Purification and characterization of the inactive MoFe protein (NifB-Kp1) of the nitrogenase from *nifB* mutants of *Klebsiella pneumoniae*

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The inactive MoFe protein of nitrogenase, NifB-Kp1, from two distinct *nifB* mutants of *Klebsiella pneumoniae*, Kp5058 (a *nifB* point mutant) and UNF1718 (a *nifB*, *nifJ* double mutant) has been purified and characterized. NifB-Kp1 can be activated by reaction with the iron-molybdenum cofactor, FeMoco, extracted from active MoFe protein. NifB-Kp1 purified from either source had similar properties and was contaminated with an approximately equimolar amount of protein of mol.wt. 21000. Like active wild-type Kp1, it was an $\alpha_2\beta_2$ tetramer, but it was far less stable than Kp1, deteriorating rapidly at temperatures above 8°C or on mild oxidation. NifB-Kp1 preparations contained 0.4–0.9 Mo and 9.0 ± 0.9 Fe atoms $\cdot \text{mol}^{-1}$ and, when activated by FeMoco, had a specific activity of approx. 500 units $\cdot \text{mg}^{-1}$. The Mo in our preparations was not associated with the e.p.r. signal normally observed from FeMoco. All preparations exhibited a weak $g_{\text{av.}} = 1.95$ e.p.r. signal which was probably not associated with activatable protein.

The enzyme nitrogenase consists of two proteins: the MoFe protein, and the Fe protein. The most active preparations of the MoFe protein contain 2 Mo and about 30 Fe atoms $\cdot \text{mol}^{-1}$ (Eady & Smith, 1979; Mortenson & Thorneley, 1979) and its biosynthesis involves the products of the *nifK*, *D*, *V*, *B*, *N* and *E* genes (Dixon *et al.*, 1981). Mutants in *nifB*, *N* or *E* synthesize inactive MoFe protein, which can be activated by adding the FeMo cofactor (FeMoco) extracted from active MoFe protein (Shah & Brill, 1977). FeMoco probably includes the site(s) for binding reducible substrates (Smith, 1977) and has a characteristic e.p.r. signal, with *g*-values near 4.3, 3.7 and 2.01 in the $\text{Na}_2\text{S}_2\text{O}_4$ -reduced protein (Eady & Smith, 1979). This e.p.r. signal integrates to 1-electron spin/Mo atom and 6 Fe atoms are associated with each electron spin (Rawlings *et al.*, 1978). However, analyses of FeMoco after extraction into *N*-methylformamide indicated 7–8 Fe atoms per Mo atom (Shah & Brill, 1977; Smith, 1980; Burgess *et al.*, 1980). The extra 1–2 Fe atoms may be those designated 'S' in the Mössbauer spectrum (Münck *et al.*, 1975; Shah & Brill, 1981). The other Fe atoms in the MoFe protein are probably arranged as four unusual [4Fe–4S]

clusters, designated 'P' clusters, per molecule (Zimmermann *et al.*, 1978).

Isolation of the two metal groupings would assist definition of their structural properties and interactions. FeMoco can be extracted from the MoFe protein into *N*-methylformamide but this extraction either destroys the 'P' clusters, or yields an insoluble residual protein. The 'P' clusters should be present in the inactive MoFe protein from mutants unable to synthesize FeMoco. Here we present details of the purification and characterization of this protein from two distinct *nifB* mutants of *K. pneumoniae*. We have designated this protein 'NifB-Kp1' in order to specify its phenotype and to distinguish it from Kp1, the active MoFe protein from wild-type *K. pneumoniae*. A preliminary account has been published elsewhere (Smith & Hawkes, 1981).

Materials and methods

Materials

Most laboratory reagents were obtained from BDH Chemicals, Poole, Dorset, U.K., and biochemicals from Sigma (London) Chemical Co., Poole, Dorset, U.K. Other chemicals were of the highest grade available. The Fe–S clusters, $\text{Fe}_4\text{S}_4(\text{SBu}^t)_4(\text{Me}_4\text{N})_2$ and $\text{Fe}_4\text{S}_4\text{Cl}_4(\text{Me}_4\text{N})_2$ were a gift from Dr. R. Burt of this Unit.

Abbreviations used: Me, methyl; Bu^t, t-butyl; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

Sources of growth of organisms

Klebsiella pneumoniae M5al was grown and harvested as described previously (Eady *et al.*, 1972). Two *nifB* mutants strains of *K. pneumoniae* were used. Kp5058, a *hisD nifB* point mutant (Cannon *et al.*, 1977) and UNF1718, a *nifB* deletion, *nifJ* insertion double mutant [$\Delta(his^-nifB)$ *nifJ2485*, *nifJ2009::Tn10*, a gift from Dr. M. Merrick of this Unit]. In growth experiments the basic medium included glucose ($20\text{ g}\cdot\text{l}^{-1}$), MgSO_4 ($0.1\text{ g}\cdot\text{l}^{-1}$), NaCl ($0.01\text{ g}\cdot\text{l}^{-1}$), FeSO_4 ($0.025\text{ g}\cdot\text{l}^{-1}$), $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$ ($0.025\text{ g}\cdot\text{l}^{-1}$), L-histidine ($0.025\text{ g}\cdot\text{l}^{-1}$), K_2HPO_4 ($12.05\text{ g}\cdot\text{l}^{-1}$) and KH_2PO_4 ($3.4\text{ g}\cdot\text{l}^{-1}$) and was sparged with N_2 at 29°C ; the pH was maintained above 6.5. The two most successful protocols were: (a) medium containing $(\text{NH}_4)_2\text{SO}_4$ (0.021 g of $\text{N}\cdot\text{l}^{-1}$) was inoculated with cells grown anaerobically on NH_4Cl (0.26 g of $\text{N}\cdot\text{l}^{-1}$). After 6 h, L-aspartate ($0.01\text{ g}\cdot\text{l}^{-1}$) was added. The cells were harvested after a further 16 h; (b) medium containing 1 g of L-arginine $\cdot\text{l}^{-1}$ and 1% or 2.5% 'L-broth' was inoculated with cells grown anaerobically on L-broth. Cells were harvested, generally after 16–20 h, when growth had stopped or was very slow. [L-broth contained Oxoid yeast extract ($5\text{ g}\cdot\text{l}^{-1}$), Difco Bactotryptone ($10\text{ g}\cdot\text{l}^{-1}$) and NaCl ($10\text{ g}\cdot\text{l}^{-1}$)]. French-pressing of cells from 5–10 litre cultures yielded crude extracts with NifB-Kp1 specific activities of 20–30 units $\cdot\text{mg}^{-1}$ (a unit is defined under 'Analytical techniques' below). However, cells from 100, 400 or 1000 litres growth generally yielded crude extracts with specific activities of only 10–15 units $\cdot\text{mg}^{-1}$.

Purification of nitrogenase

The nitrogenase proteins from *K. pneumoniae* M5al were purified by the method of Eady *et al.* (1972) as modified by Smith *et al.* (1976).

Preparation of FeMoco

FeMoco was prepared as described by Smith (1980).

Analytical techniques

Protein concentrations, C_2H_2 reduction activities (1 unit of activity = 1 nmol of C_2H_4 produced/min), and metal contents were measured by the techniques described by Eady *et al.* (1972).

To assay NifB-Kp1 it had first to be saturated with FeMoco and then the Kp1 thus formed assayed for C_2H_2 reduction with excess Kp2. Solutions of NifB-Kp1, FeMoco and buffer were mixed to give a final volume of 0.6 ml in N_2 -flushed 7.8 ml serum vials capped with Suba-Seal rubber closures. The final buffer concentration was made 40 mM -Hepes, pH 7.5, containing 2 mM - $\text{Na}_2\text{S}_2\text{O}_4$. A positive pressure of N_2 gas was established in each vial. Activation, at 30°C , was monitored by removing

$50\mu\text{l}$ samples for C_2H_2 reduction assay with $3\text{--}5\mu\text{M}$ -Kp2, and was complete in 30 min. Activities were independent of the activation temperature between 30° and 48°C (but at 2.5°C were only 50% of the maximum even after 3.5 h), and the specific activity of the FeMoco preparations used (specific activities 177–230 units/ng-atom of Mo). Lower activities were observed if the concentration of *N*-methylformamide exceeded 10% in the activation mixture or 3% in the assay mixture.

SDS/polyacrylamide-gel electrophoresis

The gel system of Laemmli (1970) was used with fixing, staining and destaining as described by Weber & Osborn (1969). A Unicam SP.1800 spectrophotometer was used for scanning densitometry of stained gels at 600 nm .

Double immunodiffusion

Antigen-antibody interaction between the proteins and purified IgG fraction of antibodies raised against Kp1 in rabbits ('anti-Kp1', a gift from Dr. R. R. Eady of this Unit) was studied by using the technique of Ouchterlony (1948).

Immunoelectrophoresis

The technique of Laurell (1972) was modified by use of Tris/HCl in place of barbital buffer. The concentration of MoFe protein in crude extracts was measured by comparing the heights of rockets with a standard of purified Kp1 at loadings between 0 and $2.5\mu\text{g}$. The gels contained $70\mu\text{g}$ of purified anti-Kp1 $\cdot\text{ml}^{-1}$.

Analytical centrifugation

Sedimentation coefficients were measured in anaerobically filled cells at 4°C and $50000\text{ rev}\cdot\text{min}^{-1}$ on an MSE Centriscan 74 ultracentrifuge with absorption optics at 400 nm .

E.p.r. spectroscopy

E.p.r. spectra were measured on anaerobically prepared frozen samples with a Varian E9 spectrometer (Lowe *et al.*, 1972) as described in the legends to the Figures.

U.v. and visible absorption spectroscopy

Spectra were measured with a Unicam SP.1800 spectrophotometer, and fixed-wavelength measurements were recorded with a Perkin-Elmer Coleman 550 double-beam spectrophotometer.

Redox measurements

Potentiometric titrations were carried out essentially by the method of Dutton (1971) as described by O'Donnell & Smith (1978).

Results and discussion

Activation of NifB-Kp1

Titration of crude or purified preparations of NifB-Kp1 with FeMoco showed linear increases in activity followed by plateau regions, indicating tight stoichiometric binding of FeMoco. Fig. 1 shows the effect of varying the purified protein concentration in the activation reaction under conditions of saturation with FeMoco. A dilution effect was observed, the specific activity at $0.35 \mu\text{M}$ -protein being only 50% of the maximum value at concentrations above $1.5 \mu\text{M}$. The dilution effect was largely relieved, but the maximum specific activity was unaffected, when Kp2 ($5 \mu\text{M}$) was included in the activation mixture.

Purification of NifB-Kp1

Purification (Fig. 2) was at $3 \pm 2^\circ\text{C}$, since NifB-Kp1 was thermolabile. In 72 h a purified preparation lost 55% of its activity at $21 \pm 1^\circ\text{C}$, but only 14% at $6 \pm 2^\circ\text{C}$. NifB-Kp1 also lost 40–85% of its activity at 4°C during gel filtration through Sephadex G-200, pH 9.1, passage through Chelex 100, pH 6.8, and DEAE-cellulose chromatography in 50mM-P_i , pH 7.4. The protein was stored as frozen beads in liquid nitrogen. Table 1 summarizes data from a typical purification. As with Kp1 (Eady *et al.*, 1972; Smith *et al.*, 1976), good recovery of units (at least 65%) was recorded in each puri-

Fig. 1. Dilution effect on NifB-Kp1 specific activity

The concentration of NifB-Kp1 in the activation mixture was varied in the presence (□) and absence (○, Δ) of $5 \mu\text{M}$ -Kp2 and samples removed for activity measurements as described in the Materials and methods section. At high NifB-Kp1 concentrations its specific activity was $430 \text{ units} \cdot \text{mg}^{-1}$. From this activity a purity of 75% was estimated and used to calculate the NifB-Kp1 concentration on the abscissa. The FeMoco concentrations were saturating at all times. Two FeMoco preparations (specific activity $180 \text{ units} \cdot \text{ng-atom of Mo}^{-1}$) were used, one (○, □) with 2.2 and the other (Δ) with $4.0 \text{ ng-atom of Mo} \cdot \text{ml}^{-1}$.

fication step, indicating that at no stage was NifB-Kp1 sustaining gross damage. If the specific activity of the starting crude extract was $<10 \text{ units} \cdot \text{mg}^{-1}$, hydroxyapatite chromatography (Step 7) was required to obtain NifB-Kp1 of high activity.

Quantitative rocket immunoelectrophoresis and scanning densitometry of stained SDS/polyacrylamide gels of purified NifB-Kp1 (Fig. 3) indicated a purity of $\approx 90\%$. One contaminant was identified as the *nifJ* product (mol.wt. 120 000), since it was absent from *nifJ* mutant crude extracts and from NifB-Kp1 purified from UNF1718, the *nifB*, *nifJ* double mutant (Fig. 3). This observation demonstrated that the *nifJ* product was not necessary for activation of NifB-Kp1 by FeMoco. The most abundant contaminant of NifB-Kp1 from both Kp5058 and UNF1718 had a subunit mol.wt. of $\approx 21 000$ (Fig. 3) and was present at a molar ratio of about 0.75. It was not found in wild-type Kp1 preparations nor in preparations of Kp1 purified from UNF1718 crude extracts after saturation with FeMoco. We cannot rule out the possibility that this protein assists in the activation of NifB-Kp1 by FeMoco.

Before activation with FeMoco, purified NifB-Kp1 had a specific activity of $7 \text{ units} \cdot \text{mg}^{-1}$ from Kp5058 and $<1.5 \text{ units} \cdot \text{mg}^{-1}$ from UNF1718. After activation with FeMoco (all NifB-Kp1 specific activities reported below are after activation with FeMoco) the maximum NifB-Kp1 specific activity obtained (Table 1) from either organism was about $500 \text{ units} \cdot \text{mg}^{-1}$ and was low in comparison with Kp1, the bulk of which generally has a specific activity of $1000\text{--}1500 \text{ units} \cdot \text{mg}^{-1}$, with some at activities around $2000 \text{ units} \cdot \text{mg}^{-1}$. However, the final specific activity agrees well with that of $420 \pm 50 \text{ units} \cdot \text{mg}^{-1}$ calculated from the NifB-Kp1 concentration (estimated by immunoelectrophoresis) in Kp5058 and UNF1718 crude extracts. Furthermore FeMoco was equally active whether assayed with crude extracts or purified NifB-Kp1. Extrapolating from results with the most active FeMoco preparation used here ($230 \text{ units} \cdot \text{ng-atom of Mo}^{-1}$), a specific activity of $2100 \text{ units} \cdot \text{mg}^{-1}$ can be calculated for Kp1 of mol.wt. 218 000 with 2 FeMoco $\cdot \text{mol}^{-1}$. These data indicated that, within the population of NifB-Kp1 molecules in both crude extracts and purified preparations, there was a species capable of activating FeMoco to its fullest extent.

Properties of NifB-Kp1

Immunological cross-reaction with anti-Kp1. Figs. 4(a) and 4(b) are photographs of Ouchterlony plates monitoring the cross-reactions of anti-Kp1 and Kp1, two preparations of NifB-Kp1 and an extract of de-repressed UNF107 (pMF260) cells (a

Fig. 2. Purification scheme for *NifB*⁻*Kp1*

All steps were carried out anaerobically at 4°C. The buffer was 50 mM-Tris/HCl, pH 7.8, except where otherwise stated, and contained 0.1 mg of dithiothreitol · ml⁻¹ and 2 mM-Na₂S₂O₄.

nifD insertion mutant of *K. pneumoniae* which lacks *Kp1*). The *nifD* mutant control showed a single precipitin line, demonstrating that our anti-*Kp1* was not wholly specific to *Kp1*. This band was present in purified *Kp1* and in *NifB*-*Kp1* at Step (6) (Fig. 2) of purification, but was removed by hydroxyapatite chromatography (Step 7). The other precipitin line showed that *NifB*-*Kp1* had the antigenic determinants of *Kp1*.

Molecular weight and subunit composition. Densitometry of the gels in Fig. 3 indicated that, like *Kp1*, *NifB*-*Kp1* consists of equivalent amounts of α - and β -subunits. *NifB*-*Kp1* behaved similarly to *Kp1* during gel filtration through Sephadex G-200 and in sedimentation-velocity experiments and thus we

conclude that it is an $\alpha_2\beta_2$ tetramer of mol.wt. $\approx 218\,000$.

Susceptibility to damage by oxidation. *NifB*-*Kp1* was irreversibly inactivated by oxygen and, during potentiometric redox titrations, was inactivated at potentials above -350 mV, whereas *Kp1* was stable at potentials up to 0 mV (O'Donnell & Smith, 1978). However *NifB*-*Kp1* could be oxidized for short (<10 min) periods with $K_3Fe(CN)_6$ or dyes without an appreciable loss of activity.

Mo and Fe content. Mo and Fe were found in all our preparations of *NifB*-*Kp1*. The presence of Mo was surprising, since *NifB*-*Kp1* was FeMoco-deficient. The Mo content was variable and did not seem to bear any relationship to the specific activity

Fig. 3. SDS/polyacrylamide gels of (a) crude extracts and (b) NifB-Kp1 from Kp5058 and UNF1718 purified to step (7) in Fig. 2

Channel 1 contained purified Kp1; 2, *Klebsiella pneumoniae* M5al crude extract; 3, Kp5058 crude extract; 4, UNF1718 crude extract; 5-8, 5-40 μ g of NifB-Kp1 from Kp5058 (specific activity 500 units $\cdot$ mg $^{-1}$); 9-13, 5-40 μ g of NifB-Kp1 from UNF1718 (specific activity 450 units $\cdot$ mg $^{-1}$); 14-15, standard molecular-weight markers.

Table 1. Typical purification data from 500 g of cells (Kp5058)

Step no. (see Fig. 2)		Total protein (g)	$10^{-3} \times$ Total activity (units)	NifB-Kp1 specific activity [units $\cdot$ (mg of protein) $^{-1}$]*	Yield (%)
(1)		15	223	14.9	100
(2)		7.9	200	25.3	90
(3)		1.71	140.5	82.2	63
(4)		0.45	82.0	181	36.75
(5)		0.152	53.3	350	23.8
(6)	Fraction 1	0.014	5.77	410	} 15.4%
	2	0.021	10.75	500	
	3	0.018	8.55	480	
	4	0.030	9.0	300	
(7)†	Fraction 1	0.07	7	10	} 85
	2	0.123	28.25	230	
	3	0.094	35.70	385	
	5	0.063	15.91	255	

* C₂H₂ reduction measured after saturation with FeMoco and assayed with excess Kp2.
† Optional; e.g. starting material from Step 6, 0.39 g of protein, specific activity 240 units $\cdot$ mg $^{-1}$; total units = 93×10^3 .

Fig. 4. Double immunodiffusion

The Figure shows double immunodiffusion of anti-Kp1, in the centre well, against: (1) purified Kp1; (2) BifB-Kp1 purified to Step (6) (Fig. 2); (3) NifB-Kp1 purified to Step (7) (Fig. 2); (4) UNF107 (pMF260) crude extract. (a) Shows removal of an impurity from NifB-Kp1; (b) shows that the impurity is probably not specific to *nif*.

of NifB-Kp1 preparations. Since the NifB-Kp1 preparations were not 100% pure, the possibility that some of the metal was associated with proteins other than NifB-Kp1 could not be eliminated. For the most pure preparations the only contaminant at a molar concentration comparable with NifB-Kp1 was the 21 000-mol.wt. protein. The metal content of NifB-Kp1 estimated from fractions with specific activities >380 units $\cdot$ mg $^{-1}$ was 0.4–0.9 Mo and 9.0 ± 0.9 Fe-atom $\cdot$ (mol of NifB-Kp1) $^{-1}$. [Tables of data supporting these conclusions are available from B.E.S.]

Attempted insertion of extra Fe-S clusters into NifB-Kp1. A possible reason for the low NifB-Kp1 specific activities was that some molecules lacked 'P' clusters. At about 9 Fe atom $\cdot$ mol $^{-1}$ the Fe content of NifB-Kp1 was lower than expected. Attempts were therefore made to insert additional Fe-S clusters. Reconstitution under conditions similar to those described by Hong & Rabinowitz (1970) for clostridial ferredoxin, did not affect the specific activity of NifB-Kp1. The specific activity was also unaffected when synthetic 4Fe-4S clusters [$70 \mu\text{M}$ -Fe $_4$ S $_4$ Cl $_4$ (Me $_4$ N) $_2$ or Fe $_4$ S $_4$ (SBU t) $_4$ (Me $_4$ N) $_2$] were included in the activation reaction with FeMoco.

Optical absorption spectrum. The optical absorption spectrum of Na $_2$ S $_2$ O $_4$ -reduced NifB-Kp1 between 400 and 850 nm (Fig. 5) was almost featureless and similar to that from Kp1, except that the change in slope observed at about 430 nm for Kp1 (Eady *et al.*, 1972) was at about 470 nm. On the assumption of 100% purity the preparation contained 0.48 Mo and 8.6 Fe atom $\cdot$ mol $^{-1}$.

Fig. 5. Optical absorbance spectrum of Na $_2$ S $_2$ O $_4$ -reduced NifB-Kp1 from Kp5058

The protein (specific activity 430 units $\cdot$ mg $^{-1}$; concentration 3.5 mg $\cdot$ ml $^{-1}$) was dissolved in 50 mM-Tris/HCl (pH 7.4)/0.2 M-NaCl/dithiothreitol (0.1 mg $\cdot$ ml $^{-1}$)/2 mM-Na $_2$ S $_2$ O $_4$. The ordinate scale was calculated on the assumption that the protein was 100% pure (estimated purity = $87 \pm 8\%$).

The absorption spectrum of isolated FeMoco is also featureless (Shah & Brill, 1977). Subtracting this spectrum (1 Mo: 6–8 Fe) from the published spectrum of Kp1 [1 atom of Mo and 17.5 atoms of Fe $\cdot$ mol $^{-1}$ (Eady *et al.*, 1972)], the absorbance due to the 'P' centres alone (approx. 10 Fe atom $\cdot$ mol $^{-1}$) can be estimated as $\epsilon_{430}^{\text{mm}} \approx 27$ litre $\cdot$ mmol $^{-1} \cdot$ cm $^{-1}$ and $\epsilon_{470}^{\text{mm}} \approx 19$ litre $\cdot$ mmol $^{-1} \cdot$ cm $^{-1}$. These numbers are in reasonable agreement with those calculated from Fig. 5 of $\epsilon_{430}^{\text{mm}} = 23.5$ litre $\cdot$ mmol $^{-1} \cdot$ cm $^{-1}$ and $\epsilon_{470}^{\text{mm}} = 15.5$ litre $\cdot$ mmol $^{-1} \cdot$ cm $^{-1}$. The absorption spectrum (Fig. 5) appears similar to those of ferredoxins containing [4Fe-4S] $^{1+}$ clusters but not [2Fe-2S] $^{1+}$ or [2Fe-2S] $^{2+}$ clusters (Stephens *et al.*, 1978; Thomson *et al.*, 1977).

E.p.r. spectroscopy. No e.p.r. signal with g -values of 4.3, 3.7 and 2.01 was observed from our Na $_2$ S $_2$ O $_4$ -reduced preparations of NifB-Kp1, confirming the absence of normal FeMoco. However, these preparations invariably gave an almost axial e.p.r. signal (Figs. 6a and 6b) with $g_1 = 2.034$, $g_2 = 1.928$ and $g_3 = 1.896$, similar to those from reduced ferredoxins. The integrated intensity of the e.p.r. signal varied from 0.013 to 0.081 electrons/mol, did not correlate with the specific activity of the NifB-Kp1, and often decreased during purification. It is therefore unlikely that the signal arises from a form of NifB-Kp1 that can be activated by FeMoco. (Tables of data supporting these conclusions are available from B.E.S.) This signal disappeared at potentials above -360 mV. Consistent with experiments on Kp1 (O'Donnell & Smith, 1978), no other e.p.r. signals were observed from NifB-Kp1 at potentials up to 0 mV.

The $g_{\text{av.}} = 1.95$ e.p.r. signal is similar to that observed by Zumft & Mortenson (1973) from the inactive 'demolybdo' form (0.5 Mo, 8 Fe atom $\cdot$ mol $^{-1}$) of Cpl (the MoFe protein from *Clostridium pasteurianum*) and to that observed from low-specific-activity Kp1, in early fractions from DEAE-cellulose during gradient elution with MgCl $_2$. Such a preparation of Kp1 ($>90\%$ pure on SDS/polyacrylamide gels) gave the e.p.r. signal shown in Fig. 6(c), with g -values at $g_1 = 2.038$, $g_2 = 1.933$ and $g_3 = 1.881$, with an integrated intensity corresponding to ≈ 0.08 electron spin $\cdot$ (mol of Kp1) $^{-1}$. This preparation was not further activated by FeMoco. It probably contained an inactive form of Kp1, analogous to that from Cpl.

Conclusions

The Mo content and relatively low specific activities of our preparations are puzzling, but we found these properties with two distinct *nifB* mutants and therefore they seem to be a function of the phenotype. It is possible that the Mo in our preparations was inserted by the *nifN* or *E* gene

Fig. 6. E.p.r. spectra of preparations of $\text{Na}_2\text{S}_2\text{O}_4$ -reduced NifB-Kp1 and low-specific-activity Kp1 (a) NifB-Kp1 from UNF1718; specific activity $360 \text{ units} \cdot \text{mg}^{-1}$, concentration $11.4 \text{ mg} \cdot \text{ml}^{-1}$; (b) NifB-Kp1 from Kp5058; specific activity $450 \text{ units} \cdot \text{mg}^{-1}$, concentration $3.7 \text{ mg} \cdot \text{ml}^{-1}$; (c) Kp1, specific activity $550 \text{ units} \cdot \text{mg}^{-1}$, concentration $15.2 \text{ mg} \cdot \text{ml}^{-1}$. E.p.r. spectra were measured at 23 K, with a microwave frequency of 9.22 GHz and power of 10 mW using 1.0 mT field modulation at 3.3 kHz. The g-values shown are relatively comparable within ± 0.001 but are only accurate to ± 0.004 .

products in place of FeMoco, thus preventing full activation of the protein. Wild-type Kp1 preparations are a mixture of ill-defined species of differing specific activities which are only partially separated by DEAE-cellulose chromatography. It is probably that our NifB-Kp1 preparations also contained a mixture of species, with only some of them being activatable by FeMoco, since the latter was activated by our preparations to a level consistent with a Kp1 specific activity of $2100 \text{ units} \cdot \text{mg}^{-1}$. Furthermore, the $g_{\text{av.}} = 1.95$ e.p.r. signal in our NifB-Kp1 preparations was probably associated with an inactivated species.

Burgess *et al.* (1981) published a preliminary report on the purification of the MoFe protein from

a FeMoco-deficient mutant, UW45, of *Azotobacter vinelandii*. This protein, although only 70% pure, had a specific activity (after activation by FeMoco) of $1900 \text{ units} \cdot \text{mg}^{-1}$ and contained no Mo. Unfortunately the nature of the mutation in UW45 has not been established, but comparison with our data would suggest that it is not in the *nifB* gene.

Despite its low activity, the purified NifB-Kp1 described here should prove useful in investigations of the role(s) of FeMoco and the P clusters in Kp1, particularly in experiments in which they are selectively enriched with ^{57}Fe . Such experiments should allow unequivocal assignment of e.p.r. signals, seen on oxidation or during turnover (Smith *et al.*, 1972; Lowe *et al.*, 1978).

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