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The vanadium–iron protein of vanadium nitrogenase from *Azotobacter chroococcum* contains an iron–vanadium cofactor

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N-Methylformamide extracts of acid-treated precipitated VFe protein of the V-nitrogenase of *Azotobacter chroococcum* are yellow–brown in colour and contain vanadium, iron and acid-labile sulphur in the approximate proportions 1:6:5. E.p.r. spectra of the extracts exhibit a weak signal with *g* values near 4.5, 3.6 and 2.0 characteristic of an $S = \frac{3}{2}$ metal-containing centre. The *N*-methylformamide extracts activated the MoFe protein polypeptides from mutants of nitrogen-fixing bacteria unable to synthesize FeMoco, the active centre of Mo-nitrogenase. The active hybrid protein exhibited the characteristic substrate-reducing phenotype associated with the VFe protein except that it could not reduce N_2 to NH_3. The above data are interpreted as demonstrating the existence of an iron- and vanadium-containing cofactor, FeVaco, within the VFe protein. It is suggested that nitrogen fixation requires specific interactions between FeVaco or FeMoco and their respective polypeptides. The biosynthesis of these cofactors is discussed.

INTRODUCTION

The recently isolated V-nitrogenases (Robson *et al.*, 1986; Hales *et al.*, 1986; Eady *et al.*, 1987) of the azotobacters have very similar physicochemical properties to those of Mo-nitrogenases (Lowe *et al.*, 1985; Eady, 1986). Both enzymes consist of two O_2-sensitive metalloproteins, namely a dimeric Fe protein containing a [4Fe-4S] cluster and a tetrameric protein containing either molybdenum and iron (MoFe protein) or vanadium and iron (VFe protein). Studies on VFe protein of V-nitrogenase from *Azotobacter chroococcum* by V *K*-edge X-ray-absorption spectroscopy (Arber *et al.*, 1987) indicated that the vanadium in V-nitrogenase, like the molybdenum in Mo-nitrogenase, has sulphur, iron and possibly oxygen as nearest neighbours. The molydbenum of Mo nitrogenase is extractable from the MoFe protein as an iron- and molybdenum-containing cofactor, FeMoco (Shah & Brill, 1977), which is, includes or forms part of the enzyme's active site (Hawkes *et al.*, 1984). We now present evidence that the VFe protein of V-nitrogenase contains an extractable iron- and vanadium-containing cofactor, which we call FeVaco, comparable with FeMoco.

MATERIALS AND METHODS

Bacterial strains and isolation of proteins

The VFe protein (Ac1*) of V-nitrogenase was isolated from *Azotobacter chroococcum* MCD1155 as described by Eady *et al.* (1987). NifB$^-$Kp1 was isolated from *Klebsiella pneumoniae* strain 5058 (a *hisD nifB* point mutant) as described by Hawkes & Smith (1983). *Azotobacter vinelandii* strain UW45 was grown as described by Shah & Brill (1977).

Analyses

Metal concentrations were determined spectrophotometrically on acid-digested samples: iron as its 8-hydroxyquinoline complex and vanadium as its gallic acid complex (Fiskman & Skougstad, 1964) after separation from iron by a modification of the method of McKenna (1971).

Acid-labile sulphur was determined by the method of King & Morris (1967).

Extraction of FeVaco

Before extraction, VFe protein was precipitated by adding citric acid (1 M) to purified protein until the pH was 2.2, followed by incubation at 0 °C for 2 min, when 0.5 M-Na_2HPO_4, pH 9, was added until the pH reached 5.5. All operations were conducted anaerobically in a glove-box at < 1 p.p.m. O_2. After centrifugation the supernatant was discarded and the grey precipitate was washed twice with 1 ml of *NN*-dimethylformamide. The precipitate was then extracted with *N*-methylformamide (1 ml or 0.5 ml).

RESULTS AND DISCUSSION

The *N*-methylformamide extracts of Ac1* were yellow–brown in colour. Six distinct extracts were analysed for vanadium and iron, and two of these extracts were also analysed for acid-labile sulphur. These analyses demonstrated the presence of iron, vanadium and acid-labile sulphur in the approximate proportions 6:1:5 (see Table 1). One of these extracts was also analysed for molybdenum, which was found to be at the limit of detection, i.e. ⩽ 4 atom % of the vanadium. Similar data have been reported for the molybdenum contents of preparations of the VFe protein (Hales *et al.*, 1986; Eady *et al.*, 1987), where the molybdenum was thought

Abbreviations used: Ac1*, VFe protein of *Azotobacter chroococcum* V-nitrogenase; FeVaco, iron- and vanadium-containing cofactor of the VFe protein; Kp1, MoFe protein of *Klebsiella pneumoniae* Mo-nitrogenase; FeMoco, iron- and molybdenum-containing cofactor of the MoFe protein.

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Table 1. Structural data on FeVaco and FeMoco

References: 1, Arber *et al.* (1987); 2, Lowe *et al.* (1985); 3, Eady (1986); 4, Shah & Brill (1977); 5, Yang *et al.* (1982); 6, Eady *et al.* (1980); 7, Eidsness *et al.* (1986); 8, Newton *et al.* (1985). Abbreviation: e.x.a.f.s., extended X-ray-absorption fine structure.

	FeVaco			FeMoco		
	Fe/V ratio	S^{2-}/V ratio	Reference	Fe/Mo ratio	S^{2-}/Mo ratio	Reference
Stoichiometry	5.85±0.43*	4.8±0.8†	This work	7±1	5±1†	2, 3, 4, 5, 6

	FeVaco				FeMoco			
		Bond length (nm)	Co-ordination no.	Reference		Bond length (nm)	Co-ordination no.	Reference
Bond lengths and co-ordination numbers for nearest neighbours derived from e.x.a.f.s. studies on the VFe protein or MoFe protein	V–Fe	0.274	3±1	1	Mo–Fe	0.267	3±1	7, 8
	V–S	0.232	2±1		Mo–S	0.237	4±1	
	V–O(N)	0.214	4±1		Mo–O(N)	0.21	2±1	

* Metal concentrations were determined as indicated in the Materials and methods section. The data presented represent the average±S.D. for measurements on six distinct extracts of FeVaco. The vanadium concentration of these extracts varied between 9 and 85 μM.

† Acid-labile sulphur was determined as indicated in the Materials and methods section. The value for FeMoco almost certainly underestimates the total sulphur content (Nelson *et al.*, 1983), since molybdenum complexes do not yield all their sulphur as H_2S with this method. The behaviour of vanadium complexes in this respect is unknown, but the value for FeVaco may also represent a lower limit.

not to be functional since it repressed synthesis of V-nitrogenase.

E.p.r. spectroscopy of the *N*-methylformamide extracts showed a weak signal with effective *g* values near 4.5, 3.6 and 2.0 (Fig. 1), which is similar to, but 8-fold lower in amplitude than, that of FeMoco of equivalent concentration (Rawlings *et al.*, 1978). The MoFe proteins (see Lowe *et al.*, 1985; Eady, 1986) and VFe proteins also show such signals but with less rhombicity (Eady *et al.*, 1987; Morningstar & Hales, 1987). Signals of this form are characteristic of a spin system with an $S = \frac{3}{2}$ ground state. These observations strongly suggested that the $S = \frac{3}{2}$ e.p.r.-active centre of Ac1* had been extracted intact. The equivalent features in the spectrum from Ac1* also have a low intensity, which has been attributed to their arising from an excited state (D. J. Lowe, unpublished work). No feature equivalent to the line at $g_{eff.} = 5.6$ observed from the Ac1* protein preparations was detected in the spectrum of the *N*-methylformamide extract. This transition, although from the ground state, is formally spin-forbidden, and changes in the ligand environment of the centre on extraction could easily result in a decreased transition probability.

The activity of FeMoco is assayed by incubating it with the inactive MoFe protein polypeptides found in extracts of mutants unable to synthesize FeMoco (Shah & Brill, 1977; Hawkes & Smith, 1983). The MoFe protein formed, when purified, has the substrate-reducing properties associated with the MoFe protein from which the FeMoco was extracted, whether this was from a wild-type or a mutant strain (Hawkes *et al.*, 1984). No

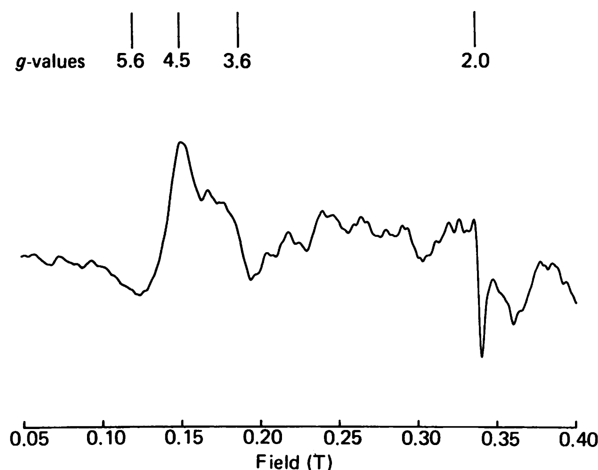

Fig. 1. E.p.r. spectrum attributed to FeVaco

A 43 mg portion of VFe protein (specific activity 1250 nmol of H_2 evolved/min per mg of protein) was treated as described in the text, and the FeVaco was extracted into 1 ml of *N*-methylformamide. The extract contained 84±3 μM-vanadium. The e.p.r. spectrum of a sample of this extract was measured, frozen in a 3 mm-internal-diameter quartz tube, on a Bruker ER200 spectrometer at 9.46 GHz and 5 K with a field modulation of 2 mT at 100 kHz and a microwave power of 51 mW; *g*-values are indicated on the Figure. The spectrum shown was obtained by computer-averaging 20 scans of the spectrum of the sample and subtracting the average of 20 scans of the spectrum of an *N*-methylformamide blank.

Table 2. Activity complementation experiments

Conditions were as follows. Expt. A: 200 μl of a crude extract of *K. pneumoniae* strain 5058 (34 mg/ml) was incubated with FeVaco in *N*-methylformamide for 30 min at 30 °C as described previously for FeMoco (Hawkes & Smith, 1983). Two different samples of FeVaco, (i) 10 μl of 43 μM-vanadium and (ii) 20 μl of 9.3 μM-vanadium, were used. After incubation the solutions were assayed for C_2H_2-reduction activity with excess Fe protein for 10 min as described previously (Dilworth *et al.*, 1988). The background activity is due to slight contamination of the added Fe protein with MoFe protein and to 'leakiness' of the KP5058 mutation. Expt. B: 200 μl of purified NifB⁻Kp1 (8 mg/ml) was incubated with FeVaco as indicated for Expt. A, sample (i), and the resultant solution was assayed with excess Fe protein for H_2-evolution (under argon) and C_2H_2-reduction activity.

Expt. A. FeVaco-activated KP5058 crude extract

FeVaco sample	C_2H_4 (nmol)	C_2H_6 (nmol)	$\frac{C_2H_6}{C_2H_4} \times 100$ (%)
None	10	0	0
(i)	359	10.7	3
(ii)	213	6.8	3.2

Expt. B. FeVaco-activated purified NifB⁻Kp1

	FeVaco addition	H_2 (nmol)	C_2H_4 (nmol)	C_2H_6 (nmol)	$\frac{C_2H_6}{C_2H_4} \times 100$ (%)	Total electron pairs (nmol)
Under argon	None	$\leqslant 1$	0	0		
	+	33	–	–	–	33
Under C_2H_2 (15 kPa)	None	0	< 1	0		
	+	13	18	1.5	8.3	34

comparable mutants containing inactive VFe protein polypeptides are yet available. We therefore tested the ability of the species in our *N*-methylformamide extracts of VFe protein to activate the MoFe protein polypeptides in crude extracts of mutants of *K. pneumoniae* (KP5058) and *A. vinelandii* (UW45), both of which are incapable of synthesizing FeMoco. In both cases C_2H_2-reducing activity had developed after 30 min incubation. Since this activity also developed when purified protein (NifB⁻Kp1) from *K. pneumoniae* KP5058 was used, no additional factors, present in crude extracts, were necessary for activation. The enzymic activities detected were characterisitic of those of the VFe protein (Eady *et al.*, 1987; Dilworth *et al.*, 1987), i.e. C_2H_6 as well as C_2H_4 was produced from C_2H_2, and the H_2-evolution activity was only suppressed by 60% in the presence of C_2H_2 [15 kPa (0.15 atm)] (Table 2). The difference between the C_2H_6/C_2H_4 ratio observed with KP5058 extracts and purified NifB⁻KP1 is within the range observed with purified V-nitrogenase under differing conditions (Dilworth *et al.*, 1988). No reduction products were observed unless all components of the incubation and assay were included. Comparable incubations and assays, under C_2H_2, with FeMoco in place of FeVaco gave no detectable C_2H_6. No reduction products of C_2H_2, nor H_2 evolution, were detected when FeVaco was incubated with bovine serum albumin and then assayed for activity.

The above observations provide very strong evidence that *N*-methylformamide extracts of acid-treated Ac1* contain a cofactor (FeVaco) that is a complex of vanadium, iron and acid-labile sulphur, that is includes or forms part of the active site of the VFe protein comparable with the FeMoco of MoFe protein. Further evidence that the vanadium is part of such a cluster has been provided by the vanadium X-ray-absorption data obtained previously (Arber *et al.*, 1987), which demonstrated that the vanadium in Ac1* had iron and sulphur among its nearest neighbours (Table 1).

In a typical extraction approx. 20% of the C_2H_2-reducing activity of the VFe protein was recovered in the hybrid product. However, in contrast with comparable experiments with FeMoco, it is difficult, at this stage, to interpret the quantitative significance of these data because of the potential mismatch between the components of the hybrid active protein. For example, the hybrid system of FeVaco plus NifB⁻Kp1 was not active in the reduction of N_2 to NH_3 as assayed either by the direct h.p.l.c. method (Corbin, 1984) or by the indophenol method after microdistillation (Chaney & Marbach, 1962). Thus, although FeVaco appears to be extracted and transferred intact, carrying with it a characteristic ability to reduce C_2H_2 to C_2H_6, we suggest that the N_2-reducing activity requires very specific interactions between the cofactor and its ligands, which are presumably provided by the VFe protein polypeptides. This suggestion is probably also valid for Mo-nitrogenase, since it has been reported that isolated FeMoco, with borohydride as a reductant, can catalyse the reduction of C_2H_2 to C_2H_4 but cannot reduce N_2 (Shah *et al.*, 1978; McKenna *et al.*, 1984). It should be noted that under our assay conditions FeVaco alone is not catalytically active.

The properties of FeMoco and FeVaco are compared in Table 1. These data imply that the chemical structures of the two cofactors are probably very similar. Therefore the pathways of their biosyntheses are also likely to be similar. FeMoco biosynthesis in *K. pneumoniae* is a

complex process known to require the products of the *nifB*, *nifE*, *nifN*, *nifV* and *nifH* genes (Dixon, 1984; Filler *et al.*, 1986). *A. vinelandii* (Hales *et al.*, 1986) as well as *A. chroococcum* (Robson *et al.*, 1986) have both the V-nitrogenase and Mo-nitrogenase systems. In the *A. vinelandii* mutant UW45 (probably a *nifB* mutant) neither system is functional (Joerger *et al.*, 1986), whereas in a defined *nifN* mutant only the V-nitrogenase system functions (Kennedy *et al.*, 1986). In *A. chroococcum* two regions of DNA, one outside the main *nif* cluster, hybridize to a *nifEN* probe (Evans *et al.*, 1988). It is probable, therefore, that there are two distinct biosynthetic pathways for the different cofactors in azotobacters with *nifB* common to both but utilizing different *nifE*-gene and *nifN*-gene products.

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