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*Phil. Trans. R. Soc. Lond. B* 1987 **317**, 131-146
doi: 10.1098/rstb.1987.0052

*Phil. Trans. R. Soc. Lond.* B **317**, 131–146 (1987)
*Printed in Great Britain*



# Biochemistry of nitrogenase and the physiology of related metabolism

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The properties of the newly discovered vanadium nitrogenase are compared with those of the better-known molybdenum nitrogenase and some aspects of the physiology of the latter are discussed. Both nitrogenases have dimeric Fe proteins of relative molecular mass ($M_r$) *ca.* 65000 containing a single [4Fe-4S] cluster. These act as MgATP-activated electron transfer agents to the MoFe or VaFe proteins, which include the substrate binding and reducing site. Both enzymes reduce $H^+$ to $H_2$, $N_2$ to $NH_3$ and $C_2H_2$ to $C_2H_4$, but the vanadium enzyme is less efficient in the last two reactions.

The MoFe protein is an $\alpha_2\beta_2$ tetramer of $M_r$ *ca.* 220000 and containing 2 Mo atoms and about 30 Fe atoms and $S^{2-}$ ions per molecule. The VaFe protein has a similar polypeptide structure and may also have an additional, small ($M_r \simeq 6000$) ferredoxin-like subunit. Current preparations contain 2 Va atoms and about 20 Fe atoms and $S^{2-}$ ions in a molecule of $M_r$ *ca.* 210000. The active site of the MoFe protein is an iron–molybdenum cofactor of unknown structure and complex biosynthesis.

The Lowe–Thorneley model for nitrogenase function is summarized. Ferredoxins or flavodoxins are the physiological electron carriers to molybdenum nitrogenase. Many aerobic diazotrophs have an uptake hydrogenase to recycle the electrons and energy wasted by the obligate $H_2$ evolution that accompanies $N_2$ fixation. Both nitrogenases are damaged by $O_2$, but many diazotrophs are aerobes or generate $O_2$ from photosynthesis. Some of the complexities of the interactions between $O_2$ and $N_2$-fixation are discussed.

## 1. Introduction

There are at least two enzyme systems (nitrogenases) capable of reducing dinitrogen to ammonia. Both recognized nitrogenases consist of two, oxygen-sensitive metalloproteins. Each system includes an iron protein (Fe protein) and one has a molybdenum–iron (MoFe) protein whereas the other has a vanadium–iron (VaFe) protein. The vanadium nitrogenase has only recently been discovered and as yet only limited data on its nature and physiology are available. However, the molybdenum nitrogenase has been studied for a number of years and in some organisms aspects of its physiology are relatively well understood.

In this article we review current knowledge on both nitrogenases and on the related physiology of the molybdenum enzyme. To give a contemporary view, references will be to reviews or recent papers rather than to initial observations.

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## 2. Molybdenum nitrogenase

The properties of molybdenum nitrogenase have been extensively reviewed elsewhere (see Lowe *et al.* 1985; Orme-Johnson 1985; Eady 1986) and only a summary will be given here. A convenient shorthand nomenclature utilizes the initials of the source and the numbers 1 and 2 for the MoFe and Fe proteins: the Fe protein from *Klebsiella pneumoniae* is Kp2 and the MoFe protein from *Clostridium pasteurianum* is Cp1. *Azotobacter vinelandii* is Av; *Azotobacter chroococcum*, Ac; *Bradyrhizobium japonicum*, Bj.

### (*a*) *The iron protein* (Fe *protein*)

All Fe proteins are dimers of $M_r$ *ca.* 57000–72000. The subunit is encoded by the *nifH* gene in *K. pneumoniae*. The amino acid sequences of the proteins from nine bacterial sources have been determined either directly or by deduction from the nucleotide sequence and show considerable homology (see Eady 1986 and references therein) with five invariant Cys residues and three potential nucleotide-binding regions (Robson 1984).

Most Fe protein preparations have been reported to contain about 4 Fe and $4S^{2-}$ ions, although higher metal contents have been reported for Av2 (Braaksma *et al.* 1983; Haaker *et al.* 1985*a*). The extra Fe does not seem to correlate with higher activities. The inorganic ions are thought to be combined in a [4Fe–4S] cubane-like cluster bonded to the polypeptides through ligation of the Fe atoms, probably to conserved Cys residues (e.g. Cys97 and Cys132 of Av2 (Hausinger and Howard 1983)). All reduced Fe proteins exhibit a rhombic electron paramagnetic resonance (EPR) signal with $g_{av} = 1.94$, typical of [4Fe–4S] containing proteins and arising from the cluster in a $S = \frac{1}{2}$ spin state but always integrating to less than one electron per molecule. Hagen *et al.* (1985), Lindahl *et al.* (1985) and Watt & McDonald (1985) have now identified an additional EPR signal, at $g \approx 5$, which arises from the [4Fe–4S] cluster in a $S = \frac{3}{2}$ spin state. The sum of the intensities of the two EPR signals was close to one spin per [4Fe–4S] cluster. The ratio of the two spin states could be altered by adding urea or ethylene glycol to the protein solutions.

Free Fe proteins have an $E_m$ in the range $-240$ to $-393$ mV, depending on the source. A negative shift in $E_m$ occurs on addition of MgADP or MgATP. With MgATP the Fe protein acts as a very specific electron donor to the MoFe protein during enzyme turnover. After electron transfer the $g = 1.94$ EPR signal is bleached. The number of electrons involved in this process is the subject of some dispute, (see Lowe *et al.* 1985).

All Fe proteins are extremely $O_2$-sensitive with half-lives for activity of about 45 s in air. MgATP and MgADP bind to the Fe protein, modifying its $g_{av} = 1.94$ EPR spectrum and increasing its sensitivity to $O_2$ and iron chelators. However, quantitation of this binding is complicated by contamination of the protein preparations with inactive material and by the observation that Fe protein with MgATP decomposes the reductant, sodium dithionite, with eventual 'self-oxidation' of the protein. Consequently, for many of the published data, the oxidation state of the protein is in doubt (see Lowe *et al.* 1985). The general consensus is that two MgATP molecules bind to each Fe protein molecule, but the reported binding constants vary between 16.7 and 560 μM (Eady 1986).




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(i) *The activation factor*

*Rhodospirillum rubrum*, several other non-sulphur purple bacteria, and the non-phototroph *Azospirillum lipoferum* all lose nitrogen-fixing activity, on the addition of ammonia to cultures, owing to covalent modification and inactivation of the Fe protein (see Eady 1986). This inactivation is reversible both *in vivo* and *in vitro* and in *R. rubrum* involves the addition of an adenosine diphosphoribose group, linked through the terminal ribose, to a guanidino nitrogen of an arginine residue (Pope *et al.* 1985). Reactivation involves MgATP, $Mn^{2+}$ and an activating enzyme isolated from the chromatophores, and involves removal of at least part of the modifying group (see Eady 1986).

### (*b*) *The structure of the* MoFe *protein*

MoFe proteins from all sources are $\alpha_2\beta_2$ tetramers of $M_r = 200000$ to 240000 with subunits, encoded by *nifD* and *nifK* genes in *K. pneumoniae*, of $M_r$ *ca.* 55000 and 60000.

The amino acid sequences of *nifD* from eight, and *nifK* from six, bacterial species have been determined (Eady 1986; Thöny *et al.* 1985; Brigle *et al.* 1985; I. Ioannidis & M. Buck, personal communication; A. Zamir, personal communication), all of them, except those for *Clostridium pasteurianum* (Hase *et al.* 1984) by prediction from the DNA base sequence. The *nifD* sequence has five, and the *nifK* sequence three, conserved Cys residues. The ferredoxin-like CysXXCys sequence, characteristic of many iron–sulphur proteins, is not observed. Subunits from different species show considerable homology and there is limited homology between the α and β subunits (Thöny *et al.* 1985). This homology apparently induces some structural homology in crystals of Cp1 in which a 6Å† resolution rotation function indicates a 2-fold relationship between the α and β chains (Yamane *et al.* 1982).

The general consensus (see Lowe *et al.* 1985) is that fully active MoFe protein preparations contain 2 Mo atoms, about 30 Fe atoms and a slightly lower number of $S^{2-}$ ions per molecule ($M_r \approx 220000$). Most preparations contain fewer inorganic ions but are thought to be mixtures of fully and partly active species. The inorganic ions can be extruded from the MoFe protein as two distinct metal cluster types. The Mo and some of the Fe can be extracted from precipitated protein as the iron–molybdenum cofactor, FeMoco. About 50% of the total Fe with an equivalent amount of $S^{2-}$ ions can be extruded as [4Fe–4S] clusters after denaturing the protein with an organic solvent in the presence of *o*-xylyl-α-α′-dithiol. The above data, together with Mössbauer spectroscopic data, suggest that the MoFe protein molecule contains two FeMoco centres, four [4Fe–4S] clusters, known as the P centres, with an additional two Fe atoms designated 'S'. The function and nature of these 'S' atoms is obscure (Smith 1983; Orme-Johnson 1985). This hypothesis is open to criticism, particularly on the basis of redox titration experiments. The FeMoco centres and the P clusters may be oxidized and reduced independently, the P clusters having the lower redox potential. Several workers have attempted to quantify the number of electrons associated with each redox step and thus the number of each type of centre. Unfortunately the results obtained differ, with 1–3 electrons being associated with the redox of the FeMoco centres and 3–4 electrons being required for the redox of the P clusters (Lowe *et al.* 1985; Watt 1985). Further experiments are required to rationalize these data.

† $1Å = 10^{-10}$ m $= 10^{-1}$ nm.




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### (c) *The iron–molybdenum cofactor (FeMoco)*

#### (i) *Biosynthesis*

Biosynthesis of FeMoco is complex, requiring the action of 5–6 *nif* genes (see Dixon *et al.*, this symposium). The *nifQ* gene product is only required if the availability of Mo is limited. Mutations in the *nifB*, *nifN* or *nifE* genes result in the formation of inactive MoFe protein that can be activated by addition of FeMoco. Mutations in the *nifV* gene result in the formation of MoFe protein with an altered substrate-specificity, which transferred with the FeMoco when the latter was extracted and used to activate the protein from a *nifB* mutant (Hawkes *et al.* 1984). These data strongly suggest that FeMoco is, includes or forms part of the enzyme's substrate-binding and -reducing site. Finally the *nifH* gene (encoding the Fe protein polypeptide) is required for FeMoco biosynthesis (Filler *et al.* 1986) but mutations in *nifH* do not result in the formation of MoFe protein that is activatable by added FeMoco.

Synthesis of FeMoco *in vitro* has recently been described (Shah *et al.* 1986); molybdate, ATP and the protein products of at least the *nifB*, *nifN* and *nifE* genes were required. One oddity was that it required mixtures of extracts from mutants of both *A. vinelandii* and *K. pneumoniae*. Mixtures of extracts from exclusively *K. pneumoniae* mutants were inactive. A factor of low molecular mass, apparently produced by the *nifV* gene product, is also required for the synthesis *in vitro* (Hoover *et al.* 1986).

#### (ii) *Structure*

The stoichiometry of FeMoco is not well defined ($MoFe_{6-8}S_{4-9}$) and its structure is unknown. It contains no significant quantities of amino acids, common sugars, coenzyme A or lipoic acid (see Smith *et al.* 1985) but other organic constituents cannot be ruled out.

Analysis of extended X-ray absorption fine-structure (EXAFS) data indicates that the nearest neighbours to the Mo are 4–5 sulphur atoms at 2.37 Å; 2–4 iron atoms at 2.67 Å; and perhaps two low-$Z$ atoms (C, N, or O) at 2.1 Å (Newton *et al.* 1985; Eidsness *et al.* 1986). This environment apparently does not change significantly in the MoFe protein from *nifV* mutants (Eidsness *et al.* 1986). Comparison of the Mo X-ray absorption near-edge structure (Xanes) region of the MoFe protein's X-ray spectrum with that of a variety of Mo complexes indicated that the ligands to Mo were probably coordinated through three sulphur and three oxygen atoms (Conradson *et al.* 1985). Together with the EXAFS interpretation these data imply that the immediate environment of molybdenum in FeMoco is very similar to that in a $MoFe_3S_4$ cubane complex with three oxygen ligands on the molybdenum.

The dithionite-reduced FeMoco centres in the MoFe protein exhibit a characteristic EPR signal, with $g$ values near 4.3, 3.7 and 2.01, which arises from the $M_z = \pm\frac{1}{2}$ Kramer's doublet of an $S = \frac{3}{2}$ spin system. Isotope substitution studies with $^{57}Fe$ and $^{95}Mo$ demonstrated interactions between the unpaired electron and the $^{57}Fe$ nuclei but not with the $^{95}Mo$ nucleus (Lowe *et al.* 1985). However, careful ENDOR (electron-nuclear double resonance) studies (Venters *et al.* 1986) have now demonstrated interactions of the unpaired spin with $^{57}Fe$, $^{95}Mo$, $^{1}H$ and $^{33}S$. These workers concluded that most of the interacting protons were on ligands from the protein. However some were exchangeable and were assigned to $OH^-$ or $H_2O$ bound to the metal centres, ligands which could be readily displaced by reducible substrates. The ENDOR study revealed at least five distinct iron environments in the FeMoco centres. Mössbauer studies concluded that there were six or eight *paramagnetic* iron atoms in FeMoco (Orme-

Johnson 1985; Dunham *et al.* 1985). N-methylformamide (NMF)-extracted FeMoco preparations contain 7–8 Fe atoms per Mo (see Smith 1983). However Shah & Brill (1981) extracted a $MoFe_6$ cluster from Av1 into methylethylketone and showed that, in NMF, this cluster exhibited the EPR spectrum of normal FeMoco but was unable to activate FeMoco-less mutant extracts. These data indicate that some of the Fe in FeMoco preparations is essential for its activity but is not paramagnetic.

Extracted FeMoco is anionic, extremely sensitive to oxygen but more stable to iron-chelating reagents than [4Fe–4S] and [2Fe–2S] clusters. Its EPR spectrum is more rhombic ($g$ values near 4.8, 3.3 and 2.0) than that of the cluster in the protein. Binding thiophenol (one molecule per Mo) sharpens the EPR spectrum and decreases its rhombicity to closer to that of the protein (see Lowe *et al.* 1985). Isolated FeMoco will also bind two ions of the reducible substrate cyanide per Mo atom (Smith *et al.* 1985), sharpening the EPR spectrum until it is almost axial. Fourier-transform infrared spectroscopy of FeMoco preparations indicates that it is bound to the NMF extractant, displacing the proton of the amide group (Walters *et al.* 1986). This could at least partly explain the anionic nature of FeMoco.

It is possible, with care, to oxidize extracted FeMoco to an EPR-silent form. Cyclic voltammetry then reveals two reduction waves; one, at $-0.32$ V against the normal hydrogen electrode, corresponds to regeneration of the EPR-active form; the other, at $-1.00$ V, is less well characterized but was suggested by the authors to correspond to the formation of the fully reduced, EPR-silent centre observed during enzyme turnover (Schultz *et al.* 1985).

### (*d*) *The P clusters*

The extrusion data described above indicated that about 50% of the iron in the MoFe proteins is present as [4Fe–4S] clusters. If this is so, then they have very unusual properties. Mössbauer spectroscopy indicates that they are in $[4Fe–4S]^0$ oxidation state (i.e. that all the Fe atoms are ferrous), a state very hard to reach with model complexes. Oxidation of the P clusters fleetingly gives rise to a $g_{av} = 1.93$ EPR signal (typical of the $[4Fe–4S]^{1+}$ oxidation state) but the clusters then relax to an EPR-inactive form with a very complex Mössbauer spectrum. Low-temperature magnetic circular dichroism of these clusters indicates that they have an $S = \frac{5}{2}$ or $S = \frac{7}{2}$ spin state. These unusual properties may indicate that not all the ligands binding the Fe atoms of the P clusters to the polypeptide chain are cysteine residues (see Lowe *et al.* 1985).

### (*e*) *Reducible substrate, inhibitor and* MgATP *interaction with the* MoFe *protein*

The association (§2(*c*) (i)) of the altered substrate specificity of *nifV* mutants with their MoFe protein and its FeMoco provides the most convincing evidence available that the MoFe protein includes the site for binding reducible substrates. Other evidence includes: (i) that $C_2H_2$ binds to the MoFe protein and perturbs an EPR-observable $pK_a$; (ii) that an EPR signal, detected during enzyme turnover in the presence of the product $C_2H_4$, is from FeMoco although no direct interaction between $^{13}C_2H_4$ and the unpaired electron could be demonstrated; (iii) that EPR signals from the MoFe protein during turnover have been observed in the presence of CO and acetylene (see Lowe *et al.* 1985). An uptake-hydrogenase activity in the presence of some oxidizing dyes has been attributed to Av1 (Wang & Watt 1984); the preparations were apparently free from contaminating hydrogenase.

Kp1 binds four $^{14}$C-ATP molecules per molecule of protein with $K_d = 600 \pm 100$ μM. Water




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proton NMR relaxation studies on the interaction of $Mn^{2+}$ and $Mg^{2+}$ with Kp1 indicate that the number of divalent metal-ion (and possibly MgATP) binding sites is directly proportional to the specific activity of the protein and extrapolate to four sites for Kp1 of specific activity 2900 (see Lowe *et al.* 1985; Eady 1986). The amino acid sequences of MoFe proteins include a site associated with ATP-hydrolysing enzymes (Robson 1984).

### (*f*) *The mechanism of molybdenum nitrogenase*

#### (i) *Substrate reduction*

Nitrogenase function requires the Fe protein, the MoFe protein, a source of low potential electrons (usually sodium dithionite *in vitro*), MgATP, which is hydrolysed to MgADP, and an anaerobic environment. EPR and Mössbauer spectroscopy have established that the Fe protein acts as a very specific MgATP-activated reductant for the MoFe protein, which binds reducible substrates (see also §2*e*). In addition to reducing $N_2$ to $NH_3$ the enzyme will reduce $H^+$ to $H_2$, $C_2H_2$ to $C_2H_4$ and a number of small triple-bonded substrates. CO inhibits the reduction of all substrates except the proton. $H_2$ is a competitive inhibitor of $N_2$ fixation only. Here we shall discuss only the physiologically important roles of nitrogenase, namely the reduction of $N_2$ and the evolution of $H_2$. The reduction of other substrates has been discussed elsewhere (Lowe *et al.* 1985; Burgess 1985; Jensen & Burris 1986).

In the absence of other reducible substrates, all the reducing equivalents from nitrogenase go into the evolution of $H_2$. With most substrates extrapolation of the experimental data to infinite substrate concentration, predicts total inhibition of $H_2$ evolution. However when $N_2$ is the substrate, even at 50 atm† pressure, a minimum of 25% of the available electrons go into $H_2$ evolution (Simpson & Burris 1984). Thus $H_2$ evolution is apparently an obligate part of the $N_2$ fixation reaction:

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2. \quad (1)$$

A further important facet of $N_2$ reduction is its competitive inhibition by $H_2$. When $D_2$ in $H_2O$ or $H_2$ in $D_2O$ was used as the inhibitor, HD was formed. When $^3H_2$ was used as the inhibitor, negligible amounts of $^3H$ were exchanged into $H_2O$.

There is some dispute over whether or not the formation of HD requires the presence of $N_2$ (Burgess *et al.* 1981; Guth & Burris 1983). The reaction in the absence of $N_2$ certainly seems to be of minor importance. Electron balance data indicate that one electron was consumed for the formation of each HD molecule. Burgess *et al.* (1981) suggested that HD formation occurred by reaction of $D_2$ with an enzyme-bound diazene-like (NH=NH) intermediate formed during $N_2$ reduction. This suggestion has been criticised (Lowe 1983) because it is not consistent with the observed competitive inhibition of $N_2$ reduction by $H_2$. An alternative suggestion (see below; see also Chatt (1980)) is that hydride–deuteride exchange occurs on a metal centre and is facilitated by the displacement of metal-bound $N_2$.

A number of mechanisms of nitrogenase action have been proposed but by far the most comprehensive is that by Lowe & Thorneley (see Thorneley & Lowe 1985 and references therein). Their model consists of a computer simulation of eight sequential one-electron reductions of the MoFe protein with concomitant side-reactions. It was developed from pre-steady-state stopped-flow and rapid-quench experiments and is able to simulate all the available experimental data on $N_2$ reduction.

† 1 atm = 101325 Pa.




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Each one-electron step consists of a cycle in which a reduced Fe protein, with MgATP bound, first complexes with and then reduces a MoFe protein molecule. MgATP is hydrolysed in this reaction. The protein–protein complex then dissociates in the rate-determining step of the cycle, which is completed by reduction of the Fe protein and the replacement on it of MgADP by MgATP. In the model each FeMoco centre of the MoFe protein acts independently and cannot release products or interact with substrates until dissociated from the oxidized Fe protein–MgADP) complex.

The first two electrons (plus protons) of the eight-electron cycle are postulated to form metal hydrides. After transfer of the second electron and dissociation of the Fe protein the enzyme can evolve $H_2$, but in the presence of excess reduced Fe protein the enzyme is recomplexed and reduced by a further electron, in a diffusion-controlled reaction. In the model, only at the three- and four-electron reduced oxidation levels can $N_2$ be bound. The binding of $N_2$ displaces $H_2$ from the metal site, thus explaining the stoichiometry of $N_2$ reduction (reaction (1)). Chemical analogues of this reaction are well known. Furthermore, the reaction is reversible, leading to competitive inhibition of $N_2$ reduction by $H_2$. In the presence of $D_2$ this reversible reaction would be expected to yield HD with protons from solution (Thorneley & Lowe 1985).

Rapid-quench studies of nitrogenase during turnover under $N_2$ demonstrated the presence of a hydrazine-yielding intermediate (Thorneley *et al.* 1978). Similar yields of hydrazine were obtained when the enzyme was quenched with acid or base, consistent with the intermediate being a hydrazido(2−) complex, $M{=}N-NH_2$. Chemical complexes of this type are relatively stable intermediates in the reduction of $N_2$ on metal sites and have the correct chemistry on reaction with acid or base. The pre-steady-state kinetics of formation of this intermediate indicated that it was formed at the four-electron reduced stage of the cycle. The pre-steady-state kinetics of ammonia release from the enzyme after an acid quench were consistent with it being formed at the five- or six-electron reduced stage. At these stages the bound $N_2$ would be expected to be reduced by only three or four electrons (two are lost with the displaced $H_2$). However if, as suggested by Chatt (1980), the $N_2$ triple bond is progressively weakened by protonation of the terminal N atom the relevant intermediates would be expected to be $M{=}N{=}NH_3^+$ and the nitrido species $M{\equiv}N$. Both these species would yield ammonia rapidly in acid. This suggestion is consistent with the observation that hydrazine is not a product. Under physiological conditions the second ammonia molecule may only be released after further reduction of the MoFe protein.

The obligate evolution of $H_2$ during $N_2$-reduction is obviously wasteful and in some organisms is partially compensated for by an uptake hydrogenase (§4*b*). Simulations with the Lowe-Thorneley model show that $H_2$ evolution by the enzyme is minimized and total $NH_3$ production maximized by the observed slow dissociation of the Fe protein–MoFe protein complex after electron transfer and by having a high concentration of both proteins at about a 2.5:1 Fe protein:MoFe protein ratio. These observations explain why the enzyme is so slow and why it can make up 10–40% of the soluble protein of diazotrophic bacteria (see Postgate 1982; Jouanneau *et al.* 1985).

There is an interesting parallel with the cyclic, electrochemical system of Pickett & Talarmin (1985). To maximize ammonia production with this system it is important to control the availability of protons, usually by temporal separation of the reduction and protonation of the metal site, otherwise excess $H_2$ is formed. Nitrogenase seems to have similar limitations. The




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model of Lowe & Thorneley, although conceptually simple, necessarily includes many partial reactions and it is difficult to check its validity with new data without running computer simulations. However, with such simulations the model can be a very powerful tool. An example of potential misunderstandings comes from recent data on product formation at saturating electron flux in $D_2O$ under 50 atm $H_2$+2 atm $N_2$ or in $H_2O$ under 50 atm $D_2$+2 atm $N_2$ (Simpson *et al.* 1985). The authors compare their product ratios with those predicted by two models: that of Guth & Burris (1983) and that of Cleland (reported in the Guth & Burris paper). The Cleland model is apparently a subset of the Lowe–Thorneley model but does not include all possible reactions. The data showed product ratios close to $1D_2:6HD:0NH_3$ or $1H_2:6HD:0NH_3$ for the two experimental conditions. The Guth & Burris model predicted these ratios, whereas the Cleland model predicted $1D_2:2HD:0NH_3$. However, a full simulation on the Lowe–Thorneley model predicted product ratios close to the experimental data thus demonstrating the importance of the partial reactions not included in the Cleland scheme.

### (ii) *The role of* MgATP

The details of the role of MgATP in nitrogenase function are still obscure. It apparently binds to both proteins and may bridge them during turnover. MgADP is a potent inhibitor of electron transfer. Rapid-quench studies have indicated that MgATP hydrolysis is concomitant with electron transfer from the Fe to the MoFe protein (see Eady 1986). However, more recent data (Haaker *et al.* 1985 *b*) showed a burst of ATP hydrolysis with oxidized proteins, indicating that ATP hydrolysis may precede and trigger electron transfer. The authors suggested that the MoFe protein is phosphorylated in the burst phase. Steady-state kinetic measurements indicate that two MgATP molecules are hydrolysed for every electron transferred to substrate. It is therefore possible that MgATP has a role in addition to the activation of protein–protein electron transfer.

## 3. Vanadium nitrogenase

The 'alternative' nitrogenase of azotobacters and the recent discovery of a second, Va-based nitrogenase in *A. chroococcum* are described by Kennedy *et al.* (these proceedings). These new nitrogenases have been studied in mutant azotobacters in which the structural genes for Mo-nitrogenase (*nifHDK*) have been deleted. The gene products of an alternative nitrogenase of *A. vinelandii* were apparently Mo-free (Hales *et al.* 1985). Extracts, prepared in our laboratory, of the $N_2$-fixing cells of the *nifHDK* deletion strain of *A. chroococcum* were irreversibly damaged by $O_2$ and the activity did not sediment when the extracts were centrifuged at 110000 *g* for 90 min. Both of these properties differ from those of crude preparations containing molybdenum nitrogenase and indicate that the vanadium enzyme does not form an $O_2$-tolerant, high-molecular-mass complex with a protective protein.

The components of the vanadium enzyme have now been purified and partly characterized. Their properties are described below.

### (*a*) *The* Fe *protein*

The Fe protein (Ac2*) of the vanadium enzyme, probably specified by the gene, *nifH**, (see Kennedy *et al.* this symposium) has been purified by conventional techniques (Robson *et al.*




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1986*a*) and its amino acid composition matches that predicted from the DNA base sequence of *nifH**.

The properties of Ac2* are very similar to those of the Fe proteins from molybdenum nitrogenases. It is an oxygen-sensitive dimer ($M_r \approx 63000$) of subunits of $M_r$ *ca.* 31500 containing about four Fe and four $S^{2-}$ ions per molecule but no significant amounts of Mo or Va. Its predicted amino acid sequence is very similar to that of Ac2, differing in only 30 out of 289 residues (Robson *et al.* 1986*b*).

### (*b*) *The* VaFe *protein*

The VaFe protein (Ac1*) purified by conventional anaerobic techniques has a half-life in air of about 40 s as opposed to about 8 min for Ac1. Its $M_r$ (210000) is very similar to that of Ac1; on stained 10% SDS polyacrylamide gel electrophoresis it gives rise to two bands of equal intensity with apparent relative molecular masses of 50000 and 55000. These data indicate that the VaFe protein is an $\alpha_2\beta_2$ tetramer. However, when Ac1* polypeptides were electrophoretically separated on 20% SDS polyacrylamide gels an additional band of $M_r$ *ca.* 6000 was observed. This component was detected in a number of Ac1* preparations despite gel filtration on Sephacryl S200 and S300. It is possible that it represents an additional subunit of Ac1*; and it could be the small, ferredoxin-like protein encoded by the DNA immediately following *nifH** (Robson *et al.* 1986*b*).

Ac1* preparations contained about 2 Va atoms, 20 Fe atoms and 20 acid-labile sulphide ions per molecule ($M_r \approx 210000$) their Mo content being less than 0.06 atoms per molecule.

The EPR spectrum of dithionite-reduced Ac1* at low temperatures shows resonances near $g = 5.6, 4.35, 3.73$ and a rhombic signal with $g_{av} = 1.93$. The low field region of this spectrum is consistent with that expected from an $S = \frac{3}{2}$ spin system with the $M_z = \frac{3}{2}$ Kramers doublet as the ground state and the $M_z \pm \frac{1}{2}$ doublet being thermally accessible. This interpretation is supported by the temperature dependence and low intensity of the spectrum. The signal with $g_{av} = 1.93$ is typical of that expected from a [4Fe–4S] ferredoxin; it may be attributable either to the additional subunit or contaminant in Ac1* preparations or to differences in the P clusters or equivalents compared with those in the MoFe protein.

### (*c*) *Vanadium nitrogenase*

The vanadium nitrogenase apparently reduces similar substrates to the molybdenum nitrogenase. With preparations so far available, however, a greater proportion (*ca.* 50%) of electrons are directed towards $H_2$ evolution under $N_2$; $C_2H_2$ is a relatively poor substrate, with a maximum of 60% of the available electrons being used for its reduction at 30 °C. MgATP is essential for the enzymic reaction; the ATP hydrolysed:2e ratio is similar to that of Ac1/Ac2 nitrogenase. These relatively minor differences from the molybdenum enzyme suggest that the vanadium enzyme may have an active site similar in structure to FeMoco but with Va in place of Mo.

## 4. Physiology

### (*a*) *Electron transport*

Nitrogenase function requires a supply of electrons at low potential. Ferredoxins or flavodoxins are implicated as the Fe protein reductants in a number of cases (Yates 1980). When




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dealing with electron carriers of low $E_m$, purely biochemical studies can rarely indicate a definitive physiological role because, like redox dyes of low $E_m$, non-physiological interactions are not only possible but likely. Convincing criteria for physiological function require not only biochemical activity *in vitro* but genetic and physiological evidence: a negative phenotype in mutants deficient in the electron carrier in question and, if appropriate, coordinated formation of the carrier with nitrogenase. These criteria have only been satisfied in one case. In *K. pneumoniae* the *nifF* product is a flavodoxin (Deistung *et al.* 1985) which mediates electron transfer from pyruvate via the *nifJ* product, a pyruvate-flavodoxin oxidoreductase (Shah *et al.* 1983), to nitrogenase (Hill & Kavanagh 1980). The amino acid sequence of the flavodoxin predicted from the *nifF* gene sequence (Drummond 1985) has been superimposed (Drummond 1986) on the crystallographically determined structure (Smith *et al.* 1983) of the flavodoxin from *Anacystis nidulans* and a tertiary structure predicted with a distinctive region of positive charge which is probably important in determining the kinetics of electron transfer interactions with the *nifJ* product and/or the Fe protein.

In azotobacters both flavodoxins and ferredoxins are present. Yates (1972) presented biochemical evidence that the hydroquinone form of a flavodoxin in *A. chroococcum* donates electrons to Ac nitrogenase and similar observations have been made with *A. vinelandii* by Scherings *et al.* (1977). This organism has three flavodoxins, the synthesis of one of which (flavodoxin II) is coordinate with nitrogenase. Thus it is the probable donor to nitrogenase (Klugkist *et al.* 1986*a*). In this organism a direct relation between the rate of electron transfer through the respiratory chain and whole-cell nitrogenase activity has been demonstrated (Klugkist *et al.* 1986*b*). In the absence of $NH_4^+$ a membrane-bound NADPH dehydrogenase activity was derepressed and two new polypeptides of $M_r$ *ca.* 29000 and 30000 were detected. However, a direct relation between these observations and electron transfer to nitrogenase was not established. As the genetics of the azotobacters develops (see Kennedy *et al.*, this symposium) it may be possible to define the electron transfer path(s) genetically. However, flavodoxin reduction may depend on membrane potential and hence membrane integrity (Haaker *et al.* 1974) and so this approach may not succeed.

A ferredoxin is probably the immediate donor to nitrogenase in the heterocysts of *Anabaena* but the rest of the electron transfer path is not established. Pyruvate can act as the source of electrons via a pyruvate:ferredoxin oxidoreductase, but other carbon sources can be used in conjunction with light-activated photosystem I (Neuer *et al.* 1985). In *C. pasteurianum*, ferredoxin carries electrons to nitrogenase from pyruvate through pyruvate:ferredoxin oxidoreductase (see Yates 1980). When the organism was grown under Fe-limited conditions, a flavodoxin replaced a ferredoxin.

### (*b*) *Uptake hydrogenase*

At least 25% of the ATP and electrons consumed by nitrogenase is apparently 'wasted' in the evolution of $H_2$ (§2*f*(i)). Many aerobic diazotrophs contain a membrane-bound $H_2$-uptake hydrogenase that is essentially unidirectional and which is believed to improve metabolic efficiency by recycling this $H_2$. These enzymes in *B. japonicum* (Evans *et al.* 1985), and in *A. chroococcum* probably contain nickel and FeS as do other membrane hydrogenases (see Cammack & Yates 1986). However, the soluble uptake hydrogenase from *C. pasteurianum* apparently contains no nickel (Adams & Mortenson 1984).

Dixon (1972) has suggested that the possible benefits to an organism of an uptake hydro-




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genase could include recovery of ATP through $H_2$-linked respiration, recycling of electrons, respiratory protection of nitrogenase against $O_2$ and relief of inhibition of the enzyme by $H_2$. However it has proved very difficult to establish effects, beneficial or otherwise, of such a hydrogenase in plants, doubtless because of the physiological complexity of the symbiosis. In *A. chroococcum* the experimental system is more manageable: Aguilar *et al.* (1985) showed that carbon-limited cultures of the wild type at high dilution rates gave higher steady-state yields than three hydrogenase minus (*hup*$^-$) mutants. Furthermore, in carbon-limited mixed cultures of a *hup*$^-$ mutant and a *hup*$^+$ recombinant strain, the *hup*$^+$ strain rapidly became dominant. The data demonstrate a clear metabolic advantage to the *hup*$^+$ strain, particularly in the initiation of diazotrophic carbon-limited growth.

Despite the experimentally difficult nature of the *Bradyrhizobium*–legume symbiosis, Evans *et al.* (1985) have shown that careful experimentation with strains which are isogenic except for the *hup* determinants provides strong evidence for the beneficial effects of *hup* (see Evans *et al.*, this symposium). The relative importance of the various mechanisms detailed by Dixon (1972) has yet to be assessed.

### (*c*) *Oxygen interactions*

Nitrogen fixation occurs in a wide variety of physiological types, from strict anaerobes to obligate aerobes, and a number of ways of protecting their nitrogenase from damage from $O_2$ have evolved (Robson & Postgate 1980). The anaerobes and facultative anaerobes generally only fix $N_2$ in the absence of $O_2$. Filamentous cyanobacteria generate $O_2$ from photosynthesis and most of them solve the problem of $O_2$-sensitivity by compartmentalization: the cells differentiate into non-diazotrophic vegetative cells and diazotrophic heterocysts (see Haselkorn *et al.* and Stewart *et al.*, this symposium).

Evidence for two mechanisms of oxygen protection in azotobacters has been reviewed by Robson & Postgate (1980). In the first, respiratory protection, the organism increases respiration, consuming more carbon source than necessary for growth, to decrease the dissolved-oxygen tension (DOT). No simple mechanism is apparent but the organisms apparently exploit changes in the absolute and relative amounts of the components of a branched electron transport route.

The second, conformational protection, comes into effect when $O_2$ stress is greater than can be managed by respiratory protection. In such conditions, the nitrogenase becomes inactive and 'switched-off' but somehow protected from $O_2$-damage. Not all authorities accept this interpretation of the reversible $O_2$-induced switch-off phenomenon (see Robson & Postgate 1980) but proteins able to protect nitrogenase from $O_2$-damage have been identified in *A. vinelandii* (Scherings *et al.* 1977) and *A. chroococcum* (Robson 1979). They are iron–sulphur proteins which combine with the two nitrogenase proteins in a 1:1:1 complex and render them $O_2$-stable.

The hypothesis of respiratory protection has been challenged (Post *et al.* 1983; Dingler & Oelze 1985) on the basis of continuous-culture experiments under differing oxygen régimes. However, the discovery (Ramos & Robson 1985) of mutants of *A. chroococcum* deficient in various steps in intermediary metabolism and whose $N_2$-fixing ability is unusually sensitive to $O_2$ (see Kennedy *et al.*, this symposium) strongly supports the idea of respiratory protection.

Hochman *et al.* (1985) observed $O_2$-induced reversible 'switch-off' phenomena in *Rhodopseudomonas capsulata* and *K. pneumoniae* and suggested that these phenomena were due simply




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to the competition for electrons between $O_2$ and $N_2$ fixation. This suggestion was made earlier for the azotobacters by Yates & Jones (1974). However, the situation with *K. pneumoniae* is more complex than these experiments indicate. Although this organism is a facultative anaerobe its $N_2$-fixing ability was enhanced at low (30 nM) $O_2$ concentrations and nitrogenase was synthesized and active at 100 nM $O_2$, a level sufficient to inhibit expression from the *nifH* promoter by 50% (Hill *et al.* 1984). Furthermore, recent experiments in this laboratory, with a *nifL* mutant in which transcription is less sensitive to $O_2$, have shown that although cells derepressed under high (6 μM) $O_2$ do not fix nitrogen, they do so without protein synthesis when they are moved to an anaerobic environment. These experiments clearly demonstrate protection of nitrogenase, or at least of its components, against $O_2$

An interesting case is the unicellular cyanobacterium *Gloeothece*. This organism is capable of $N_2$-fixation and $O_2$-producing photosynthesis in a single, undifferentiated cell type. Furthermore $N_2$-fixation is dependent on respiration, not photosynthesis, and the optimum $O_2$ concentration for activity can be as high as 80 μM (Maryan *et al.* 1986). The mechanism of protection of nitrogenase from $O_2$-damage under these conditions is not yet understood.

A further mechanism for control of $O_2$ during nitrogen fixation has evolved in the legume–rhizobium symbiosis, where the main problem is in providing sufficient $O_2$ for respiration. Here leghaemoglobin facilitates diffusion of $O_2$ to the bacteroids (see Appleby 1985; Bergersen 1984). $O_2$ binds very tightly to leghaemoglobin but has a reasonably fast 'off' rate. Without leghaemoglobin, virtually all of the available $O_2$ would be respired close to the surface of each nodule cell.

The above are a few of a variety of oxygen-excluding processes which have emerged among diazotrophs. Others include clustering, gum formation, vesicle formation, micro-aerophily, enhanced oxygenase or peroxidase activity (see Postgate 1982; Evans *et al.*, this symposium). Most of these processes are imperfectly understood and some may be illusory; undoubtedly there are some that are not recognized. The involvement of $O_2$ in the regulation of nitrogenase synthesis is described elsewhere in this volume (see Dixon *et al.*; Kennedy *et al.*).

We thank Professor J. R. Postgate, F.R.S., for informed criticism of this manuscript, Gill Ashby, Karl Fisher, Carol Gormal and Simon Harrison for skilled technical assistance, and Miss Beryl Scutt for typing.

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## *Discussion*

A. W. B. Johnston (*John Innes Institute, Norwich, U.K.*). What are the relative availabilities of vanadium and molybdenum in nature and how do they affect the expression of the two sets of *nif* genes?

R. R. Eady (*AFRC Unit of Nitrogen Fixation, University of Sussex, Brighton, U.K.*). The *content* of V and Mo in soils is of the order of 100 p.p.m. and 2 p.p.m. (by mass) respectively, and in fresh water 0.001 p.p.m. and 0.000 35 p.p.m. Their *availability* to organisms is uncertain, because under alkaline conditions both are strongly adsorbed onto humus in the soil. As regards regulation, Mo at *ca.* 0.02 p.p.m. prevents expression of the Va nitrogenase structural genes, and is required for expression of Mo nitrogenase structural genes.

J. Chatt, F.R.S. (*University of Sussex, Brighton, U.K.*). It is interesting that an active iron–vanadium protein has been isolated even if it does have a lower nitrogen-fixing activity than its iron–molybdenum analogue. Also that a vanadium analogue (FeVaco) of the iron–molybdenum cofactor (FeMoco) has been obtained from it. Has anyone tried to enhance the nitrogen-fixing activity of a solution of the iron–vanadium protein by treating its solution with FeMoco? This might displace the FeVaco from its protein by a sort of double decomposition or metathesis reaction to give a more active molybdenum-containing protein. If purely chemical equilibria were involved, such displacement would be expected from the greater chemical stability of molybdenum sulphide species as compared with those of vanadium sulphide.




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B. E. Smith. Under $N_2$, about 50% of available electrons are used for $N_2$ fixation by current preparations of the vanadium enzyme, whereas a *maximum* of 75% of the electrons are used by the molybdenum enzyme. The enzymes have comparable specific activities so the difference in $N_2$-fixing activities is not *very* large. An alternative approach would be to examine the $C_2H_2$-reducing activity of the putatively exchanging enzyme. Here the difference between the two nitrogenases is greater. However, it should be remembered that the polypeptides of the vanadium enzyme are encoded by different genes from the molybdenum enzyme and therefore it seems probable that FeVaco will be bound more tightly than FeMoco to these polypeptides and thus exchange may not occur. Nevertheless, the experiment will be tried.

J. H. Becking (*ITAL, Wageningen, The Netherlands*). Dr Smith's results indicate that there are two separate $N_2$-ase enzymes: one containing Mo, the other containing Va. In this context, it would be interesting to know whether both enzymes occur next to each other in a cell and can operate simultaneously. Further, what is the efficiency of each enzyme? In the past we found Va far less efficient in $N_2$-fixation than Mo in replacement studies. In the latter studies, performed about 25 years ago (*Pl. Soil* **16**, 171–201 (1962)) we did growth experiments in nitrogen-free medium deprived of Mo and Va and measured the response of Mo and Va additions. We observed that some *Azotobacter chroococcum* strains were unable to utilize Va as a substitute for Mo in dinitrogen fixation. The same was the general observation in all *Beijerinckia* sp. strains tested. Moreover, if Va could replace Mo in dinitrogen fixation, its efficiency was far less as Va produced a response of only 40–70% of that produced by Mo.

Further, there were quantitative differences in the Mo requirement of various *Azotobacter* strains and species. Half-maximal growth (i.e. dinitrogen fixation) was obtained in *A. vinelandii* at 0.0004 p.p.m. (by mass) Mo, in *A. agile* at 0.002 p.p.m. Mo and in general in *A. chroococcum* at *ca.* 0.05 p.p.m. Mo.





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