadrupole interaction, and so is expected to be a prominent feature of the spectrum. The observation of only one resolved e.n.d.o.r. line is not entirely unexpected, as in many cases one half of a Larmor-split doublet is rather less intense than the other. Venters *et al.* (1986) assigned their single observed peak as the low-frequency half of the Larmor-split doublet corresponding to the $M_1 = \pm \frac{1}{2}$ levels. If, however, the observed e.n.d.o.r. peak is assigned as the high-frequency peak, then one expects the low-frequency partner to occur at less than 1 MHz (probably undetectable), and obtains (to first-order) a hyperfine coupling of approx. +4 MHz. This is more consistent with our findings.

It should also be noted that the ^{95}Mo nuclear electric quadrupole coupling reported by Venters *et al.* (1986) and McLean *et al.* (1987) depends upon the assignment of the e.n.d.o.r. transitions, and so requires re-evaluation.

Isotopic enrichment with ^{57}Fe

The e.p.r. spectrum of the nitrogenase molybdenum-iron protein from *K. pneumoniae* enriched with ^{57}Fe ($I = \frac{1}{2}$) was first reported by Smith *et al.* (1973), who observed a subtle broadening of g_z of the signal from the $\pm \frac{1}{2}$ Kramer's doublet. Fig. 3 shows the $\Delta M_s = \pm 3 g'_z$ signal from *C. pasteurianum* molybdenum-iron protein enriched with ^{57}Fe , together with that from enzyme containing naturally abundant iron. A pronounced broadening of the signal can readily be observed; the reader with a discerning eye might also deduce the presence of partly resolved structure in the enriched spectrum. Hoffman,

Orme-Johnson and co-workers (Hoffman *et al.*, 1982a,b; Venters *et al.*, 1986; True *et al.*, 1988) have investigated the e.n.d.o.r. spectra of ^{57}Fe -enriched molybdenum-iron proteins from various organisms. Their work includes an exquisitely detailed study of the *Azotobacter vinelandii* protein, where the individual hyperfine tensors of the five coupled ^{57}Fe nuclei discernible in the spectra were determined by simulation of the polycrystalline e.n.d.o.r. (True *et al.*, 1988). This work confirmed and extended earlier Mössbauer studies (Münck *et al.*, 1975; Huynh *et al.*, 1980), which had indicated two different types of hyperfine couplings for the iron (with e.n.d.o.r.-derived average couplings of -15 MHz and $+11$ MHz). The ^{57}Fe e.n.d.o.r. of the *C. pasteurianum* enzyme has not yet been completely analysed, although it apparently differs only subtly (some couplings being larger) from that of the *A. vinelandii* enzyme (Venters *et al.*, 1986). We used the e.n.d.o.r.-derived couplings from the *A. vinelandii* enzyme (True *et al.*, 1988) in our simulations of the ^{57}Fe -enriched $\Delta M_s = \pm 3 g'_z$ spectrum of Fig. 3. Using a slightly narrower linewidth than that obtained from fitting the natural-abundance spectrum (Fig. 3) resolves the hyperfine structure in the simulation, which shows some resemblance to the apparent structure of the ^{57}Fe spectrum. A similar effect could be obtained in the simulations by using slightly larger hyperfine couplings, or non-Gaussian lineshapes (results not shown). In any case, the simulation is a good fit to the overall width of the signal, and we conclude that our ^{57}Fe data are in good agreement with the conclusions derived from e.n.d.o.r. spectroscopy.

The high values for the ^{57}Fe hyperfine couplings, together with the low value for the ^{95}Mo coupling, argue for a significantly greater spin localization on the iron

Fig. 3. Effects of ^{57}Fe enrichment upon the $\Delta M_s = \pm 3 g'_z$ signal

The upper traces are the signals from ^{57}Fe -enriched (—) and natural-abundance-Fe (·····) enzyme, and the lower trace shows a simulation calculated by using the hyperfine couplings reported by True *et al.* (1988). In order to show partially resolved hyperfine structure, the simulation was calculated with a slightly narrower half-linewidth (0.2 mT) than that obtained by fitting the unenriched enzyme (0.3 mT; results not shown).

than on the molybdenum, especially when one allows for the smaller inherent couplings expected for ^{57}Fe (Goodman & Raynor, 1970). As argued by Venters *et al.* (1986), this might support an even formal oxidation state of molybdenum [e.g. low-spin Mo(IV)], although it is important to note that conclusions as to the formal valence of molybdenum (or of any other cluster component) may well be inappropriate in a highly delocalized system such as the molybdenum-iron site of nitrogenase.

Exchange into H_2^{17}O and $^2\text{H}_2\text{O}$

Venters *et al.* (1986) detected protons exchangeable with solvent water, with hyperfine couplings of 1.9 and 1.4 MHz. Such proton couplings are expected to be too small to affect the lineshape of the $\Delta M_s = \pm 3 g'_z$ feature, and in agreement with this we find that the signal is unchanged in $^2\text{H}_2\text{O}$ (results not shown). Venters *et al.* (1986) suggested that the exchangeable protons might arise from $-\text{OH}$ or $-\text{OH}_2$ ligation. If this were indeed the case, then one might expect the presence of exchangeable oxygen ligands. We have therefore examined the spectra of enzyme exchanged into ^{17}O -enriched water (approx. 40% final enrichment). ^{17}O possesses $I = \frac{5}{2}$, so hyperfine coupling to a single nucleus would yield a six-line hyperfine pattern. Despite exhaustive exchange (up to 4 h) no evidence of any hyperfine interactions could be discerned (results not shown). Simulations indicate that any ^{17}O hyperfine coupling must be less than 2 MHz in order to escape detection.

The lack of detectable hyperfine couplings to exchangeable ^{17}O means that the presence of $-\text{OH}$ or $-\text{OH}_2$ ligation remains without experimental support. Nevertheless, the presence of such ligands to cluster components with little spin density, such as molybdenum, cannot be excluded, as the ^{17}O hyperfine might be undetectable in these cases. The exchangeable protons observed by e.n.d.o.r. spectroscopy (Venters *et al.*, 1986) could be from nitrogenous ligands from the protein (e.g. $-\text{NH}-$) or from homocitrate (Hoover *et al.*, 1989). Ligation to the latter is quite consistent with our results, as oxygen atoms in carboxylic acids require quite acidic conditions to exchange at observable rates (Senkus & Brown, 1938). In addition, homocitrate apparently remains bound to the molybdenum-iron cluster even when this is extracted from the protein (e.g. Hoover *et al.*, 1989) by acidification and extraction into *N*-methylformamide, and must therefore be quite firmly incorporated into the cluster. Such a strong ligation might further hamper homocitrate oxygen exchange.

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