RESEARCH COMMUNICATION

Nitrogenase of *Klebsiella pneumoniae*: electron nuclear double resonance (ENDOR) studies on the substrate reduction site

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Proton electron nuclear double resonance (ENDOR) spectra from the iron–molybdenum cofactor (FeMoco) of *Klebsiella pneumoniae* nitrogenase bound to the enzyme show that a wide variety of substrates and inhibitors, including dinitrogen, acetylene and cyanide, do not bind at or close to FeMoco in the dithionite-reduced state of the free MoFe protein, in agreement with our previous kinetic studies. Therefore models for substrate binding to FeMoco must consider structures at a more reduced

level than that described by Kim and Rees [(1992) *Science* **257**, 1677–1682]. After the enzyme has turned over in the presence of $^2\text{H}_2\text{O}$, an additional set of protons are potentially available for exchange, namely those that can give rise to dihydrogen during enzyme turnover or generate the hydridic dinitrogen binding site; such exchangeable protons were not observed. They cannot therefore be proposed in order to explain the unusual geometry of the ‘trigonal iron atoms’ observed in the structure of FeMoco.

INTRODUCTION

Biological nitrogen fixation is catalysed by nitrogenase, which comprises two metalloproteins (Thorneley and Lowe, 1985; Orme-Johnson, 1985; Burgess, 1985; Smith, 1990; Thorneley, 1992; Smith and Eady, 1992; Lowe, 1992a). These are the MoFe protein, containing the probable substrate reduction site on a heterometallic cluster called FeMoco, and the Fe protein, the specific reductant of the MoFe protein. X-ray crystallographic studies have revealed the structure of the proteins and generated metal cluster models consistent with spectroscopic and structural data (Kim and Rees, 1992; Bolin et al., 1993a; Chan et al., 1993).

The proposed structure of FeMoco (Figure 1) raises a number of interesting questions, the most important being arguably the precise position of the substrate binding and reduction site(s). There is a variety of speculative proposals concerning the position of this site, including: (1) side-on between a pair of the six central Fe atoms at the site that may have a less well defined ligand (see Figure 1 and its legend) (Orme-Johnson, 1992); and (2) between these six Fe atoms at a site within a more reduced FeMoco, which then expands as the dinitrogen becomes protonated and reduced to ammonia (Chan et al., 1993; Rees et al., 1993a). An unusual feature of the FeMoco model is that each of these six Fe atoms is trigonally co-ordinated by sulphide, and is displaced out of the plane of the sulphurs towards the centre of the cluster to give it approximately sp^3 geometry. No ligand as heavy as sulphur is apparent at this position (Chan et al., 1993) but hydrides, or other light groups, could be present. Chan et al. (1993) have postulated the existence of weak Fe–Fe bonds, which are also considered to be important in the theoretical study of Deng and Hoffmann (1993); the latter authors consider a number of other models for dinitrogen binding.

Our kinetic model suggests that no substrates, with the possible exception of the protons that are reduced to dihydrogen, bind to the redox level of isolated MoFe protein which occurs in the presence of dithionite (Lowe and Thorneley, 1984a,b; Thorneley

and Lowe 1984a,b); this is the state of the MoFe protein on which the X-ray crystallographic and other structural work has been done. The model also indicates that dinitrogen binds at a reduced hydridic site by displacing dihydrogen. A characterization of the protons that are precursors of such hydrides, and of dihydrogen, is therefore of great relevance to the nature of the dinitrogen binding site and may enable us to distinguish between the various proposals.

Electron nuclear double resonance (ENDOR) has been used by Hoffman and his co-workers as a sensitive probe of the environment of FeMoco in nitrogenase (see Lowe, 1992b, for a review). In particular, Venters et al. (1986) have shown, using proton ENDOR in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$, that there is a class of exchangeable protons coupled to FeMoco; thus the environment of FeMoco is accessible to solvent, despite the fact that it appears, in the crystal structure, to be buried in the protein. Bolin et al. (1993b) have in fact observed at least twelve probable water-binding positions close to the molybdenum/homocitrate end of FeMoco.

Since substrates bound to FeMoco would perturb the interactions between the unpaired electrons on the cluster and neighbouring protons, we have observed the effects of various substrates on proton ENDOR from FeMoco. The technique can also characterize additional coupled protons that become exchangeable when MoFe protein is active in the presence of $^2\text{H}_2\text{O}$, protons which could be the precursors of the hydrides bound to FeMoco before being displaced by dinitrogen (or possibly the hydrides themselves) and which would be undetectable by X-ray crystallography.

MATERIALS AND METHODS

Nitrogenase proteins

Nitrogenase component proteins were purified from *Klebsiella pneumoniae* (*oxytoca*) N.C.I.B. 12204 and assayed as described by Thorneley and Lowe (1983). The MoFe protein (Kp1)

Abbreviation used: ENDOR, electron nuclear double resonance.

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Figure 1 A model fitting the X-ray crystallographic electron density profile of FeMoco from nitrogenase MoFe protein (after Kim and Rees, 1992)

Within the cluster (solid bonds), Fe-atoms are represented by solid circles and S-atoms by open circles; the Mo-atom is co-ordinated by three cluster S-atoms, an imidazole N-atom from a histidine residue and a hydroxyl O-atom and a carboxylate O-atom from homocitrate. There exists some question as to the identity of one of the central S-atoms, as according to Kim and Rees (1992) and Chan et al. (1993) this has somewhat lower than the expected electron density for an S-atom, whereas Bolin et al. (1993c) did not observe this anomaly.

contained 1.4 ± 0.1 g-atom of Mo/mol of protein. Kp1 and the Fe protein (Kp2) had specific activities at 30 °C of 2000 and 1400 nmol of ethylene produced/min per mg of protein respectively.

Reagents

Biochemicals were purchased from Sigma Chemical Co., Poole, Dorset, U.K., and all salts were from BDH Chemicals. $^2\text{H}_2\text{O}$ (99.9 atomic % ^2H) was from Goss Scientific.

ENDOR samples

Kp1 was concentrated to 0.35–0.55 mM by membrane filtration using Minicon B15 concentrators (Amicon Ltd., Upper Mill, Stonehouse, Gloucestershire, U.K.) and was in 25 mM Hepes buffer, pH 7.4, 200 mM NaCl and 10 mM $\text{Na}_2\text{S}_2\text{O}_4$ unless otherwise stated. Protein concentrations were measured by the Biuret method (Gornall et al., 1949). Gases were added by equilibration with the protein solution in 7.8 ml serum bottles at room temperature (approx. 23 °C) before carefully transferring the solution to the ENDOR tubes, using a gas-tight syringe, and freezing in liquid nitrogen. Nitrogenase samples that had turned

over in $^2\text{H}_2\text{O}$ were 0.55 mM MoFe protein, 0.30 mM Fe protein, 20 mM $\text{Na}_2\text{S}_2\text{O}_4$, 10 mM MgCl_2 , 1 mM ATP, 25 mM Hepes buffer, pH 7.4, and were incubated, in an anaerobic glove box, for 30 min under N_2 gas for ATP to become exhausted, before freezing inside the glove box in methanol cooled to -30 °C by cold dinitrogen gas as described by Thorneley and Lowe (1983). A similar procedure was followed to prepare the samples that had turned over in the presence of cyanide, except that 5 mM NaCN was added to the solution at the start of the incubation. ENDOR spectra were recorded in 4 mm internal diameter Suprasil quartz tubes on an updated Bruker ER 200D-SRC spectrometer with an ENDOR/TRIPLE accessory and a radio-frequency amplifier of 100 W nominal output power. Measurements were at a temperature of 2.5 K using an Oxford Instruments e.s.r. 910 pumped helium cryostat. Other recording conditions were: microwave power, 5 mW; radio frequency power, 0 dB attenuation; modulation depth, 50 kHz; scan rate, $0.24 \text{ MHz} \cdot \text{s}^{-1}$.

RESULTS AND DISCUSSION

Substrate binding

Figure 2 shows the $S = 3/2$ e.p.r. spectrum from FeMoco in Kp1 in $^1\text{H}_2\text{O}$ at the dithionite-reduced oxidation level. All ENDOR measurements reported here were made at the field value corresponding to the arrowed feature (g_3) in Figure 2. Figure 3(a) shows the first derivative proton ENDOR spectrum measured under these conditions, which is very similar to the absorption spectrum reported by Venters et al. (1986).

We have studied proton ENDOR spectra from FeMoco in nitrogenase in the presence of the following wide variety of substrates, products and inhibitors at 1 atm (100 kPa) for gases and the given concentrations for solids: N_2 , C_2H_2 , C_2H_4 , CO, NaCN (5 mM), N_2O , CH_3NC (5 mM), NaN_3 (10 mM), NaF (50 mM) (which showed no observable ^{19}F signals), NaNO_2 (50 mM), NH_4CN (5 mM), $(\text{CN})_2\text{C}=\text{C}(\text{CN})_2$ (10 mM), MgATP (9 mM), MgADP (9 mM) and MgADP (9 mM) plus Fe protein (equimolar with the MoFe protein). None of these interacted with the FeMoco in such a way as to change the proton ENDOR signal from that of Figure 3(a). Thus none of them bind close to FeMoco at this oxidation level or bind elsewhere and change the FeMoco environment significantly. We estimate that bound substrates must be beyond the secondary co-ordination shell of the metal atoms of FeMoco. In general, these results are consistent with: (1) our kinetic model that indicates that reducible substrates bind at more reduced levels of FeMoco; (2) any nucleotide binding to MoFe protein at the dithionite-reduced oxidation level being at a site remote from the FeMoco; (3) the suggestion of Rees et al. (1993b) that the Fe protein binding site is also remote from FeMoco; and (4) the effects of nucleotide or Fe protein binding not being transmitted allosterically through the MoFe protein to alter the FeMoco environment.

A number of interesting points also arise on consideration of the results from some of the specific additions. There is kinetic evidence that azide, cyanide and methylisocyanide all bind either at the dithionite-reduced, or at the one-electron-reduced, level of FeMoco in MoFe protein (see Burgess, 1985, for a review). Our results indicate that they do not bind close to FeMoco at the dithionite-reduced level and therefore, assuming that FeMoco is the reducible substrate binding site, that they must bind at the one-electron-reduced level. In addition, Lowe et al. (1989) have proposed that cyanide reduction requires a change of binding site (FeMoco) ligation. We have now shown that turnover in the presence of 5 mM NaCN has no effect on the protein-bound FeMoco proton ENDOR spectrum, so that if this change in

Figure 2 E.p.r. spectrum from FeMoco in *K. pneumoniae* nitrogenase MoFe protein in $^1\text{H}_2\text{O}$

Microwave frequency, 9.433 GHz; microwave power, 100 mW; temperature, 10 K; modulation amplitude, 0.985 mT at 100 KHz. The arrow indicates the g -value at which all the ENDOR measurements were made.

Figure 3 ENDOR spectra from FeMoco in *K. pneumoniae* nitrogenase MoFe protein in (a) $^1\text{H}_2\text{O}$, (b) $^2\text{H}_2\text{O}$, (c) turned over in $^2\text{H}_2\text{O}$

The arrows in (a) indicate the proton set that undergoes exchange with solvent protons.

ligation occurs, it must reverse after the incubation in the dithionite-reduced state needed to regenerate the e.p.r. (and ENDOR) signals.

Smith et al. (1973) observed a small change in the rhombicity of the e.p.r. spectrum of Figure 2 with pH, and reported that the apparent pK associated with this change was displaced in favour of the high-pH form in the presence of the substrate C_2H_2 . This is clear evidence that C_2H_2 binds to this form of the enzyme. However, our observation that the proton ENDOR spectrum is not affected must mean that the proton (and C_2H_2) binding site associated with this pK are remote from the FeMoco itself. Consistent with this deduction, we have also observed no effect

of pH on the ENDOR spectrum in samples prepared at pH values of 7.4 and 9.5 (Lowe et al., 1990).

Exchangeable protons

The ENDOR spectrum shown in Figure 3(b), measured in $^2\text{H}_2\text{O}$, shows the absence of the resonances (arrowed in Figure 3a) from the exchangeable class of protons with a hyperfine coupling of 1.8 MHz also reported by Venters et al. (1986). Figure 3(c) shows the spectrum from a sample of nitrogenase that has been turning over in $^2\text{H}_2\text{O}$, when the MoFe protein has been reduced by the Fe protein to the substrate binding and reduction state, and then allowed to re-oxidize to the dithionite-reduced level, potentially introducing a new set of exchangeable protons. Figures 3(b) and 3(c) are indistinguishable, indicating that no further protons become exchangeable under these conditions. Therefore none of the 'non-exchangeable' protons that can be observed by ENDOR of FeMoco in MoFe protein at the dithionite-reduced level give rise to dihydrogen or ammonia under turnover conditions, either directly or after translocation from amino acid residue side chains or homocitrate. Under our conditions they would have been replaced by ^2H from the solvent. Thus only those protons that exchange at the dithionite-reduced oxidation level of MoFe protein are candidates for the origin of hydrides bound at or near to FeMoco, if such protons remain bound to FeMoco in this oxidation state.

These observations need to be taken into account in models of substrate reduction and FeMoco structure. In particular, it is highly unlikely that the hydrides that give rise to H_2 at the turnover oxidation level are in the centre of FeMoco as ligands to the Fe atoms at the dithionite-reduced oxidation level. These results are consistent with a recent brief report in a meeting abstract (Hoffman, 1993) that ENDOR has shown that the trigonal Fe atoms have neither hydride nor solvent as fourth ligands. Such hydrides cannot therefore be proposed in order to explain the unusual iron atom environment observed in the X-ray crystallographic studies that were performed at the dithionite-reduced oxidation level.

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