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Nitrogenase from *nifV* mutants of *Klebsiella pneumoniae* contains an altered form of the iron–molybdenum cofactor

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When the iron–molybdenum cofactor (FeMoco) was extracted from the MoFe protein of nitrogenase from a *nifV* mutant of *Klebsiella pneumoniae* and combined with the FeMoco-deficient MoFe protein from a *nifB* mutant, the resultant MoFe protein exhibited the NifV phenotype, i.e. in combination with wild-type Fe protein it exhibited poor N_2-fixation activity and its H_2-evolution activity was inhibited by CO. These data provide strong evidence that FeMoco contains the active site of nitrogenase. The metal contents and e.p.r. properties of FeMoco from wild-type and *nifV* mutants of *K. pneumoniae* are very similar.

The enzyme nitrogenase, which catalyses biological nitrogen fixation, consists of two essential metalloproteins, the Fe protein and the MoFe protein (Eady & Smith, 1979; Mortenson & Thorneley, 1979). The enzyme from wild-type organisms can reduce N_2, acetylene, several other small triple-bonded molecules and H^+ (Hardy, 1979). H_2 evolution is largely inhibited by all other substrates. Electrons are transferred from a reductant (normally $Na_2S_2O_4$ *in vitro*) to the MoFe protein via the Fe protein in a MgATP-hydrolysing reaction. The MoFe protein contains the site for binding reducible substrates. Under optimal conditions for N_2 reduction (i.e. at high rates of electron flux using a large excess of the Fe protein), 70–75% of the electrons reduce N_2 and the remainder reduce H^+ to H_2. CO inhibits the reduction of all substrates except H^+.

Abbreviations used: Kp1 and Kp2 respectively refer to the MoFe protein and Fe protein of nitrogenase from wild-type *Klebsiella pneumoniae*. The MoFe proteins from *nifB* and *nifV* mutant strains are referred to as 'NifB$^-$Kp1' and 'NifV$^-$Kp1' respectively, whereas 'NifV$^-$ nitrogenase' refers to the combination of NifV$^-$Kp1 with wild-type Kp2. The iron–molybdenum cofactor extracted from Kp1 is referred to as 'FeMoco' and from NifV$^-$Kp1 as 'NifV$^-$FeMoco'. A unit of nitrogenase activity is 1 nmol of acetylene reduced/min. Bes, 2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid.

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NifV mutants of *Klebsiella pneumoniae* contain nitrogenase with a normal Fe protein (Kp2), but an MoFe protein (NifV$^-$Kp1) that is defective (McLean & Dixon, 1981). The enzyme reduces nitrogen at a lower rate than the wild-type enzyme (N_2 reduction is limited to about 49% of the total electron flow) and evolves H_2 in a reaction which, unlike that catalysed by the wild-type, is about 70% inhibited by CO (McLean *et al.*, 1983).

The nature of the substrates, products and inhibitors of nitrogenase implies the presence of metal atoms at the active site. The most active preparation of the MoFe protein contains 2 Mo atoms and 33 ± 3 Fe atoms in a molecule of $M_r \simeq 220000$ with an $\alpha_2\beta_2$ tetrameric structure. The metal atoms are probably arranged as two iron–molybdenum cofactors (FeMoco) (with the stoichiometry $MoFe_{6-8}S_{4-9}$) and four unusual [4Fe–4S] clusters (Zimmermann *et al.*, 1978; Smith, 1983; Nelson *et al.*, 1983). FeMoco can be extracted from the MoFe protein into *N*-methylformamide (Shah & Brill, 1977; Smith, 1980) and can activate extracts of mutants which lack FeMoco. The inactive MoFe protein, NifB$^-$Kp1, of one such mutant, a *nifB* mutant of *K. pneumoniae*, has been isolated and characterized (Hawkes & Smith, 1983) and shown to contain 'P' clusters in essentially the same form as in the native protein (Robinson *et al.*, 1984).

Although it has been assumed for many years that molybdenum is at the active site of nitrogenase, the evidence for this is only circumstantial (Smith, 1977). Here we report some of the

properties of the FeMoco (NifV$^-$FeMoco) of NifV$^-$Kp1. Our data provide very strong evidence that FeMoco includes the N_2-binding site of nitrogenase.

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 50mM-Tris/HCl buffer, pH 7.4, containing 0.6mM-dithiothreitol and 2mM-$Na_2S_2O_4$. Protein concentrations, acetylene reduction activities (1 unit of activity = 1 nmol of acetylene reduced/min), H_2-production activities, and metal contents were measured by the techniques described by Eady *et al.* (1972).

Wild-type Kp1 and Kp2 from *K. pneumoniae* strain M5a1 (Eady *et al.*, 1972; Smith *et al.*, 1976), NifB$^-$Kp1 from *K. pneumoniae* strain 5058 (Hawkes & Smith, 1983) and NifV$^-$Kp1 from *K. pneumoniae* strain UNF1613 (McLean & Dixon, 1981) were purified as previously described. NifB$^-$Kp1 was only partially purified to a specific activity (after saturation with FeMoco) of 250units/mg, but was free from ATP-independent hydrogenase activity.

Purified Kp1 and NifV$^-$Kp1 were precipitated with dimethyl sulphoxide, washed with dimethylformamide and their FeMoco extracted with *N*-methylformamide as previously described (Smith, 1980). Extraction efficiency was usually about 35% for Kp1 and 10–20% for NifV$^-$Kp1. One sample of NifV$^-$FeMoco was chromatographed on Sephadex G-100 in *N*-methylformamide. The wild-type and NifV$^-$FeMoco preparations were incubated (Hawkes & Smith, 1983) with a 1–4-fold excess of NifB$^-$Kp1 (based on activity units) at 30°C, pH 7.5, for 30min. The activated protein was then assayed for H_2-evolution activity (Eady *et al.*, 1972), with an approx. 50-fold excess of Kp2, in 7.5ml assay vials, under atmospheres of Ar, N_2 or Ar + 3039Pa(0.03atm)-CO. The activity of the activated proteins was linear with concentration at least over the range 6–100nM (based on the original NifB$^-$Kp1 concentration), indicating no dissociation of the FeMoco from NifB$^-$Kp1 at concentrations well below the normal assay concentration ($\simeq$100nM). Oxygen was rigorously excluded from all of the above operations.

E.p.r. spectra were measured as previously described (Lowe *et al.*, 1972) with a Varian E9 or a Bruker ER200D-SRC spectrometer, both fitted with variable-temperature probes.

Results and discussion

FeMoco and NifV$^-$FeMoco were extracted into *N*-methylformamide and incubated with partially purified (hydrogenase-free) NifB$^-$Kp1 to form

Table 1. *H_2-evolution properties associated with the MoFe proteins formed from combining NifB$^-$Kp1 with either wild-type FeMoco or NifV$^-$FeMoco*

The NifB$^-$Kp1 was about 50–100% activated by wild-type FeMoco but was only 25–50% activated by NifV$^-$FeMoco, since the efficiency of extraction of the latter was lower (see the Materials and methods section) and the concentration of *N*-methylformamide in the incubation mixture was limited to 8% (v/v) (Hawkes & Smith, 1983). The MoFe protein formed in the incubations was assayed with an excess of Fe protein as described in the Materials and methods section. The H_2-evolution values for preparations 4–7 have been corrected for the small, residual, wild-type activity of the Kp2 protein caused by contamination with traces of Kp1.

FeMoco preparation	Source of FeMoco	H_2 (nmol/assay) evolved under: Ar	Ar + CO	N_2	Inhibition (%) of H_2 evolution by: CO	N_2	No. of assays averaged
1	Wild-type Kp1	78.9 ± 0.9	77.9 ± 4.4	23.8 ± 0.8	< 1.5	70	3
2	Wild-type Kp1	32.9*	39.9	10	None	70	2
3	Wild-type Kp1	86.8* ± 1.3	93.2 ± 3.4	26.1 ± 0.4	None	72	3
4	NifV$^-$Kp1	37.3 ± 3.9	16	27.9 ± 0.9	58	26	2
5	NifV$^-$Kp1	19.5 ± 0.8	7.3 ± 0.4	14.2	63	27	2
6(*a*)	NifV$^-$Kp1 (pre-column)	26.9 ± 1.5	7.1 ± 0.6	15.8 ± 0.6	74	41	3
6*b*)	NifV$^-$Kp1 (after Sephadex G-100 chromatography)	17.9 ± 1.2	4.6 ± 0.1	12.5 ± 1.2	74	30	3
7	NifV$^-$Kp1	36.3 ± 0.4	14.5 ± 1.3	20.5 ± 0.9	60	34.5	3

* These low activities (relative to the Ar + CO values) were due to contamination of the Ar with N_2 as observed by g.l.c. with detection by thermal conductivity. Therefore the percentage inhibition was calculated with respect to the Ar + CO value.

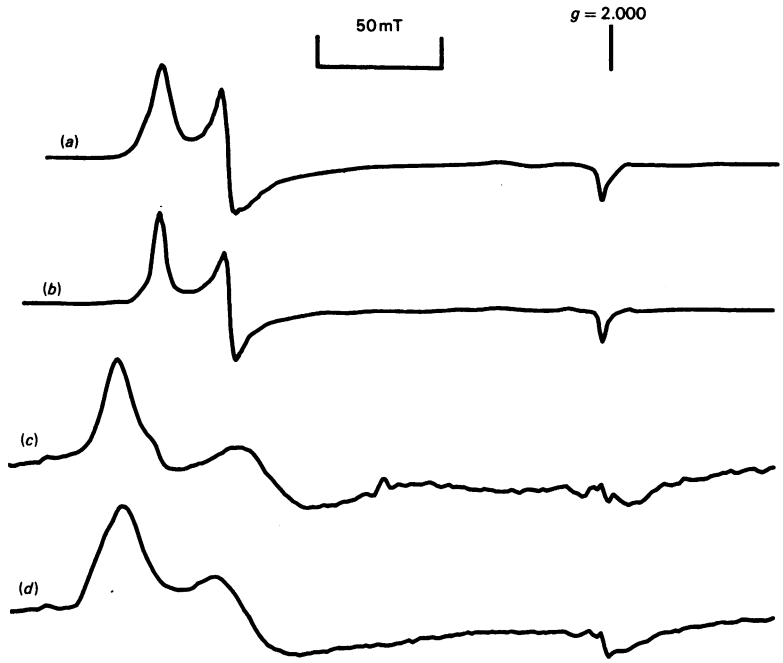

Fig. 1. *E.p.r. spectra of NifV⁻Kp1, Kp1 and their FeMoco*
(*a*) NifV⁻Kp1 (specific activity 1100units/mg); (*b*) Kp1 (specific activity 1300units/mg) were dialysed at 20°C for 15h against 50mM-Bes buffer, pH6.8, containing 2mM-$Na_2S_2O_4$, and e.p.r. spectra were recorded at 9GHz on samples in 3mm-int.diam. quartz tubes at 12K with a field modulation of 1.6mT at 100KHz and a microwave power of 100mW. The spectra of (*c*) NifV⁻FeMoco and (*d*) FeMoco were recorded with similar running conditions, except that the temperature was 6K and the microwave power 20mW.

active MoFe proteins. The substrate-reducing and CO-inhibition properties of these proteins in combination with Kp2 (Table 1) indicated that Kp1 formed in this manner retained the phenotypic properties of the MoFe protein used as a source of FeMoco. Thus the MoFe protein formed from NifB⁻Kp1 and wild-type FeMoco, like all wild-type MoFe proteins (Hardy, 1979), has its H_2-evolution activity inhibited 70–75% by N_2, but not at all by CO, whereas when NifV⁻FeMoco was combined with NifB⁻Kp1 the resultant protein, like NifV⁻Kp1 (McLean & Dixon, 1981), had its H_2-evolution activity inhibited $\leqslant 41\%$ by N_2 but 58–74% by CO. Sephadex G-100 gel filtration (Shah & Brill, 1977; Smith, 1980) of NifV⁻FeMoco resulted in a single peak of cofactor activity which retained this property of transferring the NifV⁻ phenotype. These results show that impaired N_2 reduction by *nifV* mutants is due to a defective FeMoco and strongly suggest that FeMoco is the site of N_2 (and CO) binding.

Further evidence linking substrate reduction by the enzymes from wild-type and mutant strains with their FeMoco and indicating impaired activity in the latter comes from an analysis of acetylene-reduction activities. The reported activities (Smith, 1980; Burgess *et al.*, 1980; Shah, 1980) of wild-type FeMoco preparations (after incorporation into a FeMoco-deficient protein such as NifB⁻Kp1) have ranged from 250 to 300units/ng-atom of Mo. Similarly, ten fractions of Kp1 with specific activities between 1090 and 1770units/mg of Kp1 gave activities of 275 ± 27units/ng-atom of Mo, indicating that the specific activity of Kp1 preparations is linearly related to their Mo content. However, an analysis of six NifV⁻Kp1 fractions with specific activities between 1050 and 1550units/mg of NifV⁻Kp1 gave activities of only 181 ± 10units/ng-atom of Mo, a value only two-thirds that of the wild-type. The impaired N_2-fixing ability of NifV⁻ nitrogenase is reflected in its apparent K_m of $\simeq 60$kPa(0.6atm)-N_2 (results not shown) compared with $\simeq 5$kPa(0.05atm)-N_2 for the wild-type enzyme.

FeMoco exhibits a characteristic e.p.r. spectrum both when bound to, and when extracted from, the protein (Rawlings *et al.*, 1978; Eady & Smith, 1979; Mortenson & Thorneley, 1979; Smith, 1980). The e.p.r. spectra of $Na_2S_2O_4$-reduced Kp1 and NifV⁻Kp1 at pH6.8 and of the FeMoco extracted

from these proteins are shown in Fig. 1. They arise from transitions within the $M_s = \pm\frac{1}{2}$ Kramers doublet of a $S = \frac{3}{2}$ spin system (Eady & Smith, 1979). The linewidths of the e.p.r. signals from the extracted FeMoco samples are broad, and the exact g-values of the spectral features varied slightly between preparations. Therefore we are unable, at present, to discern any subtle differences between the spectra of wild-type and NifV$^-$FeMoco. However, the linewidths of the signal from NifV$^-$Kp1 are broader than those from Kp1, with shoulders on all features. The signal from NifV$^-$Kp1 is apparently composed of two overlapping spectra, the major component of which has g-values within ± 0.01 of the wild-type spectrum. Two species of wild-type Kp1 are also observable by e.p.r., but at a higher pH. They are related by a pK_a of 8.7, and the equilibrium between them is perturbed by the substrate, acetylene (Smith *et al.*, 1973). Thus the e.p.r. signal of FeMoco can act as a sensitive monitor of protein conformation, and its seems likely that the two species contributing to the NifV$^-$Kp1 e.p.r. spectrum also reflect a conformation equilibrium, but one that is not observed in Kp1 at pH6.8. Our NifV$^-$Kp1 preparations are not mixtures of the wild-type and mutant enzymes, since their H_2-evolution properties (McLean *et al.*, 1983) are distinct from those of the wild-type, and our *nifV* mutant cultures did not fix N_2.

Mössbauer and e.p.r. studies on active MoFe proteins have indicated that six Fe atoms share the unpaired electron associated with $Na_2S_2O_4$-reduced FeMoco (Rawlings *et al.*, 1978). Another iron–molybdenum cluster with an Fe/Mo ratio of 6:1 has been isolated from the MoFe protein of *Azotobacter vinelandii*. It had the characteristic e.p.r. signal of the $S = \frac{3}{2}$ centre, but was unable to activate FeMoco-deficient MoFe proteins (Shah & Brill, 1981). Active FeMoco preparations are reported to contain seven to eight Fe atoms per Mo atom; presumably the extra one to two Fe atoms are essential for activity (Shah & Brill, 1977; Burgess *et al.*, 1980; Smith, 1980; Nelson *et al.*, 1983). We prepared FeMoco samples in parallel experiments from NifV$^-$Kp1 and Kp1 using both acid treatment (Shah & Brill, 1977) and precipitation by dimethyl sulphoxide (Smith, 1980). We found no significant difference in the metal ratios in these preparations; the Fe/Mo ratio in the wild-type FeMoco samples was $6.6(\pm 1.0):1$ and in the NifV$^-$FeMoco samples $7.1(\pm 1.1):1$.

In conclusion we have established that NifV$^-$Kp1 contains an incompletely processed form of FeMoco that is responsible for its altered substrate-reducing properties. The structural differences between wild-type and NifV$^-$FeMoco must be subtle, since in the latter the $S = \frac{3}{2}$ centre remains intact (although in a slightly modified environment) and the Mo/Fe ratio is unchanged. However, these structural differences are transferred when FeMoco is extracted from NifV$^-$Kp1 and bound to NifB$^-$Kp1. Chemical-model studies indicate that the nature of the ligands in Mo complexes greatly influences their ability to form N_2 complexes. Electron-donating ligands favour N_2 binding (Chatt *et al.*, 1978). These observations suggest that a probable role for the *nifV*-gene product is to modify the ligation of the metals by adding or subtracting ligands or reorientating existing bonds. However, wild-type FeMoco does not contain amino acids (Smith, 1980; Yang *et al.*, 1982), and the presence of any other endogenous organic component has not been confirmed (Yang *et al.*, 1982). Since the unpaired electron associated with the e.p.r. spectrum of FeMoco is largely located on the Fe atoms (Smith *et al.*, 1973; Orme-Johnson *et al.*, 1981) and the e.p.r. spectrum of NifV$^-$Kp1 is not very different from that of Kp1, modification by the *nifV* gene product seems likely to involve the molybdenum atom of FeMoco. *In vivo*, such a modification might be expected to occur at a late stage in FeMoco biosynthesis, either just before or after its insertion into the MoFe protein.

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