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The inactive MoFe protein (NifB$^-$Kp1) of the nitrogenase from *nifB* mutants of *Klebsiella pneumoniae*

Its interaction with FeMo-cofactor and the properties of the active MoFe protein formed

Timothy R. HAWKES and Barry E. SMITH
A.F.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, U.K.

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The inactive MoFe protein (NifB$^-$Kp1) of nitrogenase from *nifB* mutants of *Klebsiella pneumoniae* may be activated by addition of the iron–molybdenum cofactor (FeMoco) extracted from active MoFe protein (Kp1). However, when apparently saturated with FeMoco, our preparations of NifB$^-$Kp1 yielded activated protein, Kp1-asm, with a specific activity that was at best only 40% of that expected. This was not due to degradation of Kp1-asm, NifB$^-$Kp1 or FeMoco during the activation reaction. Nor could activation be enhanced by addition of other *nif*-gene products or other proteins. Whereas fully active Kp1 contains 2 FeMoco/molecule, apparent saturation of our NifB$^-$Kp1 preparations required the binding of only 0.4–0.65 FeMoco/molecule. By using chromatography Kp1-asm could be largely resolved from NifB$^-$Kp1 that had not been activated. However, we were unable to isolate fully active MoFe protein (i.e. Kp1-asm containing 2 FeMoco/molecule) from solutions of NifB$^-$Kp1 activated with FeMoco. The maximum activity/ng-atom of total Mo obtained for our purified Kp1-asm was approximately half the maximum activity for FeMoco. Since all NifB$^-$Kp1 preparations contained some Mo, we suggest that FeMoco activated only those NifB$^-$Kp1 molecules already containing one atom of (non-FeMoco) Mo, thus forming Kp1-asm with 2 Mo but only 1 FeMoco/molecule. Kp1-asm was identical with normal Kp1 in terms of its M_r, stability, e.p.r. signals, pattern of substrate reductions, CO inhibition and ATP/2e ratio. In addition, for preparations of differing specific activity, there was a constant and identical relationship between the e.p.r. signal intensity (from FeMoco) and the activity of both Kp1 and Kp1-asm. Assuming the above hypothesis on the structure of Kp1-asm, these data demonstrate that the two FeMoco sites in wild-type Kp1 operate independently.

Nitrogenase, the enzyme responsible for the biological reduction of N_2 to NH_3, consists of two O_2-sensitive metalloproteins. During enzyme turnover the smaller protein, the Fe protein, passes electrons, in an MgATP-hydrolysing reaction, to the MoFe protein, which contains the enzyme's site for binding reducible substrates. The enzyme can reduce N_2 to NH_3, acetylene to ethylene, and a number of other small triply bonded molecules. It can also reduce protons to hydrogen, a reaction largely suppressed by other reducible substrates. CO is a potent inhibitor of all reductions except that of the proton to hydrogen (Eady & Smith, 1979; Mortenson & Thorneley, 1979; Hardy, 1979.

The most active preparations (2300–3000 units/mg) of the MoFe protein, from a number of sources, contain 2 Mo and approx. 30 non-haem Fe atoms in an $\alpha_2\beta_2$ molecule of M_r approx. 220000 (Smith, 1983). These metal atoms are probably arranged as two iron–molybdenum cofactors (FeMoco), four 'P' clusters and two 'S' iron atoms. We have recently shown (Hawkes *et al.*, 1984) that FeMoco almost certainly includes the enzyme's

Abbreviations used: Kp1 and Kp2 are the MoFe protein and Fe protein respectively of nitrogenase from wild-type *Klebsiella pneumoniae*; NifB$^-$Kp1 is the MoFe protein from *nifB* mutant strains, and FeMoco is the iron–molybdenum cofactor extracted from Kp1; Kp1-asm is the active MoFe protein assembled from NifB$^-$Kp1 and FeMoco.

site for binding reducible substrates. FeMoco is reported to contain 4–9 acid-labile S and 6–8 Fe atoms per Mo atom (Shah & Brill, 1977; Rawlings *et al.*, 1978; Burgess *et al.*, 1980; Smith, 1980; Nelson *et al.*, 1983). It is not clear whether the minor 'S' species of Fe atoms is included within FeMoco (Shah & Brill, 1981). The 'P' clusters are probably best described as unusual [4Fe-4S] clusters that in the dithionite-reduced state are in the very unusual zero oxidation level where all four Fe atoms are Fe^{2+} (Smith & Lang, 1974; Zimmermann *et al.*, 1978; Smith *et al.*, 1980, 1983).

Preparations of the MoFe protein (Kp1) from *K. pneumoniae*, although readily obtained pure in terms of polypeptide composition, remain heterogeneous with respect to their activities and metal contents (Eady & Smith, 1979). Generally, the bulk of any preparation has a specific activity of 1000–1500 units/mg of protein and contains 0.8–1.2 Mo atoms/molecule. This compares with an average specific activity, before purification, of approx. 900 units/mg for Kp1 in crude extracts (supplemented with Kp2) as estimated by rocket immunoelectrophoresis (Hawkes, 1981). Some protein, approaching full activity (with a specific activity of 1800–2000 units/mg and containing 1.4–1.6 Mo/molecule), can be resolved from less-active species by careful chromatography on DEAE-cellulose. However, all purified fractions (varying in specific activity from 700 to 2000 units/mg) have the same specific activity (approx. 275 ± 30 units/ng-atom of Mo) when expressed on the basis of their Mo contents (Hawkes, 1981; Hawkes *et al.*, 1984). Thus, according to the simplest hypothesis, all of the Mo in Kp1 is catalytically active (i.e. contained within functional FeMoco) and the species that are partly resolved by chromatography on DEAE-cellulose differ in their FeMoco contents. However, the problem may be more complex, particularly since the specific activity of Kp1 preparations is also proportional to the number of Mg^{2+} (and probably MgATP)-binding sites on the protein (Kimber *et al.*, 1982).

FeMoco may be extracted from active MoFe protein (Shah & Brill, 1977). At least four *nif*-gene products are involved in its processing. Lesions in the *nifV* gene lead to defective FeMoco (Hawkes *et al.*, 1984), which results in an enzyme deficient in N_2-fixing ability (McLean & Dixon, 1981). Lesions in the genes *nifB*, *nifN* or *nifE* result in inactive cell extracts, which may be activated by adding FeMoco and which, presumably, contain a MoFe protein that only lacks FeMoco (Roberts *et al.*, 1978). We have purified and characterized such a protein, NifB$^-$Kp1, from two distinct *nifB* mutants of *Klebsiella pneumoniae* (Hawkes & Smith, 1983).

NifB$^-$Kp1 was always contaminated with a protein of M_r 21000, but could otherwise be obtained almost pure in terms of polypeptide composition. Preparations always contained some Mo (0.4–0.9 Mo/molecule) and 8–10 Fe atoms/molecule. The Mo was not associated with FeMoco, since (*a*) the characteristic e.p.r. signal of FeMoco was absent, (*b*) the protein was inactive and (*c*) on the basis of magnetic circular dichroism (m.c.d.) spectroscopy (Robinson *et al.*, 1984) probably all of the Fe atoms were contained within 'P' clusters.

Purified NifB$^-$Kp1 combines with FeMoco *in vitro* to form an active MoFe protein (Kp1-asm). This reaction has been exploited to provide information about the metal-containing centres in the MoFe protein (Smith *et al.*, 1983; Hawkes *et al.*, 1983, 1984; Robinson *et al.*, 1984). However, many aspects of the activation reaction were not fully elucidated. In particular, from our earlier work, the specific activity (approx. 500 units/mg) of the Kp1-asm formed with excess FeMoco corresponded to only one-third to one-half of the specific activity of Kp1 isolated from wild-type cells (see above). NifB$^-$Kp1 was stable at 4°C under the conditions used for its purification and, from rocket immunoelectrophoresis, its specific activity in crude extracts was estimated to be approx. 420 units/mg. Thus it appeared not to be inactivated during purification (Hawkes & Smith, 1983). In addition, its low activity could not be explained on the basis of a shortfall in its complement of 'P' clusters (Robinson *et al.*, 1984).

In the present study, with particular emphasis on understanding its low activity, we have further examined the optimal conditions for the activation of NifB$^-$Kp1 and the kinetics of this activation, and have compared the properties of the assembled protein, Kp1-asm, with those of Kp1.

Materials and methods

Materials

Chemicals and biochemicals were generally of the purest grades available from BDH Chemicals (Poole, Dorset, U.K.), and Sigma (London) Chemical Co. (Poole, Dorset, U.K.). DE-52 DEAE-cellulose was from Whatman (Maidstone, Kent, U.K.), Sephadex gels were from Pharmacia (U.K.) (Hounslow, Middx., U.K.) and hydroxyapatite H.A. from L.K.B. (South Croydon, Surrey, U.K.).

Strains of Klebsiella pneumoniae

The wild-type strain M5a1 (Eady *et al.*, 1972) and *nifB* mutant strains 5058 and UNF1718 (Hawkes & Smith, 1983) have been described previously. *K. pneumoniae* strain UNF3860 (Merrick *et al.*, 1980) contains the plasmid pMF260 in the *nif* deletion background UNF107. This plas-

mid carries all the *nif* genes but has a Tn5 transposon inserted into the *nifD* gene. The *nifK* and *nifY* genes are located promoter-distal from *nifD* and are within the same operon. Thus extracts of UNF3860 should have contained all the *nif*-gene products except for the MoFe protein polypeptides (from *nifD* and *nifK*) and the *nifY*-gene product (a protein of M_r approx. 24000 of unknown function; Pühler & Klipp, 1981).

Growth of organisms, preparation of extracts and purification of proteins

Wild-type Kp1 and Kp2 from *K. pneumoniae* strain M5a1 (Eady *et al.*, 1972; Smith *et al.*, 1976) and NifB⁻Kp1 from *K. pneumoniae* strains 5058 and UNF1718 (Hawkes & Smith, 1983) were purified as described previously. *K. pneumoniae* strain UNF3860 was grown in liquid culture and crude extracts were prepared as described for the *nifB* mutant strains (Hawkes & Smith, 1983). For UNF3860, de-repression was indicated by the presence of Kp2 (specific activity 11 units/mg) in the crude extract. Except where otherwise stated, protein solutions were handled under an atmosphere of N_2 and were in 50mM-Tris/HCl buffer (usually pH8.7 for Kp1; pH7.4 for other proteins) containing 0.6mM-dithiothreitol and 2mM-$Na_2S_2O_4$. (Note that for solutions at 4°C pH values were approx. 0.4 unit higher than indicated above.)

Analytical techniques

The activities of the individual nitrogenase components (Kp1 and Kp2) were measured in the presence of an optimal amount of the complementary protein (Eady *et al.*, 1972). Specific activities of the proteins generally refer to acetylene-reduction activities/mg of protein (1 unit of activity = 1nmol of acetylene reduced/min), although activities/ng-atom of Mo in the protein are also sometimes used. NifB⁻Kp1 (at a concentration greater than 1.5μM except where otherwise stated) was first activated with excess FeMoco and then assayed as Kp1 (Hawkes & Smith, 1983).

Protein concentrations, acetylene-reduction activities, H_2-evolution activities, N_2-reduction activities and metal contents were measured by following the techniques described by Eady *et al.* (1972). The ATPase activity of nitrogenase was measured as described by Imam & Eady (1980). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and subsequent staining and destaining were carried out by the methods described by Laemmli (1970) and by Weber & Osborn (1969). E.p.r. spectra were measured on anaerobically prepared frozen samples with a Varian E9 spectrometer (Lowe *et al.*, 1972).

Preparation of FeMoco

FeMoco was prepared as described by Smith (1980). It was assayed by incubating it with an excess of crude extract from *nifB* mutant cells followed by assay of the Kp1 formed. Activities are reported as acetylene-reduction activity units/ng-atom of Mo in the FeMoco preparation.

Results and discussion

Optimal conditions for the activation of NifB⁻Kp1 by excess FeMoco

We first examined the conditions under which the reaction of NifB⁻Kp1 with excess FeMoco was carried out. We had established previously (Hawkes & Smith, 1983) that the optimal conditions for activation were at 30°C in aqueous buffer (pH7.5) containing less than 10% (v/v) *N*-methylformamide with the NifB⁻Kp1 concentration above 1.5μM. Here we report investigations on (1) the kinetics of the activation reaction relative to the stabilities of the reactants and product and (2) the possibility that some undefined component was required for activation but was present in limiting amounts.

(*1*) *Kinetics of activation.* Under the conditions normally used for activation, both NifB⁻Kp1 and FeMoco were, to some extent, unstable, losing respectively 15 and 35% of their activities when incubated alone for 35min. The product of the reaction, Kp1-asm, was, however, fully stable under these conditions.

Since there was no obvious means of quenching the activation reaction without inactivating the Kp1-asm formed we studied the kinetics of activation and the activity of the Kp1-asm formed in the same reaction solution, which included Kp2, MgATP and an ATP-regenerating system (Eady *et al.*, 1972). The results are shown in Fig. 1. The rate of reduction of acetylene to ethylene increased for a short time before reaching a maximum steady value. This induction period reflected the time necessary for complete formation of Kp1-asm; it was marginally decreased by using a higher concentration of NifB⁻Kp1, but was always so short (less than 1.5min) that degradation of the reactants could not have accounted for the low specific activity of the Kp1-asm produced. This specific activity (400 units/mg) was the same as that obtained for this preparation after our normal 30min activation reaction in the absence of MgATP and Kp2.

(*2*) *Requirement for additional factors in the activation of NifB⁻Kp1.* There remained the possibility that some unknown component was present in limiting amounts in our reaction mixtures. We can discount the involvement of additional low-M_r

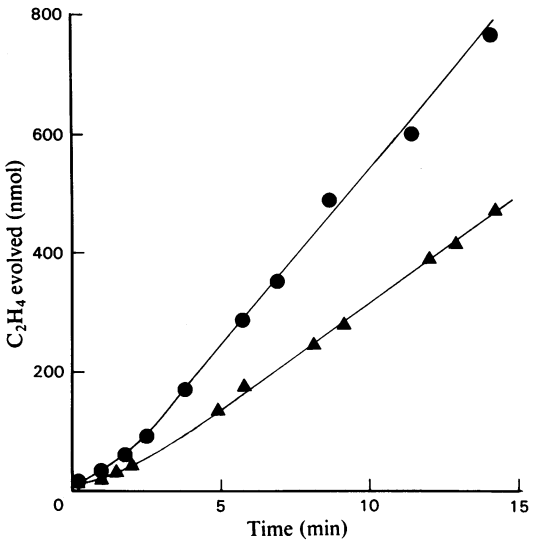

Fig. 1. *Time course of ethylene evolution after addition of FeMoco to acetylene-reduction assays containing NifB⁻Kp1 and Kp2*

Standard acetylene reduction assay mixtures (see the Materials and methods section) at 30°C contained 5 μM-Kp2 and purified NifB⁻Kp1 (75% pure by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) at (●) 0.185 mg/ml or (▲) 0.093 mg/ml. Assays were started by addition of FeMoco, to a final concentration of 0.4 ng-atom of Mo/ml [and less than 1% (v/v) *N*-methylformamide] and after various times (horizontal axis) were stopped by addition of trichloroacetic acid. Data points indicate the amount of ethylene formed (vertical axis) in separate assays. The maximum specific activity of the protein formed in the reaction with the lower concentration of NifB⁻Kp1 (▲) was 400 units/mg.

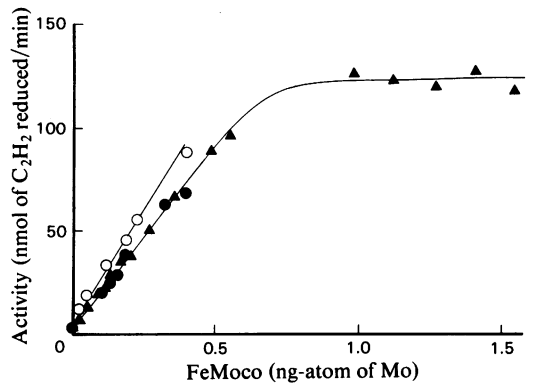

Fig. 2. *Activation of NifB⁻Kp1 by addition of FeMoco*

The data are from three separate experiments. In each experiment different amounts of FeMoco were added to a number of identical solutions of NifB⁻Kp1 in aqueous buffer at pH 7.5. The final volume of the solution was 0.6 ml and the final concentration of *N*-methylformamide did not exceed 8% (v/v). The solutions were incubated for 30 min at 30°C before samples (25–50 μl) were removed to acetylene-reduction assay mixtures (see the Materials and methods section) including 3–5 μM-Kp2. The vertical axis represents the total acetylene-reducing activity of the 0.6 ml solutions, the horizontal axis the amount of FeMoco (ng-atoms of Mo; see the Materials and methods section) that they contained. NifB⁻Kp1 was (○ and ▲) 0.243 mg (approx. 1.1 nmol) of a preparation (approx. 90% pure by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) with a maximum specific activity of 504 units/mg (see the Materials and methods section) or (●) 5.64 mg of a crude extract of strain UNF1718, with a maximum specific activity of 21.5 units/mg. The initial slopes of the graphs indicate that the two preparations of FeMoco used (○, and ● and ▲) had specific activities of 230 and 185 units/ng-atom of Mo respectively.

organic or inorganic components, since these would have been removed from NifB⁻Kp1 during purification. We investigated the involvement of the proteins that contaminated our purest preparations of NifB⁻Kp1 by adding an extract of strain UNF3860 (to a final concentration of approx. 3 mg/ml), with and without 0.5 mM-MgATP, to the activation reaction. This extract would have been expected to enrich the solution in all the proteins in a de-repressed *K. pneumoniae* extract except for the MoFe protein polypeptides and the *nifY*-gene product (see the Materials and methods section). No enhancement of activity was observed. Furthermore, addition of a Kp5058 crude extract, presumably containing the *nifY*-gene product, also did not enhance the activity (after allowing for the extra NifB⁻Kp1 added).

We still cannot discount the possibility that a protein of M_r approx. 21 000, which was present in almost equimolar proportions with NifB⁻Kp1 in our purified preparations, is involved in activation. However, the data presented above indicate that we are not underestimating the potential of NifB⁻Kp1 to be activated by excess FeMoco.

Activation of a fixed quantity of NifB⁻Kp1 with various amounts of FeMoco

Fig. 2 shows the activation of NifB⁻Kp1, in crude extracts and in purified preparations, by addition of increasing amounts of FeMoco. The form of the titration curves, a sharp increase followed by a plateau region, is most simply understood in terms of the tight binding of FeMoco to a single class of site on NifB⁻Kp1. Samples

taken from the activation reaction were diluted approx. 40-fold for the measurement of their acetylene-reduction rates, which were always linear. We can thus set a lower limit to K_S, the apparent dissociation constant of Kp1-asm, of $\geqslant 2 \times 10^9 M^{-1}$. Similarly normal Kp1 does not, apparently, dissociate at low concentrations to yield free FeMoco.

In the early part of the titration curves the activity was linearly dependent on the amount of FeMoco added (ng-atoms of Mo). The slope of this region of the curves (Fig. 2) yields the specific activity of FeMoco (Shah & Brill, 1977; Smith, 1980). For the effective incorporation of FeMoco into NifB⁻Kp1 this activity would be expected to approach 275 ± 27 units/ng-atom of Mo, corresponding to the catalytic activity of Mo (FeMoco) within wild-type Kp1. In previous work (Shah, 1980; Burgess *et al.*, 1980; Smith, 1980) with crude extracts containing NifB⁻Kp1 or related proteins, such activities were indeed observed, indicating that preparations of NifB⁻Kp1 contained molecules capable of binding and activating FeMoco to its fullest extent. Activities of approx. 185 and 230 units/ng-atom of added Mo (initial slopes of curves in Fig. 2) were obtained for the FeMoco preparations used here. These activities were identical for either crude extracts or purified NifB⁻Kp1 (see Fig. 2). We presume that the lower activity was due to limited O_2-damage during the extraction of FeMoco (which is notoriously O_2-sensitive) rather than because of damage to NifB⁻Kp1 during its purification. The higher of these two activities (230 units/ng-atom of Mo) was within 2S.D. of the expected maximum value (275 ± 27 units/ng-atom of Mo).

For the assembly of Kp1 molecules of M_r approx. 218000 containing 2 FeMoco/molecule our activities extrapolate to a specific activity of 1700–2100 units/mg of Kp1-asm (similarly, fully active FeMoco with a specific activity of 275 units/ng-atom of Mo corresponds to the assembly of protein with a specific activity of approx. 2500 units/mg). However, from the plateau region in Fig. 2 the specific activity of the purified NifB⁻Kp1 when apparently saturated with FeMoco was only 500 units/mg. The intercepts of the initial slopes with the plateau of the graphs in Fig. 2 indicate that this apparent saturation required the binding of only 0.4–0.65 molecules of FeMoco/molecule of NifB⁻Kp1 (depending on the assumptions of the purity of the components). Thus, although FeMoco could apparently be fully activated by NifB⁻Kp1, the converse was not true, and it appeared that only some of the molecules in our NifB⁻Kp1 preparations were activated by FeMoco.

Possibly, a minor species (approx. 30% of the total protein) was fully activated (with a specific activity >2000 units/mg) by the binding of 2 FeMoco/molecule while the rest of the molecules remained unactivated. If this hypothesis were true then the non-activatable molecules must bind FeMoco (if at all) much less tightly than do the activatable ones.

Purification of Kp1-asm

When crude extracts of strain 5058 or UNF1718 were saturated with FeMoco and the Kp1-asm was purified, by following a normal purification scheme for Kp1 (Smith *et al.*, 1976), the most active fraction obtained from the final step of gradient elution from DEAE-cellulose, although virtually pure on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, had a specific activity of only 450 units/mg. Further chromatography on hydroxyapatite (Hawkes & Smith, 1983) yielded some protein that had a specific activity of 750 units/mg, but there was insufficient of this material for metal analyses or further purification.

Some inactivation could have occurred during the multiple steps required in purification. Also, to obtain more information, we wished to follow the partitioning of the metal (particularly molybdenum) in NifB⁻Kp1 after addition of FeMoco and resolution into activated and non-activated molecules. Therefore we took samples of purified NifB⁻Kp1, treated them with 58, 72 or 120% of the amount of FeMoco required for complete activation and attempted to resolve the reaction products by using chromatography on DEAE-cellulose.

Fig. 3 shows the elution profile (at 4°C) of the activities obtained after 58% saturation with FeMoco. The excess NifB⁻Kp1 was largely resolved from the Kp1-asm, but there was overlap of the activity profiles with all but the first and last fractions containing both proteins. The recovered NifB⁻Kp1 could still activate FeMoco to the same extent as previously. Some 86% of the original FeMoco activity units and 106% of the original NifB⁻Kp1 activity units were recovered from the column. The highest specific activity of Kp1-asm obtained was 300 units/mg (fractions 4 and 5) and the highest activity/Mo (results not shown) was 110 units/ng-atom of Mo (fraction 4).

Table 1 contains the data from the 72%-saturation experiment. In this experiment no attempt was made to recover NifB⁻Kp1 that had not reacted, but the Mo contents, as well as the activities of the reactants and products, were monitored. The highest specific activity of Kp1-asm obtained was 520 units/mg. Recovery of FeMoco activity units was 80% and that of total protein was 83%. Recovery of total molybdenum was 76%, and the activity/Mo in the Kp1-asm-containing fractions rose steadily as the gradient

progressed, reaching a maximum in the final fraction of 125 units/ng-atom of Mo.

We attempted no detailed fractionation of the Kp1-asm from the experiments in which 120% of the required FeMoco was used. The protein, bound to DEAE-cellulose, was washed with 0.16M-NaCl and eluted with 0.35M-NaCl. The Kp1-asm had a specific activity of 580 units/mg with an average Mo content of 1.38 Mo/molecule (92 units/ng-atom of Mo). Recovery of protein activity units was 86% and that of total protein was 45%.

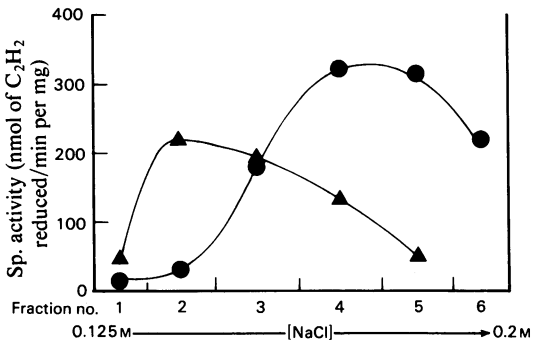

Fig. 3. *Separation by chromatography on DEAE-cellulose of Kp1-asm and NifB⁻Kp1 after partial (58%) activation with FeMoco*

A 407mg portion of partially purified NifB⁻Kp1 was activated with a limited amount of FeMoco to about 58% of the specific activity observed (265 units/mg) with saturating FeMoco. The conditions for activation were similar to those described in the legend to Fig. 2. The protein was applied to a column (1.6cm × 12cm) of DEAE-cellulose and eluted, at 4°C, in 50mM-Tris/HCl buffer, pH7.8, with a linear gradient of 180ml of 0.05–0.25M-NaCl. The Figure shows the specific activities (units/mg) of Kp1-asm (●) and unchanged NifB⁻Kp1 (▲) in the six fractions taken from the column. All protein solutions were kept anaerobic and contained 0.6mM-dithiothreitol and 2mM-$Na_2S_2O_4$. Analytical techniques were as described in the Materials and methods section.

Finally, we carried out a second stage of chromatography on hydroxyapatite of the more-active fractions from DEAE-cellulose in the 72%-saturation experiment (fractions 4–7, Table 1). Less than 30% of the total activity units was lost, and some Kp1-asm with an activity of about 800 units/mg (106 units/ng-atom of Mo) (similar to that purified from activated strain-NifB⁻ extracts) containing 1.64±0.08 Mo/molecule was isolated.

Several conclusions may be drawn from the above data. The recovery of activity units was consistently high, indicating that little damage occurred during activation and the subsequent chromatography. The data in Fig. 3 indicate that, after partial activation with FeMoco, the activated material chromatographed differently from the bulk of the remaining NifB⁻Kp1. The sample of NifB⁻Kp1 used in the 72%-saturation experiment was a (relatively narrow) fraction eluted from DEAE-cellulose by a linear gradient of NaCl (Hawkes & Smith, 1983). Thus any non-activatable molecules that were present might be expected to behave similarly to NifB⁻Kp1 and be largely resolved from Kp1-asm. However, this expectation was not full realized. The increase in the maximum specific activity of Kp1-asm with increasing saturation of NifB⁻Kp1 with FeMoco supports the observation (Fig. 3) that separation of NifB⁻Kp1 from Kp1-asm on DEAE-cellulose was incomplete.

Nevertheless, by itself, poor resolution cannot account for the discrepancy between the maximum specific activities that were obtained (520 and 580 units/mg in the 72%- and 120%-saturation experiments) and that expected (2000–2500 units/mg) on the basis of the formation of a distinct species of Kp1-asm containing 2 FeMoco/molecule. Given the above specific activities and the observation

Table 1. *Isolation of Kp1-asm from partially activated (72%) purified NifB⁻Kp1*

A 304mg portion of NifB⁻Kp1 of specific activity 360 units/mg (after saturation with FeMoco; see the Materials and methods section), containing 0.58 Mo and 6.67 Fe atoms/molecule was allowed to react, for 30min at 30°C, with 356ng-atom of Mo contained in FeMoco of specific activity 220 units/ng-atom of Mo. The product was bound to a column of DEAE-cellulose and chromatographed with a linear gradient of 0.05–0.25M-NaCl, as described in the legend to Fig. 3 but at room temperature. Seven fractions were collected from the column. Analyses for protein, metal contents and enzyme activities were carried out as described in the Materials and methods section. All protein solutions were kept anaerobic and contained 0.6mM-dithiothreitol and 2mM-$Na_2S_2O_4$.

Fraction (0.126–0.212M-NaCl) ...	1	2	3	4	5	6	7
Protein (mg)	30.8	49	43.7	53.5	38.0	24.1	14.9
Kp1 specific activity (units/mg of protein)	8.8	86.5	230	250	377	520	470
Mo (ng-atoms)	60	159	170	184	149	110	56
Activity (units/ng-atom of Mo)	4.5	27	59	76	96	114	125
Mo content (atoms/molecule)		0.71	0.85	0.75	0.85	1.0	0.82

that saturation of our NifB⁻Kp1 preparations required the binding of only 0.4–0.65 FeMoco/molecule of protein, it seems probable that, for the most part, only 1 FeMoco was bound to each activatable NifB⁻Kp1 molecule. On the basis of this half-sites model, still fully consistent with the ability of preparations of NifB⁻Kp1 to activate FeMoco fully, the theoretical maximum activity expected for the Kp1-asm would be only approx. 1000–1250 units/mg. Some protein with a specific activity (800 units/mg) close to this value was isolated by further purification on hydroxyapatite. This procedure removed at least one inactivatable species, that associated with the e.p.r. signal near $g = 1.94$ and found in all our preparations of NifB⁻Kp1 (Hawkes & Smith, 1983). The remaining shortfall (about one-third) in specific activity is consistent with that observed for our normal Kp1 preparations relative to the theoretical maximum (approx. 2500 units/mg) for protein with 2 FeMoco/molecule

A further significant observation concerns the molybdenum content of Kp1-asm. From all of our experiments, the best resolved fractions of Kp1-asm had, on the basis of their total Mo contents, activities of between 92 and 125 units/ng-atom of Mo (see, e.g., Table 1). This approaches half of the value (275 ± 27 units/ng-atom of Mo) observed with preparations of wild-type Kp1 (Hawkes *et al.*, 1984). In the 72%-saturation experiment (Table 1) recovery of total Mo was high. Thus in fractions 5, 6 and 7 of the Kp1-asm (96–125 units/ng-atom of Mo) at least half of the total Mo (0.8–1.0 Mo/molecule) was catalytically inactive (non-FeMoco) Mo carried through from the original NifB⁻Kp1 (0.58 Mo/molecule). Furthermore, non-FeMoco Mo continued to co-purify with Kp1-asm activity on hydroxyapatite chromatography, the most active material having an activity on the basis of total Mo of 106 units/ng-atom of Mo, since the increase in specific activity (to 800 units/mg of protein) was paralleled by an increased Mo content (1.64 Mo/molecule).

An attractive hypothesis therefore is that FeMoco activates only those molecules of NifB⁻Kp1 that contain 1 non-FeMoco Mo/molecule. This Mo might in fact stabilize the activatable form of the molecule. Half-sites activation (see above) of these molecules would follow if the non-FeMoco Mo blocked the binding of a second FeMoco. In principle, on the above hypothesis, it should be possible to isolate Kp1-asm containing 2 Mo/molecule with an activity of only half the maximum obtainable with Kp1, but we have not yet achieved this objective. Our failure to do so is perhaps not surprising, in view of the difficulty experienced in isolating fully active Kp1 (Smith *et al.*, 1976; Eady, 1980).

Our best preparations of NifB⁻Kp1 (500 units/mg) were clearly heterogeneous and contained molecules that could not be activated by FeMoco. We observed previously (Hawkes & Smith, 1983) that the non-FeMoco Mo content of our preparations (0.4–0.9 Mo/molecule) did not correlate with their activation by excess FeMoco. We suggest that these preparations include species with 0, 2 or 1 non-FeMoco Mo atoms/molecule, with only the last of these being activatable by FeMoco.

Finally we note that the protein of M_r approx. 21000 that was the major contaminant in our preparations of NifB⁻Kp1 was absent from our purified Kp1-asm. Thus our observation that much of the non-FeMoco Mo co-purified with Kp1-asm confirms that the Mo in our preparations was not associated with that protein of M_r approx. 21000.

Binding of thiomolybdate to NifB⁻Kp1

It is possible that the Mo in our NifB⁻Kp1 preparations is a precursor of FeMoco, lacking reaction with the *nifB*-gene product, but retaining the ability to bind to the MoFe protein polypeptides. This species would have only transient existence in wild-type cells and therefore would not be expected to be present in normal Kp1. Thiomolybdate (MoS_4^{2-}) is a possible precursor and/or degradation product of FeMoco (Zumft, 1978; Cramer *et al.*, 1978). We therefore investigated the interaction between NifB⁻Kp1 (15 μM; 80% pure by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) and $[(CH_3)_4N]_2MoS_4$ (1 mM). After incubation (15 min) and anaerobic gel filtration on Sephadex G-50, the Mo content of the NifB⁻Kp1 had increased from 0.45 to 1.9 Mo atom/molecule and the specific activity had decreased by 60%. These data demonstrate the principle that molybdenum species other than FeMoco can bind to NifB⁻Kp1, and in doing so can, at least partly, hinder its activation by FeMoco. Note that the Mo species involved in this experiment was not necessarily MoS_4^{2-}, since this ion degrades under aqueous conditions.

Properties of Kp1-asm

Kp1-asm was as temperature-stable as Kp1, retaining its full activity for at least 72 h when kept at 20–22°C. In addition, it was not inactivated by gel filtration through Sephadex G-200 at pH 9.1 (3°C) (a process that inactivated NifB⁻Kp1). In common with Kp1 and NifB⁻Kp1, it behaved as the $\alpha_2\beta_2$ tetramer of M_r approx. 220000 during gel-exclusion chromatography.

If, as we suggest above, Kp1-asm contains only 1 FeMoco bound to each $\alpha_2\beta_2$ tetramer, then its properties, compared with the properties of normal Kp1, should indicate the importance of any

interactions between the two FeMoco centres in the normal protein. Some of these properties are described below.

E.p.r. spectroscopy. The e.p.r. spectrum of dithionite-reduced FeMoco is unique in biology, with *g*-values near 4.3, 3.7 and 2.01 when it is bound to the protein (Smith *et al.*, 1972, 1973), and near 4.8, 3.3 and 2.0 when it is extracted into *N*-methylformamide (Rawlings *et al.*, 1978; Smith, 1980; Hawkes *et al.*, 1984). The spectrum arises from transitions between the $M_s = \pm\frac{1}{2}$ Kramer's doublet of an $S = \frac{3}{2}$ system in which the unpaired electron is mostly located on the Fe atoms (rather than the Mo) in the cluster (Eady & Smith, 1979). The spectrum of Kp1 is compared with that of Kp1-asm in Fig. 4. The spectra from the FeMoco centres are indistinguishable. This indicates that FeMoco in Kp1-asm is in a very similar environment to that in normal Kp1. The Kp1-asm sample also exhibited a weak additional signal with $g_{av.} = 1.95$, which virtually disappeared after chromatography on hydroxyapatite and which we assigned to a minor inactivatable species present in the original NifB$^-$Kp1 preparation (Hawkes & Smith, 1983).

Our e.p.r. instrumentation was unable to attain temperatures ($\leqslant$4.2K) low enough to enable us to integrate the main spectrum from the FeMoco centres. However, the height of the feature near $g = 3.7$ is a practical comparative measure of the intensity of the spectrum for samples measured under identical conditions (Smith *et al.*, 1973). Ten preparations of normal Kp1, with specific activities ranging from 550 to 2000 units/mg, had a ratio of $g = 3.7$ signal height (arbitrary units) to activity of 1.55 ± 0.31; six preparations of Kp1-asm with specific activities ranging from 97.5 to 540 units/mg exhibited a similar ratio of 1.64 ± 0.3. These data indicate an identical and direct relationship getween the FeMoco content (e.p.r. signal intensity) and the protein specific activity within the two classes of proteins. This observation confirms our conclusion (cf. Fig. 2) that FeMoco is activated effectively by NifB$^-$Kp1, and is also in complete accord with the now considerable evidence that FeMoco is the active site of the enzyme and our demonstration of the relationship between the Mo content and the specific activity of Kp1 (Hawkes *et al.*, 1984).

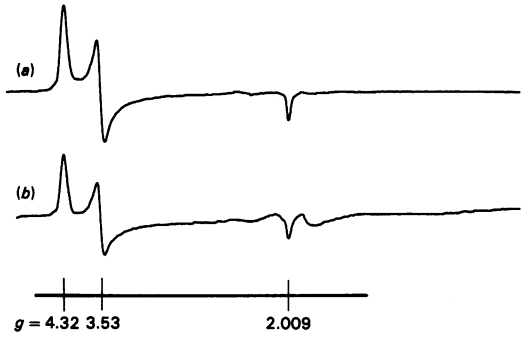

Fig. 4. *E.p.r. spectra of Kp1 and Kp1-asm*
(*a*) Kp1 (specific activity 1300 units/mg) and (*b*) Kp1-asm (specific activity 340 units/mg) were in 50mM-Bes {2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid} buffer, pH6.8, containing 2mM-$Na_2S_2O_4$. E.p.r. spectra were recorded at 9GHz on samples in 3mm-internal-diameter quartz tubes at 12K with field modulation of 1.6mT at 100KHz and a microwave power of 100mW. *g*-Values are represented on a stick diagram.

Pattern of H^+, acetylene and N_2 reduction and of CO inhibition. Our preparations of NifB$^-$Kp1 had very low or zero substrate-reducing activities before activation with FeMoco (Hawkes & Smith, 1983). A preparation of Kp1-asm, assayed with a 50-fold molar excess of Kp2, had an H_2-evolution activity of 400nmol of H_2 evolved/min per mg. This activity was not inhibited by CO. Under an atmosphere of Ar plus 10% acetylene 87% of the reducing power of the enzyme (350 units/mg) was diverted to the reduction of acetylene and the H_2-evolution activity was decreased to 53nmol of H_2 evolved/min per mg. Under an N_2 atmosphere 130 ± 40nmol of NH_3/min per mg and 100nmol of H_2/min per mg were formed, i.e. 65–75% of total electron flow was diverted to N_2 reduction.

The above pattern of substrate reduction is indistinguishable from that observed with normal *K. pneumoniae* nitrogenase (Eady *et al.*, 1972) or any other nitrogenase containing normal MoFe protein (Hardy, 1979).

ATP hydrolysis. NifB$^-$Kp1 alone did not hydrolyse ATP, although in combination with Kp2 a weak ATPase activity was observed. Kp1-asm alone did not hydrolyse ATP, but did in combination with a 14-fold molar excess of Kp2 while evolving H_2 under an atmosphere of Ar. An ATP/2e ratio of 4.7 ± 2:1 was observed in this reaction, very similar to the ratio observed with nitrogenase containing normal Kp1 (Imam & Eady, 1980).

Conclusions

Kp1-asm was apparently identical with normal Kp1 in terms of its M_r, stability, e.p.r. signals (c.f. also Hawkes *et al.*, 1983; Smith *et al.*, 1983), relationship between FeMoco e.p.r.-signal intensity and activity, pattern of substrate reductions, CO inhibition and ATP/2e$^-$ ratio. These data place on a firmer footing the earlier evidence (Roberts *et al.*, 1978; Hawkes & Smith, 1983) that the major

difference between NifB⁻Kp1 and Kp1 is only the absence of FeMoco from the former and that, in MoFe-protein processing, the *nifB*-gene product is involved solely with FeMoco biosynthesis.

However, whereas NifB⁻Kp1 could activate FeMoco fully, in the converse experiment activation of NifB⁻Kp1 by excess FeMoco appeared to be incomplete. We were unable to find any evidence that this was due to an inappropriate choice of experimental conditions, and saturation of NifB⁻Kp1 apparently required the binding of only 0.4–0.65 FeMoco/molecule. Attempts to separate activated molecules from the rest by making use of their altered chromatographic behaviour yielded Kp1-asm, with a specific activity of 800 units/mg (which contained 1.64 Mo/molecule). This is consistent with the activated molecules of NifB⁻Kp1 having bound only 1 FeMoco/molecule, leading to a maximum specific activity of 1000–1200 units/mg. Furthermore the activity/Mo of the Kp1-asm that we isolated was always low and approached half that of normal Kp1 (or of FeMoco activated with excess NifB⁻Kp1). The hypothesis that best fits our data is that FeMoco does not activate all NifB⁻Kp1 molecules but only those already containing 1 non-FeMoco Mo atom/molecule. Assuming this hypothesis to be true, our data would indicate that the two sites in Kp1 operate independently. It is possible that the non-FeMoco Mo could be a precursor of FeMoco that lacks modification by the *nifB*-gene product.

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