Nitrogenase from *Klebsiella pneumoniae*

An e.p.r. signal observed during enzyme turnover under ethylene is associated with the iron–molybdenum cofactor

Timothy R. HAWKES, David J. LOWE and Barry E. SMITH

School of Chemical and Molecular Sciences and A.R.C. Unit of Nitrogen Fixation, University of Sussex,
Brighton BN1 9RQ, U.K.

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During turnover at 10°C at pH 7.4 in the presence of ethylene, the MoFe protein of *Klebsiella pneumoniae* nitrogenase (Kp1) exhibited an electron-paramagnetic-resonance signal with g -values at 2.12, 1.998 and 1.987. ^{57}Fe isotopic substitution demonstrated that this signal arose from the Kp1 FeMo-cofactor in an $S = \frac{1}{2}$ spin state.

The enzyme nitrogenase consists of two components, the Fe protein and the larger MoFe protein (Kp2 and Kp1 respectively from *Klebsiella pneumoniae*), both of which are necessary for activity. The MoFe proteins are $\alpha_2\beta_2$ -tetramers of M_r about 220 000 and contain the enzyme's site(s) for binding reducible substrates. The most active preparations contain 2 Mo and 33 ± 3 Fe atoms per molecule, with an acid-labile sulphur content generally slightly lower than the Fe content (Eady & Smith, 1979; Mortenson & Thorneley, 1979). Evidence from Mössbauer and e.p.r. spectroscopy has been interpreted as indicating that these metal atoms are arranged as four P clusters and two FeMo-cofactors (FeMoco), where P clusters are a novel type of [4Fe-4S] cluster and FeMo-cofactors are unique clusters containing Mo, Fe and S^{2-} (for a review see Smith, 1983).

In dithionite-reduced Kp1 the P clusters are e.p.r.-silent and the isomer shift of their iron atoms indicates that all are Fe^{2+} (Smith & Lang, 1974); it is therefore suggested that they are at the [4Fe-4S] 0 oxidation level. The FeMoco centres give rise to a characteristic $S = \frac{1}{2}$ e.p.r. signal (g values near 4.3, 3.7 and 2.01), which integrates to 1 electron spin per Mo atom (Rawlings *et al.*, 1978). Comparison of the e.p.r. spectra of ^{95}Mo - and ^{57}Fe -substituted protein indicated that the unpaired electron is associated with Fe rather than Mo atoms (Smith *et al.*, 1973). FeMoco can be extracted into *N*-methylformamide (Shah & Brill, 1977) to yield a solution capable of activating the inactive Kp1 from mutant strains, such as *nifB* mutants (Hawkes & Smith, 1983), which are defective in FeMoco synthesis. Chemical analyses of extracted FeMoco indicate a stoicheio-

metry of 1 Mo to 7–8 Fe atoms (Shah & Brill, 1977; Smith, 1980; Burgess *et al.*, 1980). However, quantitative analyses of Mössbauer and e.p.r. spectra (Rawlings *et al.*, 1978) and electron–nuclear double-resonance (ENDOR) data (Hoffmann *et al.*, 1982) suggest that only 6 Fe atoms per Mo are associated with the $S = \frac{1}{2}$ centre. This discrepancy appears to have been resolved by the extraction of a fragment that contains only 6 Fe atoms per Mo. This fragment, as isolated in butan-2-one, has an e.p.r. signal with g values of 2.05 and 2.01, but recovers the e.p.r. signal characteristic of the $S = \frac{1}{2}$ FeMoco centre when exchanged into *N*-methylformamide. However, it is incapable of activating extracts of *nifB* mutants (Shah & Brill, 1981). It therefore appears that, in addition to the $S = \frac{1}{2}$ centre, FeMoco contains one or two Fe atoms that are not associated with the unpaired electron but are necessary for activity.

During slow enzyme turnover at 10°C, it is possible to detect several transient e.p.r. signals, particularly in the $g = 2$ region (Lowe *et al.*, 1978). These originate from the MoFe protein and are generally of low intensity (≤ 0.06 electron spin/molecule). The intensities of different species of these signals responded to variations in the concentrations of substrates, products or the inhibitor CO.

Recently we purified the inactive (FeMoco-deficient) Kp1, from an *nifB* mutant (UNF1718), and showed that it contained normal P clusters and that it combined with FeMoco to yield holoenzyme similar to native Kp1 (Hawkes, 1981; Smith & Hawkes, 1981; Hawkes & Smith, 1983). We now describe the activation of this protein with either $^{56}\text{FeMoco}$ or $^{57}\text{FeMoco}$ followed by comparative e.p.r. studies to determine whether a particular e.p.r. signal (signal VI) (Lowe *et al.*, 1978), observed from enzyme turning over under an atmosphere of

Abbreviations used: Kp1 and Kp2, MoFe protein and Fe protein components respectively of *Klebsiella pneumoniae* nitrogenase; FeMoco, FeMo-cofactors.

ethylene, originated from the FeMo-cofactors or from the P centres.

Materials and methods

All chemicals were from BDH Chemicals (Poole, Dorset, U.K.) and all biochemicals from Sigma Chemical Co. (Poole, Dorset, U.K.). Except where otherwise stated, protein solutions were handled under an atmosphere of N_2 and were in 50 mM-Tris/HCl buffer, pH 7.4, containing 0.6 mM-dithiothreitol and 2 mM- $Na_2S_2O_4$. Kp1 and Kp2 from wild-type *K. pneumoniae* and the inactive Kp1 (NifB-Kp1) from the *nifB* mutant strain (UNF1718) were purified and assayed as described previously (Eady *et al.*, 1972; Smith *et al.*, 1976; Hawkes & Smith, 1983). Kp1 enriched in ^{57}Fe (^{57}Fe -Kp1) was obtained from cells grown on medium enriched in ^{57}Fe (Smith & Lang, 1974). Stock solutions of Kp1, ^{57}Fe -Kp1, NifB-Kp1 and Kp2 had specific activities of 1600, 1300, 300 and 1100 nmol of acetylene reduced/min per mg respectively. FeMoco was extracted from Kp1 or ^{57}Fe -Kp1 into *N*-methylformamide by a standard method, but the final step of gel filtration through Sephadex G-100 was omitted (Smith, 1980).

Kp1 with the FeMoco centres specifically enriched in ^{57}Fe (^{57}Fe Moco-Kp1) was prepared by incubating an excess of ^{57}Fe Moco with NifB-Kp1 for 30 min at 30°C. Care was taken that the final concentration of *N*-methylformamide in the incubation medium did not exceed 10% (v/v). The protein was then bound to a column of DEAE-cellulose, washed with 0.16 M-NaCl and eluted with 0.35 M-NaCl. ^{56}Fe Moco-Kp1 was prepared from ^{56}Fe Moco and NifB-Kp1 in a similar manner. Stock solutions of ^{57}Fe Moco-Kp1 and ^{56}Fe Moco-Kp1 thus prepared had specific activities of 580 and 341 nmol of acetylene reduced/min per mg respectively.

Reaction mixtures contained final concentrations of 18 mM-phosphocreatine, 18 mM-ATP, 0.1 mg of creatine kinase/ml, 28 mM- $MgCl_2$, 30 mM- $Na_2S_2O_4$ and 110 mM-NaCl in 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] / NaOH with 18 mM-Tris/HCl buffers at pH 7.4 to which were added the premixed proteins to give final concentrations of 5 μ M-Kp2 with 32 μ M- ^{56}Fe Moco-Kp1 or 28 μ M- ^{57}Fe Moco-Kp1 or 20 μ M- ^{57}Fe -Kp1 in a volume of 0.56 ml. Reactions, at 10°C in a shaking water bath, were in 7.8 ml serum vials containing an atmosphere of ethylene and sealed with rubber closures.

After an appropriate reaction time the liquid content of each vial was transferred to an e.p.r. tube, maintained anaerobically at 10°C, by using a pre-cooled Ar-flushed syringe fitted with a 30 cm stainless-steel needle. The e.p.r. tube was then plunged into isopentane at -140°C. At the same

time 0.5 ml of 30% (w/v) trichloroacetic acid was injected into the assay vial to quench the activity of residual protein. The H_2 content of each vial was then measured.

E.p.r. spectra were measured as previously described (Lowe *et al.*, 1972), with a Varian E9 spectrometer fitted with a variable-temperature probe.

Results and discussion

E.p.r. signal VI was elicited from functioning enzyme essentially as previously described (Lowe *et al.*, 1978). Mixtures of Kp2 with a 4–6-fold molar excess of ^{56}Fe Moco-Kp1, ^{57}Fe Moco-Kp1 or ^{57}Fe -Kp1 were added to the other components required for activity as described in the Materials and methods section. The reaction mixtures containing ^{56}Fe Moco-Kp1, ^{57}Fe Moco-Kp1 or ^{57}Fe -Kp1 evolved H_2 at rates of 19, 22 or 40 nmol/min per mg of Kp2 respectively. H_2 -evolution activity remained constant for at least 100 min. Samples for e.p.r. spectroscopy were taken after 12–15 min. The e.p.r. spectra that were recorded from these samples are shown in Fig. 1.

The spectrum (a) from ^{56}Fe Moco-Kp1 shows partial resolution of the g_x and g_y features, whereas the spectra from ^{57}Fe -Kp1 (c) and ^{57}Fe Moco-Kp1 (d) do not, because of broadening due to interaction of the unpaired electron with the ^{57}Fe nucleus. This broadening is apparent if the combined width of the

Fig. 1. E.p.r. signal VI from Kp1 during enzyme turnover under ethylene

The experimental signals were elicited as described in the text with (a) ^{56}Fe Moco-Kp1, (c) ^{57}Fe -Kp1 and (d) ^{57}Fe Moco-Kp1, and were obtained from samples at 18.3 K with 1.0 mT field modulation at 100 kHz. The microwave power and frequency were 20 mW and 9.222 GHz respectively. Spectra (b) and (e) are simulations of spectra (a) and (d) respectively made by using the parameters detailed in the text.

g_x and g_y features in spectrum (a) is compared with those of spectra (c) and (d). It was not possible to estimate the magnitude of the broadening directly from the spectra owing to the overlap of g_x and g_y . However, we have simulated the normal and ^{57}Fe -broadened spectra [see (b) and (e) respectively] by using $g_z = 2.12$, $g_y = 1.998$ and $g_x = 1.987$ (the absolute values of g_x and g_y are only accurate to three significant figures). These simulations indicate broadening of the half-linewidths of the g_x and g_y features from 0.53 to 0.83 mT. This demonstrates that e.p.r. signal VI originates from the FeMoco factors and not the P centres in Kp1. Thus, during enzyme turnover under ethylene, a proportion of the FeMoco centres are in a novel $S = \frac{1}{2}$ spin state. An $S = \frac{1}{2}$ -type e.p.r. signal (g values at 2.05 and 2.01) has also been observed from the inactive MoFe₆ fragment isolated from the MoFe protein in butan-2-one (Shah & Brill, 1981). This fragment can also exhibit the typical $S = \frac{3}{2}$ e.p.r. signal after its exchange into *N*-methylformamide.

Our assignment of signal VI to the FeMoco centres is consistent with the catalytic scheme originally proposed (Lowe *et al.*, 1978), although at that time the extraction of FeMoco had not been reported and its unique character was not appreciated. In general, the family of e.p.r. signals with g values near 2 that are observed from Kp1 during enzyme turnover bear similarities to the spectra of conventional [4Fe-4S] clusters. Of the two types of cluster now thought to be present in Kp1, the P clusters seem to be the most conventional, and indeed under appropriate conditions they can apparently be extruded as [4Fe-4S] cores (Kurtz *et al.*, 1979). P clusters have been observed in various oxidation levels: an e.p.r.-silent [4Fe-4S]⁰ state in Na₂S₂O₄-reduced protein, a transient, e.p.r.-active, $S = \frac{1}{2}$, [4Fe-4S]¹⁺ state (Smith *et al.*, 1983), and an e.p.r.-silent, $S = \frac{1}{2}$, [4Fe-4S]¹⁺ state (Johnson *et al.*, 1981) in partially oxidized protein. Oxidation as far as the [4Fe-4S]³⁺ level during enzyme turnover is one mechanism whereby P centres could give rise to HiPIP-type e.p.r. signals ($g_{\text{av.}} > 2.0$). However, oxidation of the P centres to such a level would require a relatively high potential, which is unlikely to be reached during enzyme turnover. This fact, coupled with our discovery that one such signal (signal VI) arises from FeMoco, suggests that the other intermediate e.p.r. signals with $g_{\text{av.}} > 2.0$ (signals IV and V) also arise from FeMoco, the differing g values indicating slightly different environments for the unpaired electron. Such slight variations in the environment could arise by binding different substrates, products or CO to the Mo atom of FeMoco, thereby causing slightly different changes in the detailed geometry of the nearby Fe atoms with which the unpaired electron is associated.

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