Isolation and characterization of nitrogenase MoFe protein from the mutant strain pHK17 of *Klebsiella pneumoniae* in which the two bridging cysteine residues of the P-clusters are replaced by the non-coordinating amino acid alanine

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Nitrogenase MoFe protein (Kp1) from the mutant strain pHK17 of *Klebsiella pneumoniae* has been purified to give three catalytically active fractions. In this mutant, each of the two bridging cysteine ligands to the P-clusters, α -Cys-89 and β -Cys-94, has been replaced by a non-coordinating residue, alanine. SDS/PAGE and earlier native gels showed that the three fractions retained the normal $\alpha_2\beta_2$ tetrameric form of wild-type Kp1; therefore we conclude that in each of the fractions the subunits are folded differently, thus resulting in different surface charges and allowing separation of the fractions on ion-exchange chromatography. Earlier EPR and magnetic CD data had shown that the mutant fractions contain P-clusters, and thus the mutated residues are not as essential for maintaining the integrity of the P-clusters as they appear from the X-ray structure. The specific activity of each of the three fractions was less than that of wild-type Kp1, the most active fraction having only 50 % of wild-type activity. No change in substrate specificity or in the relative distribution of electrons to various substrates was found. The relationship between ATP hydrolysis and substrate-reducing activity, the EPR spectra of the $S = 3/2$ spin state of the iron–molybdenum cofactor (FeMoco) and the pH profile of

acetylene-reduction activities of the three fractions did not differ significantly from those exhibited by wild-type Kp1. The specific activities of the three mutant fractions and of wild-type Kp1 were linearly proportional to the intensity of the $S = 3/2$ EPR signal from the FeMoco centres. This implies that those molecules of the three mutant fractions and the wild-type protein that contain EPR-active FeMoco are fully active, i.e. that the Cys to Ala substitution of the P-cluster ligands does not affect the specific activity of the protein. This in turn implies that the P-clusters are not directly associated with the rate-limiting step in enzyme turnover. We conclude that the lower specific activities of the mutant fractions are observed because the fractions are mixtures of species containing a full complement of FeMoco and P-clusters and species lacking some or all of these clusters. On the basis of the Mo contents and EPR spectroscopy of the mutant fractions, we propose that the loss of the P-clusters causes (i) the physical loss or inhibition of binding of some FeMoco; (ii) the EPR and catalytic inactivation of some FeMoco; and/or (iii) the incorporation of a FeMoco-like species into the FeMoco site of the mutant molecules.

INTRODUCTION

Nitrogenase catalyses the ATP-dependent reduction of atmospheric dinitrogen to ammonia according to eqn. (1):

The nitrogenase enzyme of *Klebsiella pneumoniae* consists of two proteins: the molybdenum–iron protein (MoFe protein or Kp1) and the iron protein (Fe protein or Kp2) [2,3]. Both of these proteins are required for substrate reduction, with Kp2 acting as a very specific electron donor to Kp1 in an MgATP-hydrolysing reaction. The MoFe protein, an $\alpha_2\beta_2$ tetramer with a molecular mass of ~ 224 kDa, contains 483 and 520 amino acids in the α - and β -subunits respectively. Each $\alpha\beta$ pair of subunits contains two types of metal–sulphur cluster, called the iron–molybdenum cofactor (FeMoco) and the P-clusters.

X-ray crystallographic structures of the MoFe protein [4] showed that the FeMoco clusters are bound solely to the α -subunit and that the P-clusters, as deduced from earlier site-directed mutagenesis experiments [5], are bound at the interface of the $\alpha\beta$ pairs of subunits. FeMoco can be considered as consisting of 4Fe–3S and 1Mo–3Fe–3S clusters which are bridged by three inorganic sulphur atoms (Figure 1, left panel). The cluster is attached to the protein by only two amino acids, α -Cys-275 and α -His-442. These residues bind respectively an Fe atom at one end and the Mo atom at the other end of the cofactor. Each P-cluster is composed of two 4Fe–4S cubes which are held together by two cysteine sulphur atoms, one from each of the α - and β -subunits, and a joint sulphur atom or a single disulphide bond formed between two sulphur atoms of the two cubes (Figure 1, right panel) [6,7]. Apart from the two bridging cysteine residues, i.e. α -Cys-89 and β -Cys-94, each cube of the P-clusters is attached either to the α -subunit through α -Cys-63 and α -Cys-

Abbreviations used: Kp1 protein and Kp2 protein, MoFe and Fe proteins respectively of wild-type *Klebsiella pneumoniae* nitrogenase; pHK17Kp1, MoFe protein isolated from the pHK17 strain of *K. pneumoniae*; pHK17Kp1.A, pHK17Kp1.B and pHK17Kp1.C, the three fractions of pHK17Kp1 isolated on the Q-Sepharose anion-exchange column; FeMoco, iron–molybdenum cofactor.

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Figure 1 Structures of FeMoco (left panel) and P-clusters (right panel) of MoFe proteins of *A. vinelandii* determined by single crystal X-ray analysis [3,7]

The amino acid numbering has been changed to that of *K. pneumoniae*.

155 or to the β -subunit through β -Cys-69 and β -Cys-152. Both FeMoco and the P-clusters are buried ~ 10 Å (1 nm) below the surface of the protein, and the edge-to-edge distance from FeMoco to the P-cluster is about 14 Å [8].

The physiological substrates of nitrogenase are N_2 and H^+ , but it can also catalyse the reduction of a large number of other small multiple-bond-containing molecules such as C_2H_2 , CH_3CN , N_3^- , N_2O , HCN , etc. FeMoco has been identified as the probable substrate-binding site [9], but details of the bonding of substrates and the mode of reduction are not clear. The role of the P-clusters is also unclear, although there is evidence that they may act as 'capacitors', releasing electrons at a critical point in N_2 reduction [10]. Detailed investigation is needed to confirm this hypothesis and to rationalize the structure/function relationships of these unique clusters.

Site-directed mutagenesis is a powerful technique for the investigation of the distribution of metal centres in protein molecules and the identification of ligands to these centres. The technique is also capable of uncovering individual catalytic roles for two or more metallic centres in a given enzyme. In order to understand the structural and functional roles and relationships of FeMoco and P-clusters in nitrogenase, systematic site-directed mutagenesis programmes have been undertaken. Both conserved and non-conserved cysteine residues of the MoFe proteins of *K. pneumoniae* [5,11] and *Azotobacter vinelandii* [12–17] have been replaced by other residues. One of the mutants of *K. pneumoniae*, pHK17, prepared in this laboratory, is unique. It carries substitutions in Kp1 of the two bridging cysteine residues α -Cys-89 and β -Cys-94 at the P-cluster site (Figure 1, right panel) by a non-co-ordinating residue, alanine [5]. From the Figure it appears that this replacement is likely to result in complete breakdown of the P-clusters. However, the mutant strain retained the ability to grow diazotrophically and produced catalytically active Kp1, although when only one of the two cysteine residues was mutated to alanine the resultant strain was no longer capable of growing on N_2 [11]. On account of these unique characteristics, coupled with its fascinating structural implications, we have chosen the double mutant for further investigation. In this paper we report

the isolation, purification and preliminary investigation of the MoFe protein pHK17Kp1 from this mutant strain.

MATERIALS AND METHODS

Bacterial strains

The wild-type and pHK17 mutant strains of *K. pneumoniae* were as described by Kent et al. [5,11]. Briefly, the mutations were introduced into plasmid pWF23, which carries the *nifHDK* genes, and the plasmid was introduced into strain UNF2392 which has a Tn7 insertion in *nifH*, polar on *nifD* and *K*. The integrity of the mutant strain was checked after growth in 200 litre cultures. Some colonies had lost the pWF23 plasmid but none had lost the Tn7 chromosomal insertion.

Growth and medium

An inoculant of 20 l was grown on nutrient broth (8 g/l) supplemented with glucose (2.5 g/l) to a final reading on an Evans Electroselenium Ltd. (EEL) spectrometer of 46% at 560 nm on a transmittance scale. This inoculant was added to a nitrogen-free glucose-based minimal medium at pH 7.4 (200 l) and grown for 24 h under diazotrophic conditions (N_2 ; 50 l/min; temperature, 30 °C; agitation, 150 rev./min) in a 200 l New Brunswick Scientific Fermenter. The cells (~ 500 g) were harvested at an EEL reading of 64 at 540 nm in a Westfalia SA7 disc stack centrifuge and stored in liquid N_2 .

Isolation and purification

All operations were performed anaerobically. Buffer solutions were first flushed with nitrogen, and then $Na_2S_2O_4$ (2–4 mM) was added. Columns were made anaerobic by equilibrating them with suitable buffers containing 2–4 mM $Na_2S_2O_4$. Effluents from columns were collected in conical flasks under nitrogen.

Steps 1 and 2 were carried out at 4–5 °C but the rest of the purification was at room temperature.

Step 1

In a typical purification, 600 g of cell paste of pHK17 was suspended in 600 ml of 0.1 M Tris buffer, pH 8.7, containing 4 mM $\text{Na}_2\text{S}_2\text{O}_4$ and crushed twice in an ice-cooled French pressure cell at 500–600 kPa. The crushed cells were centrifuged at 5 °C for 2 h at ~13000 g. The crude supernatant (1200 ml) containing both Kp1 and Kp2 was chromatographed on the same day or frozen in liquid nitrogen and chromatographed the next day. The latter treatment had no effect on the final yield of the protein.

Step 2

The crude extract was loaded on to a DEAE-cellulose column (9 cm × 8 cm) equilibrated with 50 mM Tris, pH 7.4, containing 4 mM $\text{Na}_2\text{S}_2\text{O}_4$ (hereafter called buffer A). The column was first washed with 1 bed volume of buffer A followed by elution of proteins with 800 ml each of 0.1 M NaCl, 0.2 M NaCl and 0.09 M MgCl_2 prepared in buffer A. Kp1 was eluted in the 0.2 M NaCl fraction and Kp2 was eluted in the 0.09 M MgCl_2 fraction. Since significant spreading of Kp1 occurred, it was sometimes worth collecting Kp1 from the 0.1 M NaCl washing and the 0.09 M MgCl_2 fraction as well.

Step 3

Kp1 solution from the DEAE-cellulose column was either dialysed against buffer A or diluted with buffer A to 50 mM NaCl. It was then purified by FPLC using a HiLoad 26/10 Q-Sepharose column. In a typical purification procedure, 700–800 mg of dialysed protein was loaded on to the column, which was equilibrated with buffer A containing 4 mM $\text{Na}_2\text{S}_2\text{O}_4$. After washing the column with 1 column volume of buffer A, the protein was eluted by a gradient of buffer A (2 l) and buffer B (1 l). Buffer B was buffer A plus 1 M NaCl and 20 mM MgCl_2 . The protein elution profile, monitored at 450 nm, together with the gradient employed, are shown in Figure 2. Altogether four bands were observed. Band 1 was discarded because both SDS/PAGE and activity tests showed that it was neither Kp1 nor Kp2. Fractions A–C (see Figure 2) were shown by SDS/PAGE and by activity tests to be Kp1 fractions. Each of these fractions was further purified by repeating the FPLC step using the same HiLoad 26/10 or a 16/10 Q-Sepharose column, depending on the amount of protein to be purified.

Assay of enzymic activity

The enzyme was routinely assayed as an acetylene reductase. However, for comparative studies, the three major activities of the enzyme, i.e. reductions of acetylene to ethylene, protons to dihydrogen and dinitrogen to ammonia, were measured. Assays were conducted at 30 °C in glass serum bottles (7.0 ml) which were capped with Suba Seals. For acetylene and proton reductions, the assay bottles were charged with 25 μmol of Hepes, 12.5 μmol of MgCl_2 , 5 μmol of ATP, 13.5 μmol of phosphocreatine and 12.5 units of creatine kinase in a final volume of 1 ml. The bottles were flushed thoroughly with argon and then 150 μl of 200 mM $\text{Na}_2\text{S}_2\text{O}_4$ was added. For acetylene reduction, 2 ml of acetylene gas was added to the bottles, while for proton reduction the gas phase was 100 % argon. Finally, the temperature and pressure of the bottles were adjusted to 30 °C and

1.01 MPa (1 atmosphere) respectively. Reactions were started either by injecting appropriate mixtures of Kp1 and Kp2 or by adding Kp2 first and then Kp1. In all cases Kp2 was in 25–32-fold molar excess over Kp1. The assays were stopped by injecting 0.1 ml of 30 % trichloroacetic acid into each bottle. The amounts of ethylene and dihydrogen produced were measured on a PORAPACK (mesh 80–100) column using a Pye 104 gas–liquid chromatograph fitted with a flame ionization detector and on a Perkin–Elmer 452 gas–liquid chromatograph fitted with a thermal conductivity detector respectively.

For measurement of N_2 reduction to NH_3 , the amounts of both phosphocreatine and creatine kinase were double those used for the acetylene and H^+ assays, and assay bottles were thoroughly flushed with N_2 rather than argon. The assays were finally stopped by adding 0.3 ml of 0.4 M EDTA solution. The method for estimation of NH_3 produced is outlined below.

Estimation of ammonia

The amount of NH_3 in the assay bottles was estimated by the indophenol method [18], but corrections were made for the interference of creatine. These corrections were made either using the published procedure of Dilworth et al. [19] or using an alternative method which is detailed below. Dilworth et al. [19] removed compounds such as $\text{S}_2\text{O}_4^{2-}$, EDTA, ATP, protein, etc., which inhibit colour development, by passing the stopped assay mixture through an anion-exchange resin. Since creatine, which also interferes, is not removed by the anion-exchange method, its effects were separately assessed and corresponding corrections made. An alternative method was developed to avoid both the use of the anion-exchange columns and the estimation of creatine needed for correction for its interference. In this method the hydrogenase activity of the protein was measured in parallel to the nitrogenase activity. A standard amount of NH_3 was added to the hydrogenase assay bottles, and that amount of NH_3 was then estimated by the usual indophenol method. Plots of NH_3 estimated against time of the hydrogenase assays were fitted with a suitable equation. The equation, or more simply the ratio of $[\text{NH}_3]_{\text{added}}/[\text{NH}_3]_{\text{estimated}}$ in the hydrogenase assays, provided a correction factor for all interfering species, including creatine, present in the nitrogenase assay.

ATP hydrolysis

The amount of ATP hydrolysed, equivalent to the amount of creatine liberated from phosphocreatine, was indirectly measured by estimating the amount of creatine using the colorimetric method of Ennor [21]. In a separate experiment it was confirmed that the estimation is not affected by the assay mixture or by the passing or non-passing of the assay mixture through the anion-exchange column.

Protein concentration

The concentrations of protein samples were determined by the microbiuret method [22] or the Folin phenol method [23]. BSA was used as a calibration standard. In order to avoid interference by Tris buffer, both the BSA standards (prepared in Tris buffer) and protein samples were diluted initially to 1 ml with the buffer, then the colouring reagents were added. In each case three different amounts of a protein were analysed, correction was made for $\text{S}_2\text{O}_4^{2-}$ and/or its oxidation products (if needed), and then the average of the three values was recorded as the final concentration.

Metal analyses

For metal analyses, protein samples were first digested in concentrated H_2SO_4 and then oxidized with HNO_3 and/or H_2O_2 . The amount of Fe in the digested samples was estimated with 4,7-diphenyl-1,10-phenanthroline [24], while Mo was estimated with toluene-3,4-dithiol by the method of Clark and Axley [25], as described by Bulen and Le Comte [26].

Mo was also determined polarographically, using the polarographic/voltametric apparatus MetrohmAG, CH-9100 (Herisau, Switzerland). The method, outlined in the Metrohm Application Bulletin [27], is based on the complexation of MoO_4^{2-} with 5-sulpho-7-nitro-8-hydroxyquinoline (H_2L) to form the $\text{MoO}_2\text{L}_2^{2-}$ complex, which produces adsorption/desorption waves at a mercury electrode. The Mo(VI) complex is first adsorbed on a hanging mercury drop electrode and then electrochemically reduced to the Mo(V) complex. The reduced Mo(V) complex is immediately oxidized by H^+ to the original Mo(VI) complex, and is thus available again for the electrochemical reduction.

EPR measurements

EPR spectra were recorded on an upgraded Bruker ER200D Spectrometer fitted with a 20 GHz microwave frequency counter 2440 (Marconi Instruments) and a Bruker ER35M NMR gaussmeter. The low-temperature measurements were made using an Oxford Instruments ESR900 cryostat. The normal recording conditions were: temperature, 10 K; microwave power, 20 mW; microwave frequency, 9.43 GHz; modulation amplitude, 2.15 mT (21.5 G); modulation frequency, 100 kHz.

RESULTS AND DISCUSSION

Isolation and purification

The elution profile of step 3 of the purification procedure is shown in Figure 2. Four bands were separated by FPLC on Q-Sepharose HR 16/10 or 26/10 columns. SDS/PAGE and activity tests showed that fraction 1 (labelled F1 on Figure 2) did not contain MoFe protein. However, the remaining three fractions, labelled A, B and C, were shown by SDS/PAGE (Figure 3) and

Figure 2 Elution profile of Kp1 from the anion-exchange Q-Sepharose column on a Pharmacia FPLC system

Elution conditions and descriptions of the peaks are given in the text.

Figure 3 SDS/PAGE of the three fractions of Kp1 isolated from the mutant strain pHK17 of *K. pneumoniae* and of wild-type Kp1 after purification on the second Q-Sepharose column

Lane 1, wild-type Kp1; lane 2, pHK17Kp1.A; lane 3, pHK17Kp1.B; lane 4, pHK17Kp1.C.

Table 1 Comparison of the specific activities of wild-type Kp1 and the three Kp1 fractions from the double mutant pHK17

Specific activities are given in nmol of product produced/min per mg of Kp1. Values in parentheses indicate the specific activities of the reduction of H^+ to H_2 that occurs at the same time as reduction of acetylene to ethylene or of N_2 to ammonia. ATP/2e indicates the molecules of ATP hydrolysed per two electrons transferred to the substrate.

Protein	Specific activity (nmol/min per mg)			ATP hydrolysis (ATP/2e)	
	Under Ar H_2	Under $\text{C}_2\text{H}_2/\text{Ar}$ C_2H_4 (H_2)	Under N_2 NH_3 (H_2)	Under N_2	Under Ar
Wild-type Kp1	2011	1907 (73)	948 (590)	4.0 ± 0.4	4.7 ± 0.4
pHK17Kp1.A	445	333 (19)	257 (130)	4.8 ± 0.8	5.2 ± 0.2
pHK17Kp1.B	1048	978 (49)	646 (329)	4.0 ± 0.3	5.0 ± 0.3
pHK17Kp1.C	107	213 (12)	60 (33)	4.2 ± 0.7	5.4 ± 0.3

activity tests (Table 1) to contain active Kp1. We called these three fractions pHK17Kp1.A, pHK17Kp1.B and pHK17Kp1.C. Under similar conditions, FPLC of wild-type Kp1 results in a broad band with a peak close to that of pHK17Kp1.B but no peaks corresponding to the other two fractions. The integrity of the mutant strain was checked at the end of its growth in 200 l cultures (see the Materials and methods section). All colonies derived from the culture retained the Tn7 chromosomal insert, i.e. no wild-type Kp1 could be synthesized in the culture and thus all three fractions must have been produced from the mutated pWF23 plasmid.

As shown in Figure 3, the electrophoretic mobilities and relative staining intensities of the subunits of the SDS-treated fractions from the mutant were the same as those of the wild-type protein, indicating no difference in the subunit molecular masses or their relative proportions between the three fractions and wild-type Kp1. Earlier [5] we showed that Kp1 from pHK17 has a mobility on native gel electrophoresis comparable with that of tetrameric wild-type Kp1. Thus we conclude that the three fractions of pHK17Kp1 are $\alpha_2\beta_2$ tetramers. Figure 3 shows that fraction C is not as pure as fractions A and B. Further purification of all these fractions did not improve their electrophoretic purity, whereas both specific activity and protein concentration were significantly decreased. There was no observable formation of one fraction from another during this treatment.

MoFe proteins from a number of mutants of *A. vinelandii* and *K. pneumoniae* which carry single mutations at the FeMoco or P-cluster sites have been isolated and purified [16,17,28]. However,

fractionation of these proteins into more than one active fraction has never been reported. Resolution of the three fractions observed here, which have the same subunit molecular mass and $\alpha_2\beta_2$ configuration, on an anion-exchange Q-Sepharose column is remarkable. The mutations in pHK17 should not contribute to the overall charge of the protein. Furthermore, if they do contribute any charge it should be the same for all three fractions. We therefore conclude that conformational differences between the three fractions of the mutant protein contribute significantly, and differently, to the net external charge of the protein, i.e. each species must arise from different folding patterns of the subunits within the tetramers, generating distinct surface charges.

The mutations substitute each of the two bridging cysteine residues, α -Cys-89 and β -Cys-94, with the non-co-ordinating residue alanine. From Figure 1 (right panel) it appears that these two Cys residues occupy structurally important positions. However, isolation of catalytically active fractions from pHK17 suggests either that P-clusters are not needed for nitrogenase activity or that the two cysteine residues are not as essential for maintaining the P-cluster structure as the structure implies. We have EPR and magnetic CD spectroscopic evidence [28a] that the mutant fractions contain P-clusters, and thus the latter presumption appears more plausible. Substitution of the two bridging cysteine residues by alanine leaves the four bridged Fe atoms co-ordinatively unsaturated. On the basis of the X-ray crystallographic data of Kim and Rees [4] we were unable to locate any suitable residues at the P-cluster site of the MoFe protein of *K. pneumoniae* which could co-ordinate the four Fe atoms of the mutated Kp1. It thus seems likely that anions such as sulphide, chloride or hydroxide satisfy the co-ordinative unsaturation of the four Fe atoms.

Catalytic activity

Under saturating conditions of all required reagents, the substrate-reduction activities of wild-type Kp1 and the three fractions from the mutant were linear for at least 20 min, indicating no activation, inactivation or inhibition of the enzyme during the 20 min of the assay. These data were obtained for the reduction of protons to dihydrogen, acetylene to ethylene and dinitrogen to ammonia, and are summarized in Table 1. Fraction B has only 50% of the activity of the wild-type protein, whereas fractions A and C have only ~25% and 5–10% of its activity respectively.

In order to assess the possibility that only one of these fractions is catalytically active while the other fractions are inactive with apparent activities due to contamination by the active fraction, each fraction was re-run on FPLC. No contamination in terms of separate peaks was detected, nor was there any evidence of dynamic equilibria between the species. Each fraction remained active after this treatment, but all lost some activity.

No change in the specificity of substrate reductions was observed for any of the three fractions compared with wild-type Kp1. For each fraction, the total number of electrons consumed under argon (i.e. reduction of H^+ to H_2), under an acetylene/argon mixture (i.e. reduction of C_2H_2 to C_2H_4 plus H^+ to H_2) and under nitrogen (i.e. reduction of N_2 to NH_3 plus H^+ to H_2) was essentially the same. Furthermore, the relative distribution of these electrons between C_2H_2 and H^+ under C_2H_2 , and between N_2 and H^+ under N_2 , was also indistinguishable from that observed for wild-type Kp1. Similar data have been reported for Av1 from the single mutant DJ122, which carries a β -Cys-153 $\rightarrow$ Ser mutation of a P-cluster ligand. This mutant of Av1 was also less active, the distribution of electrons to substrates under

various conditions was unaltered, and no change in substrate specificity was observed [16]. The MoFe protein from the corresponding single mutant strain of *K. pneumoniae*, pHK15 (β -Cys-152 $\rightarrow$ Ser), has a similar activity pattern (F. K. Yousafzai and B. E. Smith, unpublished work). On the other hand, changes in substrate specificity are commonly observed for proteins that carry mutations at the FeMoco sites. For instance, Mo-dependent nitrogenases expressed in the mutant strains DJ64, DJ178 and DJ255 of *A. vinelandii* have single amino acid substitutions near the FeMoco site, i.e. α -Gln-191 $\rightarrow$ Glu, α -His-195 $\rightarrow$ Asn and α -Gln-191 $\rightarrow$ Lys respectively (see Figure 1, left panel) [17]. The nitrogenase-derepressed crude extracts of these strains are able to catalyse the reduction of acetylene by both two and four electrons, to ethylene and ethane respectively [17]. However, the corresponding wild-type protein can only catalyse the reduction of acetylene to ethylene. Furthermore, unlike the wild-type protein, Av1 purified from the mutant strain DJ255 was incapable of N_2 reduction and catalysed H^+ reduction which was inhibited by CO [17]. Proton reduction by nitrogenase is also inhibited by CO and the substrate specificity is significantly altered when citrate, fluorohomocitrate, *threo*-isocitrate, chlorocitrate, etc., rather than homocitrate is co-ordinated to FeMoco [28,29].

The above discussion clearly shows that changes around FeMoco alter both the specific activity and the specificity of nitrogenase substrates, whereas changes around P-clusters seem to alter only the specific activity of the enzyme. This conclusion supports the consensus opinion that FeMoco is the substrate-binding site, but leaves the precise role of the P-clusters undefined.

ATP hydrolysis

According to eqn. (1), at least two equivalents of MgATP are hydrolysed for each electron transferred from reductant to substrate through the nitrogenase enzyme. Since the function of ATP hydrolysis during nitrogenase activity is unclear [30–35], we measured the ATP hydrolysis of the mutant fractions in comparison with the wild-type protein. The ATP hydrolysis data, expressed as the molecules of ATP hydrolysed per two electrons transferred to the substrate, for the three mutant fractions and wild-type Kp1 are given in Table 1. Again there are no significant differences in the data, indicating that ATP hydrolysis is coupled to electron transfer in the mutant proteins in much the same way as in wild-type Kp1, with no additional futile ATP hydrolysis cycles.

pH titrations

The redox potential ($E_m = -180$ mV) of FeMoco in Kp1, obtained by redox titration of the $S = 3/2$ signal of FeMoco, is pH-dependent [36]. This dependence was interpreted in terms of a single pK_a of 7.0 associated with the FeMoco site, but not directly involving protonation of the metal–sulphur cluster itself. More recently, studies on the Mo-nitrogenase of *A. vinelandii* showed that there is a group with a pK_a of ~6.3 which must be deprotonated and another group with a pK_a of ~9.0 which must be protonated for substrate reduction to occur [37]. On the basis of the substrate dependence of these pK_a values, it was suggested that the groups being titrated are located at or near the FeMoco site of the MoFe protein. Thus both the spectroscopic and catalytic behaviour of FeMoco in the MoFe protein depend on the pH of the solution. We therefore determined whether the lower activities observed with the mutant fractions could be associated with pH effects. The acetylene-reduction activities of the mutant fractions along with that of the wild-type protein were determined at different pH values (Figure 4). The activity of none of the fractions reached that of the wild-type protein at any

Figure 4 pH-dependence of the acetylene reductase activities of wild-type Kp1 and the three fractions of pHK17Kp1

Specific activities are given in nmol of product/min per mg of Kp1.

pH value, showing that pH is not responsible for the lower activities of the mutants, although there may be some changes in the pK_a values of the mutant fractions compared with that of wild-type Kp1.

Metal analyses

The Fe and Mo contents of the three mutant fractions and of the wild-type protein are given in Table 2. As both the Fe and the Mo contents of the mutant proteins are lower than those of the wild-type protein, lower levels of both FeMoco and P-clusters are possible, with either potentially resulting in lower activities. Assuming that the clusters remain intact, the metal analyses also imply that each of the fractions A, B and C consists of a mixture of species which contain four (two FeMoco plus two P-clusters), three, two, one or zero clusters and thus differing metal contents, but with the same polypeptide folds and consequently the same charge for each fraction.

A catalytically inactive MoFe protein with a low metal content has been isolated from *Clostridium pasteurianum* [12]. This inactive demolybdo species of *C. pasteurianum* had a characteristic EPR signal at $g = 1.94$. Only a negligible level of this signal was observed in the EPR spectra of the three mutant fractions (results not shown), indicating the absence of significant levels of this inactive species.

Some FeMoco, equivalent to the difference between the Mo content of wild-type Kp1 and those of the mutant fractions, is definitely lost. As the mutations were at the P-clusters site, loss

Figure 5 EPR spectra of the $S = 3/2$ state of wild-type Kp1 and the three fractions of pHK17Kp1 measured at 10 K

The microwave power, microwave frequency, modulation frequency, modulation amplitude and gain were 20 mW, 9.43 GHz, 100 KHz, 2.15 mT (21.5 G) and 1×10^4 respectively.

of FeMoco is perhaps unexpected. Three possible reasons can be envisaged for the loss of FeMoco. First, it could be due to changes in the protein conformation around FeMoco caused by the adjustment of the relatively remote P-clusters in their mutant sites and demonstrated by the differing charges of the species. Secondly, the sluggish growth and production of clusterless fractions of pHK17Kp1, compared with that of the wild-type protein, may arise from changes in the rate of production and/or the rate of incorporation of the metal-sulphur clusters. Thirdly, changes in characteristics of the P-clusters may affect FeMoco which might, therefore, become unstable and consequently degraded. We are currently unable to differentiate, unambiguously, between these three factors or a combination of them as the cause of the low level of FeMoco in the three fractions. However, as the pH titration of the catalytic activity and the $S = 3/2$ EPR spectra of FeMoco (see below) show only a marginal change at the FeMoco site, it seems unlikely that changes in the protein conformation around FeMoco could be responsible for its loss.

EPR spectroscopy

EPR spectra of the dithionite-reduced samples of the mutant proteins and wild-type Kp1 are shown in Figure 5. These spectra indicate that the $S = 3/2$ spin state of FeMoco is not changed and that the individual g values of its EPR signal are relatively

Table 2 Metal analysis of wild-type and mutant proteins

Fe and Mo contents are given as atoms/molecule of Kp1 or g-atoms/mol of Kp1. Activity is given as nmol of C_2H_2 reduced/min per mg of Kp1.

Protein	Fe content	Mo content	Activity (nmol/min per mg)	Mo _{mutant} /Mo _{wild-type}	Act _{mutant} /Act _{wild-type}
Wild-type Kp1	25.8 ± 0.3	1.61 ± 0.11	2000	1.00	1.00
pHK17Kp1.A	12.4 ± 0.2	1.08 ± 0.22	445	0.67	0.22
pHK17Kp1.B	22.7 ± 0.8	1.44 ± 0.15	1048	0.89	0.52
pHK17Kp1.C	5.9 ± 0.8	0.74 ± 0.06	225	0.46	0.11

Figure 6 Expanded view of the EPR spectra in Figure 5

1 G = 0.1 mT.

invariant. We interpret this as indicating no change in the structure of FeMoco itself. The expanded version of the $g = 4.3$ and 3.7 region (Figure 6) shows a systematic appearance of a new feature at $g = 3.82$, in the series wild-type Kp1 < pHK17Kp1.A < pHK17Kp1.B < pHK17Kp1.C. The precise nature and significance of this new feature is not yet clear, but it is likely to be due to minor conformational changes at the FeMoco site. This sensitivity of the $S = 3/2$ EPR of FeMoco to its environment is well known. For example, changes in the g values of the FeMoco signal are observed upon changes in pH [36] and changes in the oxidation state of the P-cluster (A. J. Pierik and D. J. Lowe, personal communication). More recently, changes in the g values of the $S = 3/2$ spin state of FeMoco were observed for a mutant protein in which Glu-191 at the FeMoco site was replaced by lysine; however, the feature that we have observed in our mutant protein fractions has not been reported before.

As discussed above, the Mo contents of the mutant fractions were lower than that of wild-type Kp1, indicating the loss of some FeMoco. These contents were, however, higher than those expected from the activity data in Table 2, which show that the ratio $\text{Mo}_{\text{mutant}}/\text{Mo}_{\text{wild-type}}$ is significantly higher than the corresponding ratios of the activity data. This indicated that a non-FeMoco Mo species or catalytically inactive FeMoco species was present in the three mutant Kp1 fractions. Quantification of FeMoco by EPR spectroscopy was thought likely to provide further insight. On the assumption that all of the Mo (1.6 Mo atoms per molecule of Kp1) in wild-type Kp1 is present as FeMoco, the FeMoco content of the mutant fractions of Kp1

Figure 7 Relationship between the total substrate-reducing activities of wild-type and mutant Kp1 fractions and the integrated intensities of the $g = 4.3$ feature of their EPR spectra

Total substrate-reducing activities were measured under argon (○), acetylene (▲) and nitrogen (■). Units of specific activity are nmol of 2 electrons used per min per mg of Kp1. EPR spectra were measured under the conditions described in the legend to Figure 5.

was estimated by comparing the integrated intensity of the $g = 4.3$ band of a mutant fraction with that of an equal concentration of the wild-type protein. The [Mo in FeMoco] content estimated in this way, along with the Mo contents determined by the metal analyses, are given in Table 3. Comparison of the two values shows that the Mo content obtained from the EPR spectra, which is a direct measure of the EPR-active FeMoco content, is significantly lower than that implied by the metal analyses. In other words, all the measured Mo is not present as EPR-active FeMoco. The most plausible explanation, as deduced above, would be that Mo is distributed as FeMoco and a non-FeMoco species. The alternative explanation, where all Mo is present as FeMoco, part of which is EPR-silent, may seem unlikely, but cannot be ruled out completely.

Figure 7 clearly demonstrates that activity is directly proportional to the EPR intensity of the $S = 3/2$ signal, which is a direct measure of FeMoco, rather than to the Mo content, which is a measure of all Mo-containing species. Our conclusion is that loss of activity is due to loss of FeMoco or, precisely speaking, loss of EPR-active FeMoco.

In order to assess the possibility that the loss of activity may also be due to loss of P-clusters, statistical analyses of the Fe

Table 3 Comparison of observed and distributed Fe, observed and EPR-based Mo/FeMoco, and observed and EPR-expressed activity

Observed Fe is given as atoms/molecule of Kp1 or g-atoms/mol of Kp1. Distributed Fe was calculated as the Fe in FeMoco present in all Mo-containing molecules ($7 \times \text{Mo/FeMoco}$ found by metal analyses) plus the Fe in P-clusters present in molecules containing EPR-active FeMoco ($8 \times \text{Mo/FeMoco}$ from EPR). The observed FeMoco content is equivalent to the Mo content found by metal analyses, whereas the EPR-based FeMoco content was obtained from the EPR signal (see the text). Observed activity is the experimentally determined catalytic activity, whereas EPR-expressed activity was calculated as $(\text{EPR intensity of mutant})/(\text{EPR intensity of wild-type}) \times 2000$ (units of nmol of C_2H_2 reduced/min per mg of Kp1). See the text for further details.

Protein	Fe content		FeMoco/Mo content		Activity (nmol/min per mg)	
	Observed	Distributed	Observed	EPR-based	Observed	EPR-expressed
Wild-type	25.8 ± 0.3	24.15	1.61 ± 0.11	1.61	2000	2000
pHK17Kp1.A	12.4 ± 0.2	10.6	1.08 ± 0.22	0.38	445	472
pHK17Kp1.B	22.7 ± 0.8	19.2	1.44 ± 0.15	1.14	1048	1416
pHK17Kp1.C	5.9 ± 0.8	6.5	0.74 ± 0.06	0.17	225	211

content were undertaken, with the observed Fe being distributed between FeMoco and P-clusters. The best fit to the data was obtained (Table 3) with the assumption that all of the experimentally found Mo is present as FeMoco but that P-clusters are present only in those molecules that contain EPR-active FeMoco. This tentative analysis implies that Mo is distributed as EPR-active and EPR-inactive FeMoco. Since the observed activity compares very well with the activity determined in terms of the $S = 3/2$ EPR signal (Table 3), the analysis suggests that it may be the lack of P-clusters that makes FeMoco both EPR- and catalytically inactive. Alternatively, an FeMoco-like species which is both EPR- and catalytically inactive may be incorporated into those molecules that do not contain P-clusters. Lack of P-clusters seems a more reasonable cause of the loss of activity as well as of EPR-active FeMoco, because the mutations occur at the P-cluster site. An unambiguous assignment will be possible only if the loss of P-clusters and the presence of an EPR- and catalytically inactive FeMoco or FeMoco-like species, present in those molecules that do not contain P-clusters, are independently verified. At the moment we are unable to do this. However, an MoFe₆ cluster which is inactive catalytically and is harder to reduce to the characteristic $S = 3/2$ EPR-active state can be isolated from the MoFe protein [38]. Contamination with such a species could explain our data. One possibility is that conformational changes that cause the changes in surface charge resulting in the separation of the three distinct species by ion-exchange chromatography may also affect the kinetics and/or specificity of incorporation of the metal clusters and thus allow binding of other Mo- and Fe-containing clusters. Alternatively, these conformational changes might destabilize FeMoco, leading to the formation of (say) an MoFe₆ species. The latter suggestion would be consistent with the instability of the activity of the mutant fractions to repeated FPLC.

Conclusion

We conclude that the partial loss of catalytic activity of the three mutant fractions of Kp1 isolated from pHK17 is due to partial loss of EPR-active FeMoco. This conclusion is supported by both metal analyses and EPR spectroscopy. Since the observed Mo contents of these fractions are higher than those indicated by EPR spectroscopy and by the catalytic activity of the enzyme, it is quite likely that another form of FeMoco which is both EPR- and catalytically inactive, or a different type of Mo-containing cluster containing significant numbers of Fe atoms, is present in these fractions. The best fit to the observed Fe content not only supports the presence of such clusters but, more importantly, suggests that such clusters are present only in those molecules that do not contain P-clusters. It is therefore quite likely that loss of P-clusters, triggered by mutation of the P-cluster ligands, alters the EPR and catalytic properties of FeMoco or allows other clusters to be incorporated at the FeMoco site of Kp1.

A further important conclusion is that since activity is proportional to the EPR-active FeMoco content then those molecules that contain this species are fully active, i.e. as active as the wild type. Therefore the Cys → Ala mutations at the P-cluster site do not affect the specific activity of Kp1 molecules that contain both FeMoco and P-clusters, i.e. they do not perturb the rate-limiting step in enzyme turnover, which is thought to be the

dissociation of the MoFe and Fe proteins after electron transfer and ATP hydrolysis [39].

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