Molybdenum-independent nitrogenases of *Azotobacter vinelandii*: a functional species of alternative nitrogenase-3 isolated from a molybdenum-tolerant strain contains an iron–molybdenum cofactor

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Nitrogenase-3 of *Azotobacter vinelandii* is synthesized under conditions of molybdenum and vanadium deficiency. The minimal metal requirement for its synthesis, and its metal content, indicated that the only transition metal in nitrogenase-3 was iron [Chisnell, Premakumar and Bishop (1988) J. Bacteriol. **170**, 27–33; Pau, Mitchenall and Robson (1989) J. Bacteriol. **171**, 124–129]. A new species of nitrogenase-3 has been purified from a strain of *A. vinelandii* (RP306) lacking structural genes for the Mo- and V-nitrogenases and containing a mutation which enables nitrogenase-3 to be synthesized in the presence of molybdenum. SDS/PAGE showed that component 1 contained a 15 kDa polypeptide which N-terminal amino acid sequence determination showed to be encoded by *anfG*. This confirms that nitrogenase-3, like V-nitrogenase, comprises three subunits. Preparations of the nitrogenase-3 from strain RP306 contained 24 Fe atoms and 1 Mo atom per molecule. Characterization of the cofactor centre of the enzyme by e.p.r. spectroscopy and an enzymic cofactor assay, together with stimulation of the growth of strain RP306 by Mo, showed that nitrogenase-3 can incorporate the Mo-nitrogenase cofactor (FeMoco) to form a functional enzyme. The specific activities (nmol of product produced/min per mg of protein) determined from activity titration curves were: under N_2, NH_3 formation 110, with concomitant H_2 evolution of 220; under argon, H_2 evolution 350; under 10% acetylene (C_2H_2) in argon, ethylene (C_2H_4) 58, ethane (C_2H_6) 26, and concomitant H_2 evolution 226. The rate of formation of C_2H_6 was non-linear, and the C_2H_6/C_2H_4 ratio strongly dependent on the ratio of nitrogenase components.

INTRODUCTION

Three genetically distinct nitrogenase systems capable of supporting growth of *Azotobacter* on dinitrogen have been identified. *Azotobacter vinelandii* and *A. chroococcum* each have a well-characterized molybdenum (Mo)-nitrogenase as well as a vanadium (V)-containing nitrogenase which is synthesized when V is present in Mo-deficient conditions. *A. vinelandii*, but not *A. chroococcum*, also has a third nitrogenase, nitrogenase-3, which supports growth when both Mo and V are deficient. Nitrogenase-3 appears to contain only iron (Chisnell et al., 1988). Nitrogenase-3 has also been purified from the phototrophic species *Rhodobacter capsulatus* (Schneider et al., 1991b; Müller et al., 1992). All nitrogenases have similar requirements for activity: MgATP, a low-potential electron donor and the absence of oxygen. They can each be separated into two essential protein components: component 2, an Fe-containing protein, and component 1. The latter is a MoFe protein ($Av1^{Mo}$) in Mo-nitrogenase, a VFe protein ($Av1^{V}$) in V-nitrogenase and a homologous protein ($Av1^{Fe}$) which contains Fe, but only low levels of Mo or V, in nitrogenase-3 [see Eady (1990), Pau (1991) and Bishop and Premakumar (1992) for reviews].

The products of *nifDK*, the genes encoding the α and β-chains of component 1 of Mo-nitrogenase, show considerable sequence similarity to the products of the corresponding genes for V-nitrogenase and nitrogenase-3 (Joerger and Bishop, 1988; Joerger et al., 1990; Pau, 1991). However, an additional small open reading frame (*vnfG* and *anfG*) is located between the genes encoding the α and β-subunits of the V-nitrogenase and nitrogenase-3. In *A. chroococcum*, *vnfG* has been shown to encode a low-molecular-mass subunit of $Ac1^{V}$. V-nitrogenase therefore has an $\alpha_2\beta_2\delta_2$ structure, in contrast with the $\alpha_2\beta_2$ structure of Mo-nitrogenases (Robson et al., 1989). The presence of a similar gene (*anfG*) in the cluster of genes encoding nitrogenase-3 suggests that this system also has a δ-subunit, although this has not been shown directly.

X-ray-absorption-spectroscopy studies (Arber et al., 1987; George et al., 1988) have shown that Mo and V in the MoFe- and VFe proteins are present in analogous cofactor centres and have a similar ligand geometry. An Fe- and V-containing cofactor (FeVaco), similar to the Fe- and Mo-containing cofactor (FeMoco) of MoFe proteins, has been extracted from the VFe protein of *A. chroococcum* (Smith et al., 1988). No comparable data are available for nitrogenase-3. However, indirect evidence suggests that nitrogenase-3 contains a cofactor centre like those in Mo- and V-nitrogenases. First, the genes *nifB* and *nifV*, which are involved in FeMoco biosynthesis, are also required for V- and nitrogenase-3 activity (Joerger and Bishop, 1988; Kennedy and Dean, 1992). Secondly, sequence comparisons of the products of the structural genes shows that amino acid residues implicated in forming part of the FeMoco-binding site in MoFe protein are conserved in all three nitrogenases. These residues have been identified by studies involving site-directed mutagenesis (Hawkes et al., 1984; Kent et al., 1989, 1990; Dean et al., 1990; May et al., 1991) and, very recently, by the determination at 0.27 nm (2.7 Å) resolution of the X-ray structure of the MoFe protein from *A. vinelandii* (Kim and Rees, 1992a,b).

We report here the purification of component 1 of *A. vinelandii*

Abbreviations used: nitrogenase component proteins are referred to by abbreviations in which the first two letters refer to the bacterial species (Av, *A. vinelandii*, Ac, *A. chroococcum*, Kp, *Klebsiella pneumoniae*, Rc, *Rhodobacter capsulatus*), the numerals 1 and 2 refer to component 1 and component 2 proteins, and the superscripts $^{Mo, V, Fe}$ to Mo-, V- or nitrogenase-3; FeMoco, iron and molydenum-containing cofactor of the MoFe protein; FeVaco, iron and vanadium-containing cofactor of the VFe protein; NMF, *N*-methylformamide; NifEN and VnfEN, biosynthetic proteins for Mo-nitrogenase and alternative nitrogenase cofactors respectively.

nitrogenase-3 from a strain with deletions in the structural genes for both Mo- and V-nitrogenases, and containing a mutation which enables the strain to grow in the presence of Mo. The N-terminal amino acid sequences of the polypeptides of this protein show unequivocally that $Av1^{Fe}$, like $Av1^{V}$, but unlike $Av1^{Mo}$, comprises three subunits. However, our preparations of $Av1^{Fe}$ contained approx. 1 g-atom of Mo per mol, which was extracted in a form able to reconstitute activity in the FeMoco assay system *in vitro*. Furthermore, Mo stimulated the growth of this strain of *Azotobacter* which only has the structural genes for nitrogenase-3. The implications of these results are discussed in the context of the original observations which indicated that nitrogenase-3 contained only Fe.

MATERIALS AND METHODS

Bacterial strains and growth

Azotobacter vinelandii strain RP206 has deletions in the structural genes for both the Mo- and V-nitrogenases (Δ*nifHDK*, Δ*vnfDGK*, Rif^r, $Spec^r$; Pau et al., 1989) and is unable to grow in the presence of Mo (Pau et al., 1989). Strain RP306 is a spontaneous Mo-tolerant mutant of strain RP206. It was obtained by selecting a colony of strain RP206 which grew on solid medium containing 10 μM Mo. Cells were grown in N-free *Azotobacter* medium (Pau et al., 1989) in 50 ml sidearm flasks incubated in air at 30 °C with rotary shaking (200 rev./min), and growth was monitored with a Klett–Summerson photoelectric colorimeter with a no. 54 green filter. For enzyme purification, strain RP306 was grown in a 400-litre all-glass fermenter in the N-free medium without added Mo and V. Cells were harvested and extracts prepared as described by Eady et al. (1987).

Assay of nitrogenase activity

Nitrogenase activity was measured as NH_3 formation or H_2 evolution as described by Eady et al. (1987). Ethylene (C_2H_4) and ethane (C_2H_6) as products of acetylene (C_2H_2) reduction were measured as described by Dilworth et al. (1988). One unit of enzyme activity is defined as the amount of nitrogenase component required to produce 1 nmol of product/min when complemented with the optimal molar ratio of the second component. Specific activities refer to the nitrogenase component limiting the assay.

Analytical methods

Fe, Mo, V and acid-labile sulphide analyses were carried out as described by Eady et al. (1987). E.p.r. spectra were measured using a Bruker ER200D spectrometer fitted with an Oxford Instruments ESR9 liquid-He cooling system.

Cofactor extraction and assay

Purified $Av1^{Fe}$ was precipitated with citric acid under anaerobic conditions and the precipitated protein treated with *NN'*-dimethylformamide before extraction with *N*-methylformamide (NMF), essentially as described by Smith et al. (1988) for the extraction of the cofactor FeVaco from $Av1^{V}$. The cofactor activity of the NMF solution was measured using nitrogenase component 1 protein (Kp1) purified from *Klebsiella pneumoniae* strain 5058 (a *hisD nifB* point mutant) as described by Hawkes and Smith (1983).

N-terminal amino acid sequence determination

Purified $Av1^{Fe}$ was electrophoresed in 20% polyacrylamide/SDS gels and the separated polypeptides blotted on to a poly(vinylidene difluoride) membrane (Immobilon, from Millipore (U.K.) Ltd., Harrow, Middx., U.K.) using a semi-dry electroblotting procedure (Kyhse-Anderson, 1984). After staining with Coomassie Blue, the portions of the filters containing the three predominant bands corresponding to 15, 51 and 58 kDa were cut out and subjected to gas-phase N-terminal amino acid sequence analysis using an Applied Biosystems 470 sequenator equipped with a 120 on-line analyser. Prior to sequencing, cysteine was converted into *S*-methylcysteine by pyridine ethylation. Densitometric analysis of the stained gels was carried out with an UltroScan XL laser densitometer (Pharmacia Ltd., Central Milton Keynes, Bucks., U.K.).

Purification of nitrogenase components

The component proteins of nitrogenase-3 were separated and purified from crude extracts of *A. vinelandii* (strain RP306) following much of the procedure of Chisnell et al. (1988), who isolated this nitrogenase from a tungstate-tolerant strain deleted for the structural genes of Mo-nitrogenase (*A. vinelandii* strain CA11.6) grown in N-free Burks medium without added Mo or V. Our procedure was modified as described below.

The 52 °C heat treatment of the fractions containing nitrogenase activity eluted from DEAE-cellulose by 0.25 M NaCl used in their procedure was omitted. Instead, the active fractions were chromatographed directly on Sephacryl S-200 (superfine grade) (essentially under the conditions described by Chisnell et al., 1988), a step which separates the Fe protein ($Av2^{Fe}$) from $Av1^{Fe}$.

In place of the preparative-electrophoresis step described by Chisnell et al. (1988), both components were purified further by NaCl-gradient elution from DEAE Sephacel equilibrated with 50 mM Tris/HCl, pH 8.0, as described for the components of V-nitrogenase of *A. chroococcum* (Eady et al., 1987, 1988). The most active fractions ($Av1^{Fe}$ specific activities: 350 nmol of H_2 evolved $min^{-1} \cdot mg$ of $protein^{-1}$ and $Av2^{Fe}$: 518 nmol of H_2 evolved $min^{-1} \cdot mg$ of $protein^{-1}$) were equilibrated with 50 mM Tris/HCl, pH 8.0, containing 10 mM $MgCl_2$ and 0.4 g/l $Na_2S_2O_4$ by gel filtration through Bio-Gel P6-DG before being used in the experiments described here.

RESULTS AND DISCUSSION

Growth of *A. vinelandii* strain RP306

Growth of the strain RP306 and the parent strain RP206 in the presence of media containing different concentrations of Mo are shown in Figure 1. Low concentrations of Mo repress growth of strain RP206, but not strain RP306, whose growth is, by contrast, stimulated by increasing concentrations of Mo.

Purity and subunit structure

Despite the similarities in physicochemical properties between MoFe- and VFe-proteins, the latter have been shown to contain an additional type of small subunit. This 13 kDa δ-subunit is encoded by *vnfG*, a gene located between *vnfD* and *vnfK*, which has no counterpart in the structural-gene cluster of Mo-nitrogenase. There is an open reading frame with sequence similarity to *vnfG* between the genes *anfD* and *anfK* encoding the α- and β-subunits of $Av1^{Fe}$, and it has been proposed that, like the $Av1^{V}$, $Av1^{Fe}$ has a δ-subunit (Joerger et al., 1989). SDS/PAGE of our

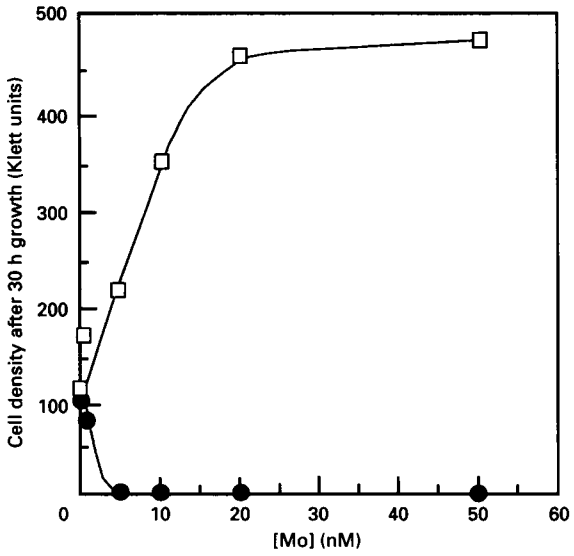

Figure 1 Effect of molybdenum on growth of *A. vinelandii* strains RP206 (●) and RP306 (□)

The relative cell densities measured after 30 h were representative of all time points during exponential growth.

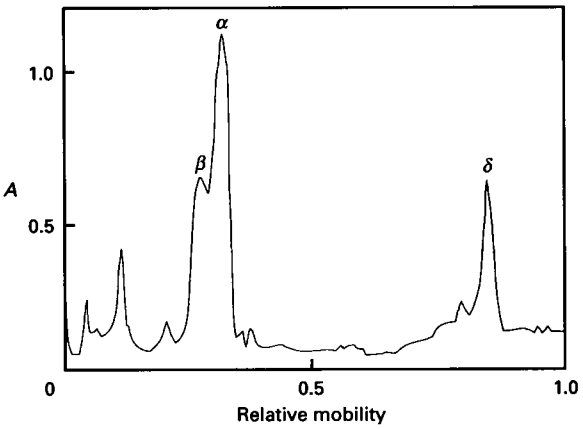

Figure 2 SDS/PAGE of *A. vinelandii* nitrogenase-3 from strain RP306

Densitometric plot of a Coomassie Blue-stained SDS/PAGE gel. The 51, 58 and 15 kDa polypeptides of the α-, β- and δ-subunits are labelled α, β and δ.

preparations of $Av1^{Fe}$ showed three bands with molecular masses of 15, 51 and 58 kDa when stained with Coomassie Blue (Figure 2). Table 1 shows that the N-terminal amino acid sequence of the small peptide in our preparations correlates with the derived amino acid sequence of AnfG, indicating that, as inferred previously from the DNA sequence data, $Av1^{Fe}$ has a δ-subunit. The data in Table 1 also confirm that the N-terminal amino acid sequences of the two larger polypeptides of $Av1^{Fe}$ isolated from SDS/PAGE gels correspond to the derived amino acid sequence of AnfD and AnfK. They are distinct from the N-terminal-amino acid sequences of the products of *nifEN* and *vnfEN* which are homologous with the nitrogenase α- and β-subunits and are involved in cofactor biosynthesis (Brigle et al., 1987; Wolfinger and Bishop, 1991). However the ratios of the intensities of the

Table 1 N-terminal amino acid sequences of polypeptides of $Av1^{Fe}$ compared with the sequence predicted for the products of *anfDGK* genes

N-terminal amino-acid sequences were determined on polypeptides of $Av1^{Fe}$ separated by SDS/PAGE as described in the Materials and methods section. Unidentified residues are represented by X, and residues which gave small or ambiguous peaks are indicated. The derived amino acid sequences are those of Joerger et al. (1989), and begin with the first amino acid encoded after the start codon.

Polypeptide	Sequence						
AnfD	P	H	H	E	F	E	
α-subunit	P	X	E?	E	F	E	
AnfG	S	T	A	S	A	A	A
δ-subunit	S	T	A	X	A	A	A
AnfK	T	C	E	V	K	E	
β-subunit	T	C or V	E	V	K	E	

Table 2 Comparison of the metal- and acid-labile sulphide (S^{2-}) contents of different species of $Av1^{Fe}$ and component 1 of *R. capsulatus* nitrogenase-3 ($Rc1^{Fe}$)

The data for $Av1^{Fe}$ were obtained as described in the Materials and methods section and have been calculated for a molecular mass of 250 kDa.

	Content (g-atom/mol)				
Species	Fe	Mo	V	S^{2-}	References
$Av1^{Fe}$	24.2	1.07	0.05	17.9	The present work
$Av1^{Fe}$ (slow species)	28.0	0.10	0.01	20.3	Chisnell et al. (1988)
$Rc1^{Fe}$	24.0	0.02	0.02	N.A.	Müller et al. (1991)

three bands of our preparations on SDS/PAGE gels, averaged from scans of several lanes, was 1.7:1.0:2.8, which approximates to a subunit ratio of $\alpha_2\beta\delta_3$, although interpreting this data is problematical owing to differential binding of the dye (see the α- and β-chains in Figure 2). Nitrogenase α- and β-subunits have been reported to stain unequally with Commassie Blue (Eady et al., 1972). $Av1^{Fe}$ purified by Chisnell et al. (1988) was isolated as electrophoretically distinct $\alpha_2\beta_2$ and $\alpha_2\beta$ forms, but under their conditions the smallest polypeptide would not be expected to be resolved. The species of $Av1^{Fe}$ isolated by us corresponds to their 'fast' form ($\alpha_2\beta$). It should be noted that, contrary to the assignment given in Chisnell et al. (1988), the faster-running subunit is the α-chain.

Metal and acid-labile sulphide content

The Fe, V, Mo and acid-labile sulphide content of $Av1^{Fe}$ are shown in Table 2, where they are compared with data for the more active of the two species of this protein isolated by Chisnell et al. (1988), and also with component 1 of nitrogenase-3 of *R. capsulatus* ($Rc1^{Fe}$) (Müller et al., 1991). The Fe- and acid-labile sulphide contents are broadly similar, but, surprisingly, our preparations have a significant Mo content. This finding was unexpected, because Mo represses expression of the structural genes for nitrogenase-3 (Jacobson et al., 1986; Luque and Pau, 1991), and previous preparations of the enzyme had low Mo

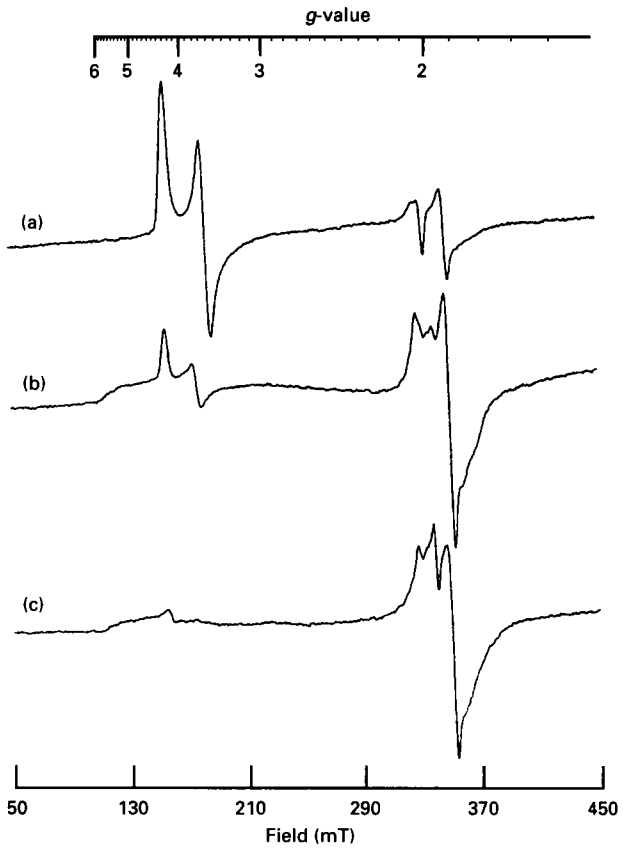

Figure 3 E.p.r. spectra of dithionite-reduced $Av1^{Fe}$ as isolated and during enzyme turnover in the presence of MgATP and $Av2^{Fe}$

The spectra were measured at 10 K with a microwave power of 20 mW at 9.47 GHz using 2.1 mT field modulation at 100 KHz. The dithionite-reduced $Av1^{Fe}$ was in 50 mM Tris/HCl buffer, pH 8.0, containing 50 mM $MgCl_2$ and 0.4 g/l $Na_2S_2O_4$. (**a**) $Av1^{Fe}$ (8.9 mg); (**b**) $Av1^{Fe}$ (3.6 mg) and $Av2^{Fe}$ (4.0 mg) in a reaction mixture containing 50 μmol of Hepes/NaOH, pH 7.4, 25 μmol of $MgCl_2$, 40 μmol of phosphocreatine, 30 μmol of $Na_2S_2O_4$ and 100 μg of creatine phosphokinase in a total volume of 0.21 ml; (**c**), as (**b**), but with 4 μmol of MgATP added and frozen after ~ 30 s by plunging the e.p.r. tube into liquid N_2.

contents (Chisnell et al., 1988). The possibility that Mo was somehow associated adventitously with our preparations, as appears to be the case with the inactive MoFe protein synthesized by a *nifB* mutant of *K. pneumoniae* (Hawkes and Smith, 1983), appears unlikely, since the e.p.r. and extrusion studies discussed below indicate that our preparations of $Av1^{Fe}$ contain FeMoco.

E.p.r. spectra

At low temperature, dithionite-reduced $Av1^{Fe}$ isolated from strain RP306 exhibited an e.p.r. spectrum with features at g values of 4.3, 3.66 and 2.01 typical of those arising from the $S = 3/2$ spin system of the FeMoco centre of the MoFe proteins (Figure 3a) (see Lowe, 1992). These data contrast with those of Müller et al. (1991), who reported a $g = 5.44$ e.p.r. signal assigned to a $S = 3/2$ spin system of maximal rhombicity in component 1 of *R. capsulatus* nitrogenase-3. Preparations of the latter protein contain negligible levels of Mo. We were unable to observe similar e.p.r. features when a sample of $Av1^{Fe}$ at 140 mg/ml was measured at 10 K at 20 mW microwave power and 2.2 mT modulation, with data accumulated over 20 min. We therefore consider that our preparations do not contain an e.p.r.-detectable centre of this type. The e.p.r. spectra of isolated $Av1^{Fe}$ also show a signal of low intensity with a $g_{a.v.} = 1.94$ attributable to a $S = 1/2$ spin system. Similar signals are found in preparations of Mo- and V- nitrogenases and nitrogenase-3 (Zumft and Mortenson, 1973; Eady et al., 1990; Müller et al., 1991). In the case of Mo nitrogenase it is associated with an inactive species (Zumft and Mortenson, 1973).

The species of $Av1^{Fe}$ from which the $S = 3/2$ e.p.r. species arises is redox-active during turnover, like the FeMoco centre of the MoFe protein. The intensity of the features around $g = 3.7$ are considerably diminished in a mixture of $Av1^{Fe}$ and $Av2^{Fe}$ when MgATP is added to initiate enzyme turnover (compare Figure 3b and 3c). The broad feature between $g = 6$ and $g = 4.3$ arises from $Av2^{Fe}$ (results not shown). By analogy with the more intensively studied Mo-nitrogenase, the diminution of the $g = 3.7$ features is consistent with the centre in $Av1^{Fe}$ being reduced beyond the dithionite-reduced paramagnetic oxidation level during substrate reduction.

Extraction of FeMoco from $Av1^{Fe}$

When our preparations of $Av1^{Fe}$ were precipitated and extracted with NMF under conditions which extract FeMoco from MoFe protein, and FeVaco from the VFe protein, a yellowish-brown solution was obtained. This NMF solution, which contained Mo and Fe in a ratio of 1:4.3, was capable of activating $Kp1^{Mo}$ from a *nifB* mutant of *K. pneumoniae*. In a typical incubation, 67% of the C_2H_2-reducing activity of $Av1^{Fe}$ was recovered in the cofactor assay, corresponding to an activity of 105 nmol C_2H_2 reduced/ng-atom of Mo based on the Mo content of the NMF solution. This compares with the maximum value of 275 nmol of C_2H_2 reduced/ng atom of Mo obtained for FeMoco isolated from $Kp1^{Mo}$ (Hawkes and Smith, 1983).

A characteristic of both V-nitrogenase and nitrogenase-3 activities is reduction of C_2H_2 to form some C_2H_6 in addition to C_2H_4, the usual product with Mo-nitrogenase (Dilworth et al., 1988; Pau et al., 1989). This characteristic activity was transferred to the hybrid enzyme formed when FeVaco extracted from $Ac1^{V}$ was combined with apo-$Kp1^{Mo}$ (Smith et al., 1988). In the present case, when C_2H_2 was used as the substrate in the cofactor assay of the NMF extract of $Av1^{Fe}$, C_2H_4 was the only product of C_2H_2 reduction detected (results not shown). The possible significance of the failure of the cofactor isolated from $Av1^{Fe}$ to transfer the characteristic of high levels of production of C_2H_6 from C_2H_2 is discussed below.

The species of $Av1^{Fe}$ which we have isolated appears to contain only one FeMoco centre per molecule. A precedent for occupancy of one of the two cofactor-binding sites (half-site occupancy) is provided by $Av1^{Mo}$ isolated from cells grown in the presence of tungstate (Hales and Case, 1987). Under these conditions, both Mo and W (tungsten) are processed and incorporated into the protein which exhibits two distinct $S = 3/2$ e.p.r. spectra. One corresponds to a normal FeMoco signal and the other is hypothesized to arise from a W-analogue of FeMoco. During turnover, only the former is bleached, indicating that it is redox-active, and the activity of the protein is suggested to arise from the FeMoco centre it contains.

Substrate reduction

The species of $Av1^{Fe}$ we have purified was active only when combined with the Fe protein of nitrogenase-3, since when complemented with purified $Av2^{Mo}$ or $Ac2^{V}$, no nitrogenase activity was detected. Similar results were reported by Chisnell et

Table 3 Comparison of the specific activities of $Av1^{Mo}$ and different species of $Av1^{Fe}$

Data for $Av1^{Mo}$ are derived from [a]Hales et al. (1986) and [b]Chisnell et al. (1988). Data for the present work are derived from assays using saturating concentrations of $Av2^{Fe}$ as determined by activity titration curves of the type shown in Figure 5. Other data are those of Chisnell et al. (1988) for the faster-running and slower-running species separated by preparative SDS/PAGE.

	Specific activity (nmol of product/min per mg of protein)					
Substrate...	H^+	N_2 and H^+		H^+ and C_2H_2		
Protein Product...	H_2	NH_3	H_2	C_2H_4	C_2H_6	H_2
$Av1^{Mo}$	2220[a]	1040[a]	nd*	2000[b]	0	303[b]
$Av1^{Fe}$	350	110	220	58	26	226
$Av1^{Fe}$ (fast)	203	30	145	18	nd	124
$Av1^{Fe}$ (slow)	253	38	213	28	nd	202

* nd, not determined.

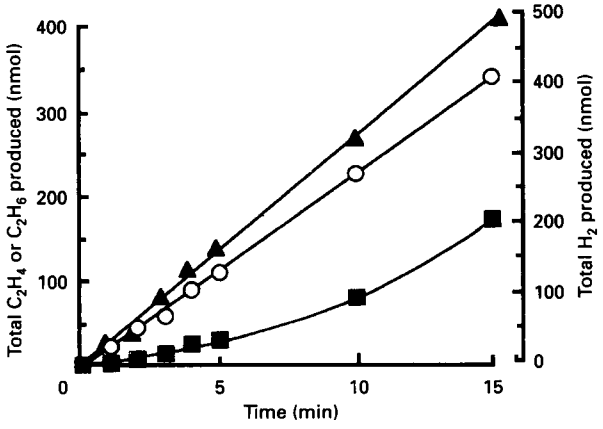

Figure 4 Time courses of C_2H_4 and C_2H_6 formation from C_2H_2 and H_2 evolution

$Av2^{Fe}$ and $Av1^{Fe}$ (molar ratio 26:1) were assayed for the times indicated under standard assay conditions before product analysis: ▲, H_2 evolution under C_2H_2; ○, C_2H_4 formation; and ■, C_2H_6 formation from C_2H_2.

al. (1988) and Premakumar et al. (1989) for their species of $Av1^{Fe}$ lacking Mo.

Previous preparations of nitrogenase-3, which contained less than 0.15 g-atom of Mo per mol, had low activities towards N_2 (Chisnell et al., 1988). The activities of Mo-independent nitrogenases are lower than that of Mo-nitrogenase. For N_2 reduction, the published values for specific activities (nmol of NH_3 produced/min per mg of protein) show a 30-fold range, from 990 for $Kp1^{Mo}$, 350 for $Ac1^{V}$ to ~35 for $Av1^{Fe}$ (see Eady, 1990). However, nitrogenase-3 is clearly capable of supporting good rates of N_2-dependent growth (Pau et al., 1989). It is not clear whether the reason for the low activity of the purified enzyme is due to loss of some essential component during purification or because the optimum conditions for assay have not been attained. Our preparations of $Av1^{Fe}$ have a higher specific activity for substrate reduction than those reported previously, in particular the activity with N_2 is some 3-fold greater (see Table 3). Furthermore, the total electron flux under N_2 is greater than

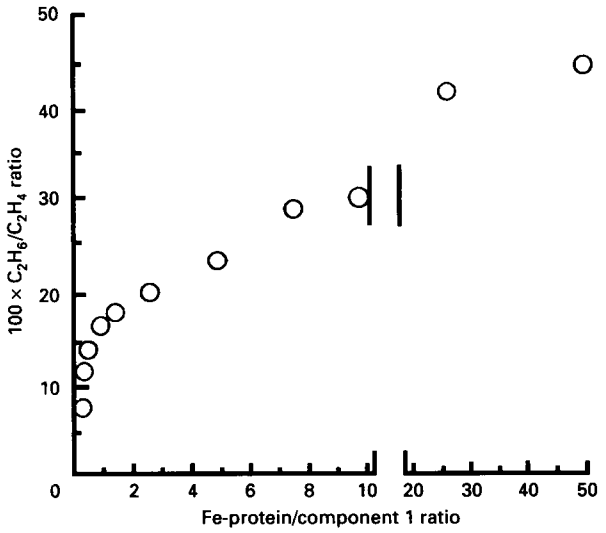

Figure 5 Effect of the $Av2^{Fe}$ and $Av1^{Fe}$ molar ratio on the electron-flux-to-C_2H_4/electron-flux-to-C_2H_6 ratio

Each assay mixture contained $Av1^{Fe}$ (500 μg for molar ratios up to 2.5 and 72.5 μg for higher molar ratios) and appropriate amounts of $Av2^{Fe}$ to give the component ratios shown. Assays were run for 10 min before quenching and product analysis.

under argon or argon plus C_2H_2. The extrusion data discussed above, and the products formed on reduction of C_2H_2, suggest that the higher activities are a consequence of our preparations of $Av1^{Fe}$ containing FeMoco.

Under a 10 kPa atmosphere of C_2H_2 in Ar, time courses for the reduction of C_2H_2 to C_2H_4 and protons to H_2 were linear (Figure 4). In contrast, the time course for the appearance of C_2H_6 as a product of C_2H_2 reduction was non-linear. Similar kinetics have been reported for the V-nitrogenase of *A. chroococcum*, where free C_2H_4 was shown not to be an intermediate in the formation of C_2H_6, and it was proposed that different routes exist leading to the formation of C_2H_6 as a product of C_2H_2 (Ashby et al., 1987) and C_2H_4 reduction (Dilworth et al., 1988; Lowe et al., 1990).

The titration curves for C_2H_2 reduction by complementation of $Av1^{Fe}$ with increasing concentrations of $Av2^{Fe}$ show that the ratio of C_2H_6 to C_2H_4 is strongly dependent on the ratio of the component proteins. It reached a limiting value of 0.45 at the highest $Av2^{Fe}$ concentration tested (Figure 5). This ratio of products is an order of magnitude larger than that observed for V-nitrogenase where titration of V-nitrogenase components of *A. chroococcum* showed a 10-fold lower limiting value of 0.045 at saturating $Ac2^{V}$ concentrations (Dilworth et al., 1988). However, we have observed that this ratio is dependent on the absolute protein concentration. Further work is required to establish the reasons for this.

Implications for cofactor of $Av1^{Fe}$

The pathway leading to synthesis of the cofactor for MO-nitrogenase and the alternative nitrogenases is branched, requiring the products of two sets of genes. NifEN is required for synthesis of FeMo cofactor, and VnfEN for the cofactors of the alternative nitrogenases (Brigle et al., 1987; Wolfinger and Bishop, 1991). We have shown previously that a strain deleted for *nifEN* and structural genes for both alternative nitrogenases (*vnfDGK* and *anfHD*) synthesizes a species of Mo-nitrogenase

which supports growth under N_2, but, unlike Mo-nitrogenase, reduces C_2H_2 to C_2H_6 (Eady et al., 1990). Thus *vnfEN*, genes normally involved solely in synthesis of alternative nitrogenase cofactors can function, as a result of deletion of *nifEN*, in combination with Mo-nitrogenase structural genes to produce a nitrogenase with altered catalytic properties. Another mutant strain, which combined *nifEN*, the genes required for FeMo cofactor synthesis, with the structural genes for V-nitrogenase, synthesized an enzyme capable of reducing C_2H_2, but not N_2. Again, the nitrogenase produced probably contains an alternative nitrogenase cofactor inappropriately inserted into Mo-nitrogenase polypeptides.

The results presented here are consistent with nitrogenase-3 being able to incorporate FeMoco into one of the two cofactor-binding sites. FeMoco was presumably incorporated into nitrogenase-3, because *A. vinelandii* concentrates Mo very efficiently from medium made from laboratory-grade chemicals without added Mo (Pienkos and Brill, 1981; Schneider et al., 1991a). Further, strain RP306 contains a spontaneous mutation which enables it to grow diazotropically using an alternative nitrogenase in the presence of Mo, which would otherwise repress transcription of alternative nitrogenase structural genes. Two classes of mutants can be expected to confer such Mo-resistance: those which affect metal-dependent gene regulation and those which reduce the intracellular levels of Mo. The spontaneous mutation in strain RP306 most likely affects metal-dependent regulation, for Mo transport is unaffected (Figure 1). Since the normal repressive effects of Mo are blocked, both FeMoco and nitrogenase-3 can be synthesized together, resulting in the incorporation of the cofactor into unusual nitrogenase polypeptides. Such novel cofactor/polypeptide combinations are the result of altered gene regulation.

The diazotrophic growth rate of strains such as RP206, which can only synthesize nitrogenase-3, is approximately a third of the growth rate of cells synthesizing Mo nitrogenase (Pau et al., 1989). However, nitrogenase-3 purified from strain CA11.6, a Mo-nitrogenase deletion strain containing a different Mo-resistant spontaneous mutation from that in strain RP306, has unexpectedly low nitrogen reducing activity relative to Mo-nitrogenase (Chisnell et al., 1988; Table 3). The higher activity of nitrogenase-3 *in vivo* is not the result of the incorporation of FeMoco, since N_2-dependent growth of strain RP206, which lacks a Mo-resistant mutation, is inhibited by Mo (Figure 1; Pau et al., 1989; Luque and Pau, 1991). However, addition of Mo (10 μM) to cultures of strain RP206 results in a change in the *in vivo* catalytic properties of nitrogenase-3, the ratio of C_2H_6 to C_2H_4 formed as products of C_2H_2 reduction by whole cells changes from 0.04 in the absence of Mo to 0.45 shortly after Mo addition. This change suggested that Mo can be incorporated into nitrogenase-3 (Pau et al., 1989). In strain RP206, nitrogenase-3 activity is subsequently lost, owing to Mo-repression of its synthesis. The species of $Av1^{Fe}$ which we have purified from strain RP306 contains Mo (present in FeMoco), and shows a ratio of C_2H_6 to C_2H_4 as products of C_2H_2 reduction similar to that achieved by Mo-treated cultures. This suggests that the species of $Av1^{Fe}$ which predominates during the N_2-dependent growth of RP306 may be the one formed when Mo is added to cultures of *A. vinelandii* strain RP206.

Studies on the enzymic properties of site-directed mutants of Mo-nitrogenase (Scott et al., 1990), on a hybrid nitrogenase with the FeV-cofactor in Mo nitrogenase polypeptides (Smith et al., 1988), and on turnover of V-nitrogenase at elevated temperature (Dilworth et al., 1993), have shown that reduction of N_2 to NH_3 requires more specific cofactor/polypeptide interactions than the reduction of other substrates. Thus mutation Gln α191, a residue which interacts with the FeMoco via homocitrate, to Lys, results in a nitrogenase which can no longer reduce N_2, though it can still reduce C_2H_2. However, the ratio of products formed by reduction of C_2H_2 is altered (Scott et al., 1990; Kim and Rees, 1992b). In the species of nitrogenase-3 isolated here, half the electron flux during C_2H_2 reduction can result in C_2H_6 formation. This hybrid nitrogenase is also competent in N_2 reduction, showing higher activity than the nitrogenase-3 so far isolated, and lower activity than Mo- or V-nitrogenases both *in vivo* and *in vitro*.

We thank Professor B. E. Smith for discussions, and P. Baker, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, for N-terminal amino acid analyses.

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Received 10 November 1992/19 January 1993; accepted 26 January 1993