Evidence for a dynamic role for homocitrate during nitrogen fixation: the effect of substitution at the α -Lys⁴²⁶ position in MoFe-protein of *Azotobacter vinelandii*

Marcus C. DURRANT*, Amanda FRANCIS†, David J. LOWE†¹, William E. NEWTON‡ and Karl FISHER†¹

*Biomolecular and Biomedical Research Centre, School of Applied Sciences, Ellison Building, Northumbria University, Newcastle upon Tyne NE1 8ST, U.K., †Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Colney Lane, Norwich NR4 7UH, U.K., and ‡Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.

Although it is generally accepted that the active site of nitrogenase is located on the FeMo-cofactor, the exact site(s) of N₂ binding and reduction remain the subject of continuing debate, with both molybdenum and iron atoms being suggested as key players. The current consensus favours binding of acetylene and some other non-biologically relevant substrates to the central iron atoms of the FeMo-cofactor [Dos Santos, Igarashi, Lee, Hoffman, Seefeldt and Dean (2005) *Acc. Chem. Res.* **38**, 208–214]. The reduction of N₂ is, however, a more demanding process than reduction of these alternative substrates because it has a much higher activation energy and does not bind until three electrons have been accumulated on the enzyme. The possible conversion of bidentate into monodentate homocitrate on this three electron-reduced species has been proposed to free up a binding site for N₂ on the molyb-

denum atom. One of the features of this hypothesis is that α -Lys⁴²⁶ facilitates chelate ring opening and subsequent orientation of the monodentate homocitrate by forming a specific hydrogen bond to the homocitrate -CH₂CH₂CO₂⁻ carboxylate group. In support of this concept, we show that mutation of α -Lys⁴²⁶ can selectively perturb N₂ reduction without affecting acetylene reduction. We interpret our experimental observations in the light of a detailed molecular mechanics modelling study of the wild-type and altered MoFe-nitrogenases.

Key words: *Azotobacter vinelandii*, homocitrate, molybdenum-iron protein of nitrogenase (MoFe-protein), nitrogenase, nitrogen fixation.

INTRODUCTION

Nitrogenase is the complex metal-containing enzyme that is responsible for the conversion of atmospheric N₂ into NH₃. The most extensively studied nitrogenase, Mo-nitrogenase, contains molybdenum and iron and consists of two separable proteins known as the MoFe-protein (molybdenum-iron protein of nitrogenase Av1) and Fe-protein (iron protein of nitrogenase Av2) [1]. The MoFe-protein is an $\alpha_2\beta_2$ tetramer that contains two copies each of a pair of highly distinctive metal-sulphur clusters known as the P-cluster and FeMoco (FeMo-cofactor), which are implicated in electron transfer and substrate reduction respectively. The second enzyme component, the Fe-protein, contains an Fe₄S₄ cluster and two MgATP-binding and -hydrolysis sites; it is the obligate electron donor to the MoFe-protein. FeMoco is an inorganic cluster formulated as MoFe₇S₉X, where X is a light atom at the centre of the cluster, plus an organic ligand identified as (*R*)-homocitrate [2,3], which is attached to the molybdenum atom giving a five-membered chelate ring. FeMoco binds within the MoFe-protein α -subunit and is covalently attached to the protein via α -Cys²⁷⁵ and α -His⁴⁴², which bind to the terminal iron and molybdenum atoms respectively (see Figure 1). The inorganic part of FeMoco is tightly packed within the MoFe-protein and surrounded by (i) residues that approach each of its three faces (α -Val⁷⁰, α -Arg³⁵⁹ and α -Phe³⁸¹), (ii) residues that have the potential to hydrogen bond to the bridging sulphide atoms (α -Arg⁹⁶, α -His¹⁹⁵, α -Gly³⁵⁶ and α -Gly³⁵⁷), and (iii) a residue that has the potential to hydrogen bond to a sulphur atom contained within the MoFe₃S₃ subcluster fragment (α -Arg³⁵⁹) [4].

N₂ reduction by wild-type Mo-nitrogenase requires an anoxic environment, an adequate supply of MgATP and a reductant of sufficiently low potential. Both component proteins are absolutely required for enzymatic activity. A detailed scheme consisting of three main phases has been developed to describe the kinetics of the interactions between the two nitrogenase component proteins [5]. First the MoFe-protein associates with the one-electron-reduced Fe-protein that has two molecules of MgATP bound, then an electron is transferred from the Fe-protein to the MoFe-protein and both molecules of MgATP are hydrolysed, and finally the two proteins dissociate, allowing re-reduction (typically by dithionite *in vitro* and ferredoxin/ flavodoxin *in vivo*) of the Fe-protein and replacement of the MgADP by MgATP. For N₂ to be reduced to NH₃, a minimum of eight of these cycles must take place. The rate at which electrons appear in the product is independent of the substrate being reduced because the rate-determining step in turnover is the dissociation of the Fe-protein from the MoFe-protein. This situation leads, under ideal conditions, to a constant ratio of the rate of MgATP hydrolysis to the rate of transfer of electron pairs to product (the ATP/2e⁻ ratio) of 4:1.

A number of resting-state structures of both nitrogenase component proteins, both separately and as complexes, have been solved by X-ray crystallography [6–9]. However, these structures have given relatively little information about how N₂ and alternative substrates interact with the active site because wild-type nitrogenase only binds substrates under conditions of enzymatic turnover [1]. The debate continues as to whether N₂ binds to iron or molybdenum at the active site, although evidence is accumulating that alternative substrates, such as acetylene

Abbreviations used: FeMoco, FeMo-cofactor (the Fe₇MoS₉X-homocitrate prosthetic group in the MoFe-protein); FeVco, FeV-cofactor.

¹ Correspondence may be addressed to either of these authors (email david.lowe@bbsrc.ac.uk or karl.fisher@bbsrc.ac.uk).

Figure 1 FeMoco and selected residues of importance in the present study

This and other structural Figures were drawn using ORTEP-3 for windows, version 1.08 [45]. Atomic coordinates taken from X-ray crystal structure 1M1N [7]. Molybdenum and nitrogen atoms are shaded dark grey, sulphur and oxygen atoms are shaded light grey, and iron and carbon are shaded white.

and propargyl alcohol, bind initially to one or more iron atoms [10].

Recent spectroscopic studies have detected species derived from hydrazine and methyldiazine, which are substrates of altered proteins and are a potential intermediate and intermediate analogue respectively in N_2 reduction [11]. Even more significantly, an enzymic state derived from N_2 itself has been observed for the wild-type protein [12], albeit under conditions with an excess of MoFe-protein over Fe-protein, where N_2 is not reduced and does not inhibit proton reduction. These studies were interpreted as supporting the hypothesis that the binding site of these nitrogenous species is on the same α -Val⁷⁰ Fe-S face of FeMoco as that assumed to bind alkynes, because the binding of hydrazine and methyldiazene is affected by mutations close to this face [11].

In the absence of any direct experimental evidence for the mode of N_2 binding and reduction, a variety of theoretical models has been developed using quantum calculations [13–17]. Although a consensus has not yet been reached from these studies, they do at least illustrate the various possibilities. A model assuming that the structure of FeMoco remains unchanged during N_2 reduction has been explored in greatest detail by Dance [16,17], which suggests that the molybdenum atom remains six-co-ordinate throughout the catalytic cycle and N_2 reduction proceeds entirely on the iron atoms of the cluster. Alternative models allow the structure of FeMoco to be more dynamic during turnover, with one or more of its bonds undergoing temporary cleavage to open up co-ordination sites for N_2 (see Scheme 1). Blochl and co-workers

have developed one such model based on cleavage of an Fe-S bond in the core cluster [14]. The N_2 ligand then bridges between iron atoms before reduction, and again the molybdenum/homocitrate moiety plays no active role in catalysis.

These models may be thought of as being compatible with the existence of two alternative nitrogenases. The better established of these is V-nitrogenase in which vanadium takes the place of molybdenum, whereas the third is more poorly characterized, but appears to contain only iron. Although molybdenum and vanadium have similar chemistries, associated with their diagonal relationship in the Periodic Table, iron is clearly different. Because all three nitrogenases reduce N_2 , it has been inferred that the heterometal, the molybdenum/homocitrate part of FeMoco, is not directly involved and only provides ‘fine-tuning’. However, all three nitrogenases contain homocitrate, and changes to the homocitrate ligand, such as its replacement by citrate in the $\Delta nifV$ MoFe-protein [19,20], drastically impair their ability to reduce N_2 [21]. The importance of homocitrate for N_2 reduction raises the possibility that the molybdenum site, or its equivalent in the alternative nitrogenases, is directly involved by cleavage of one of the bonds to homocitrate upon reduction of the enzyme [22–25]. Support for this hypothesis comes from two sources. First, studies with model complexes have shown that protonation of a carboxylate ligand upon reduction of a molybdenum complex can indeed open a chelate ring and result in subsequent binding of N_2 [22]. Secondly, experiments performed on extracts of isolated FeMoco showed a specific interaction between homocitrate and an imidazole ligand on molybdenum, which were interpreted in terms of a cluster-activating interaction between monodentate homocitrate and the protein ligand of molybdenum, α -His⁴⁴² [23]. Furthermore, critical dependence on molybdenum was shown by Hales and Case [26], who succeeded in substituting tungsten for molybdenum in the FeMoco and showed that the modified enzyme was unable to reduce N_2 .

An important feature of the homocitrate ring-opening model is that it includes an experimentally testable feature, namely a specific role for α -Lys⁴²⁶ in N_2 reduction, but not in acetylene reduction. Thus acetylene reduction can proceed on the central iron sites of FeMoco, using states of the MoFe-protein that are not sufficiently reduced to induce homocitrate ring opening, whereas the reduction of N_2 requires the more reduced MoFe-protein states that can undergo ring opening. The α -Lys⁴²⁶ side chain then acts to orientate the monodentate homocitrate ligand in two ways. First, a general electrostatic interaction between the cationic lysine side chain and the anionic $-CH_2CH_2CO_2^-$ arm of homocitrate helps to rotate the monodentate homocitrate away from the molybdenum and into the correct position to allow N_2 binding at molybdenum. Secondly, a specific hydrogen bond between these two groups orientates the homocitrate $-CH_2CH_2CO_2^-$ arm in such a way that an additional hydrogen bond is formed between the latter group and the NH group of α -His⁴⁴². Although probably quite

Scheme 1 Proposed equilibrium at Lowe-Thorneley redox state E_4 between a molybdenum hydride, A, which evolves H_2 rapidly by hydrolysis, and a relatively stable homocitrate ring-closed species, B, as described by Durrant [25]

H^+ in circles denote protons attached to amino acid side chains in the vicinity of FeMoco. Note that the charge on FeMoco is increased by one electron relative to the original model [18].

weak in energy terms, this interaction could provide a valuable enhancement of the reactivity of the molybdenum site with respect to initial reduction of N_2 . Similar effects would operate in the alternative nitrogenases, but not in the $\Delta nifV$ mutants.

In the present study, we show that substitutions at α -Lys⁴²⁶ position can impair the enzyme's ability to reduce N_2 without affecting its acetylene-reducing characteristics. We interpret the details of our observations in terms of the homocitrate ring-opening hypothesis by means of molecular mechanics calculations, and provide further evidence that the protein environment is tailored for homocitrate ring opening by analysis of the available amino acid sequences for Mo- and V-nitrogenases.

EXPERIMENTAL

Micro-organisms and site-directed mutagenesis

The wild-type bacterium used throughout the present study was strain DJ527 of *Azotobacter vinelandii*, which contained an insertion mutation within *hoxKG*, which knocks out uptake hydrogenase activity, but retains intact *nif* genes. Site-specific changes were made using overlap extension PCR methodology [27]. Mutations generated by overlap extension PCR were transformed into an *A. vinelandii* deletion strain and selected on the basis of restored Nif^+ phenotype before sequencing to confirm the presence of the desired change.

A. vinelandii cell growth and nitrogenase expression

The growth of wild-type and altered *A. vinelandii* strains was as reported by Shen et al. [28]. Culture density was monitored using a Klett-Summerson meter equipped with a no. 54 filter. Ideally, a mid-exponential-phase culture with a Klett reading of 100–150 units was used to inoculate a 28-litre fermenter. Cultures were grown overnight in 24 litres of a fixed nitrogen-rich basal liquid Burk's medium [29] in the 28-litre fermenter (New Brunswick Scientific), with a dissolved O_2 concentration of 25 % maintained by varying the flow of compressed air, N_2 gas and the stirring rate.

Nitrogenase expression was induced at a cell density of 250 Klett units by centrifuging cells at 8000 g at 30 °C and resuspending in 24 litres of a fixed nitrogen-free medium made from deionized water containing 10 μ M Na_2MoO_4 , 20 μ M $FeCl_3$ and Burk's sucrose basal medium in the fermenter. The dissolved oxygen concentration was maintained at 25 % for 3.5 h before harvesting.

Crude-extract preparation and nitrogenase protein purification

Whole-cell rupture was accomplished by osmotic shock as follows. Approx. 200 g of cells was thawed under an N_2 atmosphere and then diluted with 750 ml of anaerobic 25 mM Tris/HCl (pH 8.0), containing 4 M glycerol and 2 mM sodium dithionite. After resuspension, the cells were spun at 14 000 g for 30 min at 4 °C. The supernatant was discarded, and the cell pellets were resuspended in approx. 400 ml of 25 mM Tris/HCl, pH 8.0, containing a small amount of DNase. After violent shaking under an N_2 atmosphere, the extract was transferred anaerobically into 95-ml ultracentrifuge tubes and centrifuged at 98 000 g for 90 min at 4 °C. Unless stated otherwise, the buffer system used throughout protein purification was 25 mM Tris/HCl, pH 7.4, containing 2 mM sodium dithionite.

Purification of the MoFe-nitrogenase involved Q-Sepharose and anion-exchange chromatography, Sephacryl S-200 gel filtration and lastly phenyl-Sepharose hydrophobic-interaction chromatography exactly as described by Kim et al. [30]. The Fe-protein was purified to homogeneity via fractionation from a second Q-Sepharose anion-exchange column.

Table 1 Diazotrophic growth and purification properties of wild-type and α -426 altered MoFe-proteins

All specific activity values are nmol of H_2 /min per mg of protein with an excess of Fe-protein. EPR intensity is measured at $g = 3.7$ as a percentage of the wild-type EPR signal.

MoFe-protein	Doubling time (h)	Crude extract specific activity	Purified MoFe-protein specific activity	[Mo]/[MoFe-protein]	[Fe]/[Mo]	EPR intensity (%)
Wild-type	3.5	150	2500	1.6	16	100
α -Ala ⁴²⁶	4.1	108	1300	1.0	28	60
α -Thr ⁴²⁶	4.5	105	1400	1.3	15	70
α -Gln ⁴²⁶	4.5	64	1940	1.8	15	91
α -Ser ⁴²⁶	4.7	89	1300	1.2	16	60
α -Arg ⁴²⁶	5.6	101	2500	1.5	12	100
α -His ⁴²⁶	5.6	37	340	0.6	15	20
α -Met ⁴²⁶	5.8	56	850	0.5	28	40
α -Tyr ⁴²⁶	7.9	65	650	0.8	35	30
α -Glu ⁴²⁶	8.9	34	200	0.2	67	10
α -Asp ⁴²⁶	11.8	12	320	0.7	64	10
α -Trp ⁴²⁶	12.8	55	170	0.3	80	10

All chromatography columns were run at room temperature (21 °C) and were made anaerobic before use by flushing with dithionite-containing anaerobic buffer until the eluant was able to reduce Methyl Viologen. The elution profile of the proteins was monitored at 365 nm with an in-line Pharmacia-LKB Uvicord SII absorbance detector cell (Amersham Biosciences) coupled with a single-channel chart recorder. Coloured fractions were collected anaerobically in N_2 -flushed conical flasks.

Purified nitrogenase proteins were concentrated separately in an Amicon micro-filtration apparatus using either a 30 K or a 100 K MWCO membrane before buffer exchange into 25 mM Hepes, 10 mM $MgCl_2$ and 200 mM NaCl, pH 7.4, by passage through an anaerobic P-6DG column (Bio-Rad) in an anaerobic glove box with an O_2 concentration of less than 1 p.p.m.

Assay of nitrogenase activity

Nitrogenase-catalysed acetylene reduction [acetylene/argon (1:9)], N_2 reduction (100 % N_2) and proton reduction (100 % argon) were assayed in 7.8-ml reaction glass serum vials fitted with no. 25 Suba-Seal® rubber stoppers (William Freeman Ltd) as described previously [30]. Stock 100 % acetylene was generated from the reaction of CaC_2 (BDH) with water and subsequently diluted to 10 % in the assay. *A. vinelandii* Fe-protein used in the present study had a specific activity of 3000 nmol of H_2 /min per mg of protein, the MoFe-protein specific activities are listed in Table 1.

The apparent K_m for acetylene reduction catalysed by Av1 was determined by entering the acetylene-reduction rate as a function of acetylene concentration into SigmaPlot 8.0. Steady-state assays containing acetylene at ~0.1–20 kPa were performed under maximal flux conditions (Fe-protein/MoFe-protein ratio of 20:1).

Product analysis

Product formation was measured by gas chromatography using a GC-14B gas chromatograph with a Shimadzu Class-VP Chromatography Automated Data Software System. Ethylene was quantified on a Poropak N column using a flame-ionization detector. H_2 evolution was also measured by gas chromatography with a molecular sieve 5 Å (1 Å = 0.1 nm) column and a thermal conductivity detector. Both systems were calibrated by injection of standard gases.

NH₃ production was measured by phenol/hypochlorite colorimetry [26,28]. MgATP hydrolysis was determined as described by Dilworth et al. [31], and Dilworth and Fisher [32].

Protein estimation, metal estimation, EPR measurements and SDS/PAGE

Nitrogenase-protein concentrations were estimated by the Lowry method [33] with BSA as a standard. Absorbances were read, after colour development, at 750 nm in 1-cm pathlength cells. Both the molybdenum and iron contents of the purified nitrogenase proteins were determined by inductively coupled plasma atomic emission spectroscopy using a PerkinElmer Plasma 400 spectrometer. The EPR spectra of the purified MoFe-proteins were recorded at the X-band on a Bruker ElexSys 500 instrument with an ER094X microwave bridge and an ER4122SHQ cavity [34]. SDS/PAGE was employed to check nitrogenase expression in crude extracts and to estimate the purity of the MoFe- and Fe-proteins after each column chromatography step. Samples and gels were prepared according to the method of Laemmli [35]. Running gels were 10 % polyacrylamide with 1.35 % cross-linker, and stacking gels were 4 % polyacrylamide. Electrophoresis was carried out at 25 mA, using a mini-Protean 3 electrophoresis apparatus (Bio-Rad) and 10–250 kDa Kaleidoscope pre-stained molecular-mass markers (Bio-Rad).

Molecular modelling

Molecular modelling studies were performed using Insight II, version 2000.1 (Accelrys). The 1.16 Å X-ray crystal structure of nitrogenase [3], Protein Data Bank [36] accession code 1M1N, was edited to a working model consisting of one FeMoco plus all of protein chains α and β , residues 350–523 of chain β' , and 19 water molecules defining the 'water pool' around homocitrate (chains labelled as A, B and D respectively in 1M1N). Partial atomic charges for FeMoco were assigned based on the results of density functional theory calculations [16,18], and the central atom was assumed to be N. Hydrogen atom positions and partial atomic charges for the protein and water were calculated at pH 7.0, using the charged capping mode. The waters were aligned manually so as to maximize hydrogen-bonding with the surrounding protein. Energy minimizations on the resting-state structures were carried out using the consistent valence force field and steepest descent algorithm within the Discover module of Insight II, with the following groups kept fixed: molybdenum, iron, sulphur and nitrogen atoms of FeMoco; ND1 atom of α -His⁴⁴²; O5 and O7 of homocitrate; α -chain residues 4–55 (all atoms), 56–71 (backbone), 72–93 (all atoms), 94–98 (backbone), 99–189 (all atoms), 190–192 (backbone), 193–352 (all atoms), 353–360 (backbone), 361–378 (all atoms), 379–382 (backbone), 383–415 (all atoms), 416–420 (backbone), 431–446 (backbone), 447–480 (all atoms); β -chain residues 2–94 (all atoms), 95–106 (backbone), 107–523 (all atoms); β' -chain residues 350–523 (all atoms). For the ring-opened structures, water number 154 was replaced with an N₂ ligand attached to the molybdenum atom, the initial monodentate homocitrate geometry was constructed according to our earlier model [22], and additional fixed atom restraints were included as follows: N₂ ligand, all of α -His⁴⁴², and O4 of homocitrate; O3 was allowed to relax. Values for the distance-dependent dielectric constant of 10.0 and the Coulombic scale factor of 2.0 were chosen empirically by comparing the results of trial geometry optimizations with the original crystal structure; these values were found to reproduce the original structure to within satisfactory limits. The root mean square deviation for superimposition of all the heavy protein atoms in the optimized geometry with the crystal

structure was 0.046 Å, although a few side chains had relatively large atomic movements (up to 1 Å) owing to rotations, and some of the water molecules in the water pool had moved by up to 0.6 Å. For each mutant, as well as the wild-type structure, geometry optimizations were carried out as above on both the homocitrate-closed and homocitrate-open conformations. Where the side chain of the substituting residue was significantly smaller than the wild-type α -Lys⁴²⁶ residue, one or two extra water molecules were included in the model.

RESULTS

General physical and catalytic properties

Eleven mutant strains, each having a specific amino acid substitution at α -Lys⁴²⁶ in the MoFe-protein of *A. vinelandii*, were constructed and their proteins were expressed and purified. Table 1 summarizes the MoFe-protein proton-reduction specific activities for crude extracts and MoFe-proteins purified from nitrogenase-derepressed wild-type and mutant strains, together with their diazotrophic growth behaviour. All 11 α -Lys⁴²⁶ mutant strains were capable of diazotrophic growth, although they all showed impaired growth rates under diazotrophic conditions in fixed-nitrogen-free medium compared with the wild-type strain. Four of them, which carry the altered MoFe-protein with alanine, glutamine, threonine or serine at this position, fixed N₂ almost as efficiently as the wild-type, with doubling times of approx. 4–4.5 h. The doubling times for growth on N₂ for the other strains ranged from 5–6 h for the arginine-, histidine- and methionine-substituted strains to 8–13 h for the tyrosine-, glutamate-, aspartate- and tryptophan-containing strains.

All mutant strain crude extracts showed similar Fe-protein specific activities, indicating that nitrogenase was derepressed effectively during cell growth (results not shown). However, the crude extract H₂-evolution activities under argon are all lower than the wild-type activity, which suggests that FeMoco insertion into the MoFe-proteins has been compromised.

After purification, only the α -Arg⁴²⁶ and α -Gln⁴²⁶ MoFe-proteins contain a molybdenum complement and H₂-evolution specific activity similar to wild-type MoFe-protein. The α -Ser⁴²⁶, α -Thr⁴²⁶ and α -Ala⁴²⁶ MoFe-proteins contain 50 % or more of the expected molybdenum content, whereas the other altered proteins range from 40 to 10 % of the possible maximum. In all cases, the lowered metal content is consistent with their lowered H₂-evolution activity and S = 3/2 EPR-signal intensity. No new dithionite-reduced resting-state EPR features were observed from any of the purified altered MoFe-proteins.

Although each altered MoFe-protein was purified using exactly the same procedure as that employed for the wild-type MoFe-protein, different levels of purity were achieved as shown by SDS/PAGE (Figure 2). The α -Gln⁴²⁶, α -Thr⁴²⁶ and α -Arg⁴²⁶ MoFe-proteins were of comparable purity with that of the wild-type MoFe-protein, whereas the others contained either high- or low-molecular-mass contaminants or both. In addition, the α -Glu⁴²⁶, α -Trp⁴²⁶ and α -Tyr⁴²⁶ MoFe-proteins do not contain equal amounts of the α - and β -subunits, which is consistent with the lowered molybdenum content of these proteins.

With one exception, the addition of 10 % CO to nitrogenase assays, under conditions of high electron flux, resulted in the complete inhibition of acetylene reduction, with all electrons being directed to hydrogen formation (results not shown). However, the α -Arg⁴²⁶ MoFe-protein, when complemented with a large excess of wild-type Fe-protein, has H₂-evolution activity that is inhibited by CO. CO maximally inhibits the activity by 50 %, which is comparable with that observed previously for the $\Delta nifV$ and

Figure 2 SDS/10% PAGE of MoFe-proteins purified from the α -426 mutant strains and wild-type

Lane 1, molecular-mass markers (sizes given in kDa); lane 2, wild-type MoFe-protein; lane 3, α -Ser⁴²⁶ MoFe-protein; lane 4, α -Thr⁴²⁶ MoFe-protein; lane 5, α -Glu⁴²⁶ MoFe-protein; lane 6, α -Met⁴²⁶ MoFe-protein; lane 7, α -Ala⁴²⁶ MoFe-protein; lane 8, α -Trp⁴²⁶ MoFe-protein; lane 9, α -Tyr⁴²⁶ MoFe-protein; lane 10, α -Arg⁴²⁶ MoFe-protein; lane 11, α -His⁴²⁶ MoFe-protein; lane 12, α -Gln⁴²⁶ MoFe-protein; lane 13, α -Asp⁴²⁶ MoFe-protein. Lanes 2–13 contain approx. 0.2 μ g of MoFe-protein.

Table 2 Substrate-binding affinities and ATP/2e⁻ data for wild-type and α -426 altered MoFe-proteins under ethylene/argon (1:9) and 100% N₂

nd, not determined.

MoFe-protein	Total e ⁻ to ethylene (%)	ATP/2e ⁻ ethylene	K _m acetylene (kPa)	Total e ⁻ to NH ₃ (%)	ATP/2e ⁻ N ₂	K _m N ₂ (kPa)
Wild-type	94	4.7	0.83 ± 0.03	71	6.1	8.2 ± 0.6
α -Ala ⁴²⁶	96	6.1	0.51 ± 0.02	66	7.6	9.4 ± 1.7
α -Thr ⁴²⁶	96	6.6	0.49 ± 0.03	69	7.2	7.5 ± 0.3
α -Gln ⁴²⁶	92	6.2	0.64 ± 0.05	74	6.0	9.2 ± 0.4
α -Ser ⁴²⁶	96	6.1	0.49 ± 0.02	64	6.6	7.5 ± 0.3
α -Arg ⁴²⁶	96	5.2	0.9 ± 0.05	58	5.8	24.2 ± 3.8
α -His ⁴²⁶	94	6.8	0.52 ± 0.03	68	6.8	9.0 ± 1.0
α -Met ⁴²⁶	96	6.6	0.61 ± 0.02	64	6.6	10.3 ± 0.6
α -Tyr ⁴²⁶	95	13.5	0.53 ± 0.03	54	11.2	11.7 ± 1.1
α -Glu ⁴²⁶	92	20.4	0.43 ± 0.02	50	14.6	9.6 ± 1.0
α -Asp ⁴²⁶	78	29.7	0.31 ± 0.04	65	10.8	7.3 ± 0.6
α -Trp ⁴²⁶	75	32.3	0.61 ± 0.03	35	20.6	15.4 ± 3.1
Δ nifV	90	nd	nd	31	nd	nd

α -Lys¹⁹¹ altered MoFe-proteins [37]. Because the rate of MgATP hydrolysis is not similarly inhibited by CO, the ATP/2e⁻ ratio increases 2–3-fold for this MoFe-protein in the presence of CO. The K_i for CO inhibition of H₂ evolution for the α -Arg⁴²⁶ MoFe-protein was measured as 5.5 Pa, which is comparable with the values of 8 and 4 Pa measured previously for the α -Lys¹⁹¹ and Δ nifV MoFe-proteins respectively [37]. These values, although similar, appear to reflect directly the extent of the inhibition by CO.

EPR spectra obtained by freeze-quenching during turnover in the presence of CO were identical with those observed with the wild-type and the Δ nifV MoFe-proteins [37].

Effects of substituting at α -Lys⁴²⁶ on both acetylene and N₂ reduction by purified MoFe-protein

Table 2 shows that when the α -Lys⁴²⁶-altered MoFe-proteins were assayed under an acetylene/argon (1:9) atmosphere under high-electron-flux conditions, the primary product was ethylene. Only

the α -Asp⁴²⁶ and α -Trp⁴²⁶ MoFe-proteins produced a significantly higher percentage of H₂ than the wild-type enzyme. None of the proteins were capable of reducing acetylene through to ethane, a phenotype of the Δ nifV protein. For the wild-type (α -Lys⁴²⁶) MoFe-protein assayed under maximal flux conditions, a K_m for acetylene of 0.83 kPa partial pressure was calculated; this falls within the range reported previously [28,37,38]. Similar binding affinities were calculated for the altered MoFe-proteins, with values between 0.31 and 0.9 kPa. Surprisingly, the highest and lowest apparent binding affinities were obtained for the α -Asp⁴²⁶ MoFe-protein, which showed the lowest percentage of electrons to ethylene, and the α -Arg⁴²⁶ MoFe-protein that was our most conservative substitution respectively.

The addition of 5% or higher acetylene resulted in a 70% inhibition of electron flux through the α -Asp⁴²⁶ MoFe-protein, without affecting the rate of ATP hydrolysis. This resulted in an increase in the ATP/2e⁻ ratio from approx. 8 while reducing protons to more than 35 in the presence of saturating acetylene. The percentage of the total electrons that were diverted to H₂ remained constant in the presence of saturating acetylene. Inhibition of nitrogenase turnover by acetylene was not observed with any other MoFe-protein used in the present study. Because acetylene acted as an inhibitor of total electron flux, it was only possible to estimate the K_m for acetylene reduction from low (< 5 kPa) acetylene concentrations.

Neither the rate of complex dissociation nor the rate or amplitude of primary electron transfer from Av2 to the MoFe-protein were affected by the presence of acetylene (results not shown).

When assayed under 100% N₂, the percentage of total electrons transferred to NH₃ varied from 74% for the α -Gln⁴²⁶ MoFe-protein to 35% for the α -Trp⁴²⁶ MoFe-protein (Table 2). The α -Gln⁴²⁶ MoFe-protein was reproducibly better at fixing N₂ than the wild-type enzyme, whereas the Δ nifV MoFe-protein was more compromised with respect to N₂ reduction than any of the α -Lys⁴²⁶-altered MoFe-proteins. Only the tyrosine-, glutamate-, aspartate- and tryptophan-substituted MoFe-proteins exhibited electron transfer that was uncoupled from MgATP hydrolysis, a phenomenon mirrored in the presence of acetylene and possibly arising from the presence of apo-MoFe-protein in the enzyme preparations. By measuring the NH₃ formed at various N₂ concentrations, we were able to determine the K_m for N₂ binding to the wild-type MoFe-protein as 8.2 kPa, in agreement with literature values [5]. The altered MoFe-proteins gave similar K_m values, apart from the α -Arg⁴²⁶ MoFe-protein, which had a K_m approx. 3-fold higher; this reduced binding affinity was accompanied by a decreased electron allocation to NH₃, but not an uncoupled ATP/2e⁻ ratio.

The effect of substituting at the α -Lys⁴²⁶ position is shown clearly by a plot of percentage of electrons consumed by ethylene production against those producing NH₃ (Figure 3). The linear correlation strongly implies that N₂ reduction, but not acetylene reduction, is compromised in these altered proteins. The only exceptions are the α -Asp⁴²⁶ and α -Trp⁴²⁶ MoFe-proteins, which also show a significant effect on N₂ reduction. Furthermore, the α -Arg⁴²⁶ MoFe-protein is a surprisingly poor N₂ reducer for such a conservative alteration.

Molecular modelling

Each altered protein was modelled in two forms, namely the 'closed' form in which the homocitrate chelates the molybdenum atom as seen in the X-ray crystal structures, and an 'open' form in which the Mo–O bond to the homocitrate β -carboxylate group has broken and the homocitrate has rotated to allow co-ordination of N₂ at the molybdenum. Amino acid substitutions were introduced

Figure 3 Comparison of electron distribution to NH_3 and ethylene (C_2H_4) during N_2 and acetylene reduction by wild-type, $\Delta nifV$ and α -426 mutant MoFe-proteins

Assays contained acetylene/argon (1:9) or 100% N_2 and were initiated with a 20:1 mixture of Av2/Av1. Assays were run for 40 min and were quenched with 0.3 ml of 0.5 M EDTA, pH 7.5. Linearity was checked by quenching assays at 16 min (results not shown). Each assay contained 0.0375 mg of MoFe-protein. Mutant proteins are indicated by the single-letter amino acid code for the residue at α -426. The broken line is a best fit to the points, excluding the tryptophan (W) and aspartate (D) data which do not fit this correlation.

such that, as far as possible, the positioning of the new side chain followed that of the original. The number of waters in the homocitrate water pool was adjusted to reflect the size of the α -Lys⁴²⁶ mutant side chains; α -Ala⁴²⁶ gained two extra water molecules, α -Arg⁴²⁶, α -Trp⁴²⁶ and α -Tyr⁴²⁶ each lost one water molecule, and the rest were unchanged. Geometry optimizations of both closed and open forms were carried out using consistent procedures for each protein.

Considering the closed forms first, the only significant displacements of the protein backbone were seen for the α -Trp⁴²⁶ and α -Tyr⁴²⁶ proteins, where accommodation of these bulky side chains necessitated significant perturbations of the protein backbone (C α -426 displacements compared with the wild-type of 0.82 and 0.86 Å respectively). In the case of the α -Trp⁴²⁶ protein, the β -Ser¹⁰¹ side chain rotated through approx. 110° during the geometry optimization, in order to relieve close contacts with the tryptophan side chain. These steric clashes have significant consequences for the hydrogen bond normally seen between O3 of the homocitrate $-\text{CH}_2\text{CH}_2\text{CO}_2^-$ arm and the backbone NH of α -Ile⁴²⁵ in the resting state structure. In the wild-type structure, this interaction is characterized by an O...N distance of 2.84 Å in the X-ray crystal structure and 2.89 Å in the geometry-optimized structure. It is lost in the closed forms of both the α -Trp⁴²⁶ and α -Tyr⁴²⁶ proteins, which show O...N distances of 4.53 and 3.52 Å respectively; for the α -Trp⁴²⁶ protein, a significant movement of the homocitrate $-\text{CH}_2\text{CH}_2\text{CO}_2^-$ arm means that O4, rather than O3, of homocitrate is now weakly hydrogen-bonded to the NH group (O...N distance of 3.42 Å). The α -Tyr⁴²⁶ protein shows another interesting feature in that an hydrogen bond has developed between the tyrosine hydroxy group and the homocitrate $-\text{CH}_2\text{CH}_2\text{CO}_2^-$ arm (O...O distance of 3.35 Å; Figure 4A) with the homocitrate moving towards the tyrosine to facilitate this bond formation (homocitrate O atom is displaced by 0.83 Å). The closed forms of the other variants all showed essentially unperturbed homocitrate geometries, with

Figure 4 Stereo views of the model of the resting form of the α -Tyr⁴²⁶ (A) and α -Arg⁴²⁶ (B) mutants

Atom shading is as in Figure 1; selected hydrogen atoms and predicted hydrogen bonds are shown as small circles and grey lines respectively. In both mutants, note the hydrogen bonds between homocitrate and α -Gln¹⁹¹, as seen in the wild-type structure. (A) The α -Tyr⁴²⁶ mutant also has a hydrogen bond between homocitrate and the phenolic hydroxy group of α -Tyr⁴²⁶, formed at the expense of the hydrogen bond to the α -Ile⁴²⁵ backbone NH group. (B) The α -Arg⁴²⁶ mutant has a hydrogen bond between homocitrate and the α -Ile⁴²⁵ backbone NH group, as seen in the wild-type structure, and the α -Arg⁴²⁶ side chain has also formed a bifurcating hydrogen bond with homocitrate.

preservation of the hydrogen bond between homocitrate and α -Ile⁴²⁵ (O...N distances of 2.92–2.99 Å). Besides α -Tyr⁴²⁶, only one other altered protein shows a direct interaction between the new side chain and homocitrate; the α -Arg⁴²⁶ protein shows a strong bifurcated hydrogen bond with O4 of the homocitrate $-\text{CH}_2\text{CH}_2\text{CO}_2^-$ arm (O...N distances of 2.87 and 2.99 Å; Figure 4B) in addition to the normal hydrogen bond between O3 and α -Ile⁴²⁵. Finally, neither the glutamate- nor aspartate-altered proteins show any perturbation of the homocitrate; however the aspartate variant is noteworthy in that the side chain is orientated towards β -Arg¹⁰⁰ in the neighbouring subunit (Figure 5A). The closest contact between these two residues in our model is 3.12 Å, between the carboxylate O and the arginine N δ . Although the orientation of this interaction does not permit an explicit hydrogen bond, this would probably be possible given greater flexibility for the β -chain than allowed for in our modelling procedure, which kept all of the backbone atoms of the β -chain fixed. The extra CH_2 group in the glutamate mutant, compared with aspartate, appears to weaken the interaction with β -Arg¹⁰⁰ and orientate the α -Glu⁴²⁶ back towards homocitrate (Figure 5B).

Figure 5 Models for the resting state of the α -Asp⁴²⁶ (A) and α -Glu⁴²⁶ (B) mutants

The α -helix incorporating residues 96–107 of the β -subunit is shown in grey as an α -carbon trace, with the side chain of β -Arg¹⁰⁰ included in ball-and-stick mode. Note the orientation of the aspartate side chain towards the arginine side chain. Atom shading is as in Figure 1.

Turning to the ring-opened models, our modelling strategy was determined by two considerations. First, in the wild-type enzyme, we postulate that the α -Lys⁴²⁶ side chain promotes homocitrate ring opening through a general electrostatic effect. Secondly, the side chain promotes the specific orientation of the homocitrate $-\text{CH}_2\text{CH}_2\text{CO}_2^-$ arm required for activation of FeMoco by interaction with α -His⁴⁴². We therefore built the α -His⁴⁴²–homocitrate interaction into our models by keeping the position of homocitrate O4 fixed; the rest of the homocitrate was allowed to move. The optimized structures should then give an indication of whether the α -His⁴⁴²–homocitrate interaction is accessible or not. The results fall into several different categories. First, the alanine, histidine, methionine and threonine side chains show essentially no interaction with homocitrate (serine was not modelled because it is so similar to threonine); of these, the methionine side chain approaches closest, to within van der Waals contact ($\text{O} \cdots \text{CH}_3$ distance of 3.60 Å). This suggests that these residues would neither hinder nor enhance the interaction between homocitrate and α -His⁴⁴². The large tryptophan and tyrosine side chains are in contact with the homocitrate $-\text{CH}_2\text{CH}_2\text{CO}_2^-$ arm, suggesting that these groups will sterically hinder realization of the correct homocitrate geometry. The arginine mutant side chain also contacts the homocitrate $-\text{CH}_2\text{CH}_2\text{CO}_2^-$ arm in its open conformation, with a favourable electrostatic interaction in this case ($\text{O} \cdots \text{N}$ distance of 3.19 Å), but the geometry prevents formation of a specific hydrogen bond. Neither of the two acidic residues, glutamate and aspartate, has very close contacts with homocitrate, although glutamate has the closest approach ($\text{O} \cdots \text{O}$ distances of 4.13 and 4.25 Å for glutamate and aspartate respectively). Finally,

Figure 6 Stereo view of the ring-opened state of the α -Gln⁴²⁶ mutant

Atom shading is as in Figure 1; selected H atoms and predicted hydrogen bonds are shown as small circles and grey lines respectively. Note the N_2 ligand on molybdenum; for clarity, only the imidazole ring of α -His⁴⁴² is shown. In addition to the usual hydrogen bond between homocitrate and α -Gln¹⁹¹, there is a bifurcating hydrogen bond between homocitrate and the α -Gln⁴²⁶ side chain, and the putative functionally significant hydrogen bond between homocitrate and the imidazole NH group of α -His⁴⁴².

glutamine is the only other residue, apart from the wild-type lysine, that forms a specific hydrogen bond with O3 of homocitrate in the ring-opened conformation (Figure 6); both hydrogens of the side-chain amino group are involved in a bifurcated hydrogen bond ($\text{O} \cdots \text{N}$ distance of 3.03 Å; $\text{O} \cdots \text{H}$ distances of 2.66 and 2.79 Å). In all cases, homocitrate retains its hydrogen bond to α -Gln¹⁹¹ in both the ring-closed and -open conformations.

Sequence comparisons and homology modelling

In order to investigate the level of conservation of α -Lys⁴²⁶ in known nitrogenase sequences, we searched the Uniprot database for nitrogenase component 1 α -chain proteins, using the *Klebsiella pneumoniae* sequence as a query. Sequences returned by this search were discarded if they were significantly incomplete or if they lacked any of the four cysteine and one histidine residues required for FeMoco and P-cluster binding. The remaining sequences were screened for redundancy and gene annotation, giving 68 α -chain MoFe-protein (NifD) and six α -chain VFe-protein (VnfD) sequences. These were aligned using the MUSCLE server [39]. This showed that α -Lys⁴²⁶ is strictly conserved across all 68 NifD sequences. Interestingly, however, the equivalent residue in all of the VnfD sequences is arginine. Another significant difference is seen in the preceding residue, α -Ile⁴²⁵ in Av1; in the NifD sequences, this is isoleucine (50 sequences), valine (17 sequences) or leucine (one sequence), but proline in all six VnfD sequences. The sequence variations in the vicinity of α -Lys⁴²⁶ are summarized in Table 3.

DISCUSSION

Certain amino acid residues in FeMoco's environment within the MoFe-protein influence its orientation and/or its catalytic and electronic properties, as shown by targeted substitution (see [10] and references therein). Most of the published data focuses on the interaction of inhibitors, such as CO or the substrate acetylene, with altered nitrogenase proteins. For example, all substitutions at the α -Gln¹⁹¹ position exhibit CO-sensitive H_2 evolution and reduce acetylene poorly, with ethane as a product [40]. In the present study, we have pinpointed a residue, α -Lys⁴²⁶,

Table 3 Amino acid sequence analysis for MoFe- and VFe-protein α -chain sequences, residues 421–429

Numbering is taken from the Av1 sequence. Values in parentheses are the number of sequences incorporating each residue. One MoFe-protein sequence has an extra glutamate residue inserted between residues 426 and 427.

(a) MoFe-protein sequences (68 sequences)

Amino acid at position								
421	422	423	424	425	426	427	428	429
Val (34)	Gly (29)	Ser (57)	Gly (68)	Ile (50)	Lys (68)	Glu (61)	Lys (67)	Tyr (66)
Ile (19)	Ala (26)	Ala (9)		Val (17)		Asp (7)	Arg (1)	Phe (2)
Phe (8)	Cys (5)	Val (1)		Leu (1)				
Met (7)	Phe (4)	Gly (1)						
	Leu (4)							

(b) VFe-protein sequences (six sequences)

Amino acid at position								
421	422	423	424	425	426	427	428	429
Ile (5)	Phe (5)	Thr (6)	Gly (6)	Pro (6)	Arg (6)	Val (6)	Gly (6)	Glu (3)
Val (1)	Leu (1)							Asp (2)
								Ala (1)

which we believe could act to position homocitrate such that N_2 reduction is optimized. In addition to the functional role of this residue during nitrogenase turnover, our experimental results suggest a structural role in terms of insertion of FeMoco into the apoprotein. Thus several of our altered proteins contain low or very low levels of functional FeMoco, as is evident from their low EPR intensities, poor catalytic abilities and low molybdenum contents. The importance of α Lys⁴²⁶ in helping to orient FeMoco during its insertion into the MoFe-protein has been inferred by comparison of the fully complemented and FeMoco-free protein crystal structures [41]. The ease of FeMoco insertion into the apoprotein correlates reasonably well with the expected steric and electrostatic properties of the residue at α -426. Thus the two basic residues, lysine and arginine, have full FeMoco complements, whereas the glutamine residue, which also appears to have the potential to form an hydrogen bond to homocitrate (see above), has the next best. The unfavourable electrostatic properties of the two acidic residues, glutamate and aspartate, and the steric problems associated with the very bulky residues tryptophan and tyrosine, explain why these four mutants have very low FeMoco complements. The other residues have intermediate FeMoco complements. The only inconsistency found is for histidine, which has an unexpectedly poor FeMoco complement; we speculate that this is due to some specific but undetermined effect, perhaps arising from this residue's ability to act as a ligand in its own right. Although the proteins have a wide range of activities, the good correlation between EPR intensity and specific activity for H_2 reduction supports the assumption that only properly assembled nitrogenase molecules are catalytically active for the reduction of H_2 , N_2 and acetylene in our preparations.

Our experimental data for the α -Lys⁴²⁶ mutants are broadly consistent with the premise that a mutation that perturbs the homocitrate ring-opened state should selectively affect N_2 reduction, but not acetylene reduction. Thus, in general, the altered proteins have a much wider range of N_2 -reduction efficiencies than acetylene-reduction efficiencies. The efficiencies of the mutants for N_2 reduction span a range between that of the wild-type protein and

the Δ nifV mutant; this result was expected, because replacement of homocitrate by citrate in the Δ nifV mutant is a direct change to the FeMoco structure and should have a greater effect than the more distant perturbations from our substitutions.

There are two exceptions to the general trend, namely tryptophan and aspartate. The tryptophan variant scores poorly against every measure of its activity, including acetylene reduction, and this is readily explained by the perturbations in the protein structure required to accommodate this very bulky residue. The aspartate mutant is more interesting because it alone shows reasonable N_2 -reduction activity but poor acetylene-reduction activity. Our modelling studies suggest that this anomalous behaviour is not due to some specific interaction between α -Asp⁴²⁶ and homocitrate, but to an interaction between α -Asp⁴²⁶ and β -Arg¹⁰⁰ which lies in an α -helix containing one of the P-cluster ligands, β -Cys⁹⁵; it seems reasonable to suppose that a specific hydrogen-bonding interaction between these two residues could distort the structure of the protein. Why this should perturb acetylene reduction so dramatically but hardly affect N_2 reduction is not clear. Although other point mutations that inhibit acetylene but not N_2 reduction are known [42], there are none that inhibit electron flux through the protein in this manner.

We find that the efficiencies of the other altered proteins for N_2 reduction correlate well with the results of our modelling studies. Thus the glutamate mutant is particularly poor for N_2 reduction, and we assign this to the electrostatic repulsion between the carboxylate group of the $-CH_2CH_2CO_2^-$ arm of homocitrate and the glutamate side chain. The aspartate mutant is also negatively charged, but, in this case, the separation between the groups will be larger because of its shorter side chain and also because of the likely interaction between α -Asp⁴²⁶ and β -Arg¹⁰⁰ discussed above. Hence the glutamate-altered protein is a significantly poorer N_2 fixer than the aspartate-altered protein. After glutamate, the tyrosine- and arginine-altered proteins are the next poorest for N_2 fixation. Both of these have the opposite hydrogen-bonding pattern to that of the wild-type α -Lys⁴²⁶ residue; namely, a hydrogen bond between the mutant side chain in the resting state, but not in the ring-opened state. In addition, the bulky tyrosine side chain is likely to hinder the motion of the homocitrate upon ring opening. Hence, the equilibrium between the ring-opened, N_2 -binding state and the ring-closed state will be shifted in favour of the latter, making the tyrosine and arginine variants relatively poor N_2 fixers. The observation that the α -Arg⁴²⁶ protein is similar to the wild-type protein with respect to the efficiency of FeMoco insertion, and yet is a relatively poor N_2 fixer, strongly supports the contention that α -Lys⁴²⁶ has a wider role than simply facilitating insertion of FeMoco. The methionine, alanine, histidine and threonine substitutions are all fairly neutral in that there are no specific interactions between their side chains and homocitrate. They are all slightly inferior in their N_2 -fixing activity to the wild-type because they lack the positive effect of lysine in promoting the interaction between homocitrate and α -His⁴⁴².

Finally, the glutamine mutant is of particular interest in that it shows a moderate, and yet reproducible, enhancement in N_2 -reduction efficiency over the wild-type protein. Our modelling studies indicate that this is the only other residue that is capable of forming a hydrogen bond to homocitrate in its ring-opened, molybdenum-activating conformation (Figure 6). These experimental and theoretical results indicate that it may indeed be possible to construct better nitrogenases than wild-type, provided that the gain in functional efficiency is not offset by a loss in efficiency of FeMoco insertion.

The α -Arg⁴²⁶ protein, like the Δ nifV protein, shows inhibition of H_2 evolution (i.e. uncoupling of ATP hydrolysis from electron transfer) in the presence of CO. In the active homocitrate model for

H₂ evolution [25], this behaviour in the $\Delta nifV$ protein is explained in terms of a perturbation by CO of an equilibrium between a ring-opened molybdenum monohydride species **A**, which can rapidly evolve H₂, and a ring-closed species **B**, which is unreactive (Scheme 1). This scheme readily adapts to the α -Arg⁴²⁶ protein; the extra hydrogen bonds between the arginine residue and the -CH₂CH₂CO₂⁻ homocitrate arm that we observe in our model of the resting state of this altered protein would also help to stabilize **B** over **A**.

Sequence comparisons

The differences in amino acid sequence in the vicinity of the homocitrate ligand for the VFe-protein compared with the MoFe-protein (Table 3) are interesting in terms of potential hydrogen bonds between these residues and homocitrate. The MoFe-protein X-ray crystal structures show a hydrogen bond between the homocitrate -CH₂CH₂CO₂⁻ arm and the backbone NH group of α -Ile⁴²⁵, which must be broken if the homocitrate undergoes chelate ring opening. The substitution of isoleucine by proline in the VnfD sequences means that there is no possibility of an analogous interaction in VFe-proteins; indeed, the steric constraints of the proline side chain would demand a different conformation for the -CH₂CH₂CO₂⁻ group in the resting state. As we have seen, replacing α -Lys⁴²⁶ with arginine in the MoFe-protein reduces the efficiency of N₂ fixation, because arginine can hydrogen bond to homocitrate in the ring-closed, but not the ring-opened, conformation, the reverse pattern to the wild-type lysine. However, in the case of the VFe-proteins, the substitution of proline for the wild-type isoleucine, together with the other local sequence changes, especially in the three residues following α -Lys⁴²⁶, might be sufficient to switch this behaviour so that arginine becomes the preferred residue for interaction with homocitrate in the ring-opened state.

Further insight into this question can be gained by consideration of two hybrid proteins, namely the VFe component 1 protein complemented with FeMoco [43], and the MoFe-protein complemented with FeVco (FeV-cofactor) [44]. Whereas the VFe-protein-FeMoco combination behaves like normal MoFe-protein with respect to N₂ reduction, the MoFe-protein-FeVco combination does not reduce N₂ at all. If the homocitrate chelate ring opening is intrinsically more difficult for FeVco than for FeMoco, then the differences in the protein sequences may reflect a compensating promotion of the ring-opened state by the protein environment for the VFe-protein.

Finally, we note that α -Gly⁴²⁴ is strictly conserved across all the MoFe- and VFe-protein sequences. Modelling the effects of replacing this residue with alanine, we find that there would be a steric clash between homocitrate and α -Ala⁴²⁴ in both the ring-closed and -opened homocitrate conformations. Given that replacement of α -Ile⁴²⁵ by proline in the VFe-protein sequences must produce a similar steric clash in the resting state, and yet the VFe-protein-FeMoco hybrid is viable for N₂ reduction, it seems unlikely that the α -Ala⁴²⁴ mutation would be a serious problem for the ring-closed state. However, modelling the α -Ala⁴²⁴ ring-opened state suggests that this mutation would prevent the interaction between homocitrate and α -His⁴⁴², possibly producing similar phenotypes to those we observe for the α -Lys⁴²⁶ mutants.

In summary, we have proposed that the α -Lys⁴²⁶ residue anchors homocitrate so that it can hydrogen bond to α -His⁴⁴², which helps optimize N₂ reduction, and we have shown that disrupting the α -Lys⁴²⁶ hydrogen bond to homocitrate compromises N₂ reduction. α -Lys⁴²⁶ is more than twice the distance from the Fe-S face of FeMoco adjacent to α -Val⁷⁰ than is molybdenum. Thus we find it even more difficult to understand how the mutations at α -Lys⁴²⁶

could affect N₂ binding and reduction if this occurred at the α -Val⁷⁰ site, than do Barney et al. [12] to imagine how molybdenum could participate in N₂ binding because of the effects of mutations at α -Val⁷⁰. Further experiments are clearly necessary to resolve these apparent inconsistencies. Although there is a growing body of evidence that supports alkynes and alkenes being bound and reduced at or close to the α -Val⁷⁰ Fe-S face of FeMoco, our results support molybdenum as the favoured binding and reduction site for N₂.

Financial support of the Biotechnology and Biological Sciences Research Council (a postdoctoral award to K.F.) and the Competitive Strategic Grant to the John Innes Centre is gratefully acknowledged. We thank Dr Shirley Fairhurst for help with the EPR measurements. W.E.N. thanks the NIH (National Institutes of Health) (DK-37255) for support.

REFERENCES

- Burgess, B. K. and Lowe, D. J. (1996) Mechanism of molybdenum nitrogenase. *Chem. Rev.* **96**, 2983–3012
- Chan, M. K., Kim, J. and Rees, D. C. (1993) The nitrogenase FeMo-cofactor and P-cluster pair: 2.2 Å resolution structures. *Science* **260**, 792–794
- Einsle, O., Tezcan, F. A., Andrade, S. L., Schmid, B., Yoshida, M., Howard, J. B. and Rees, D. C. (2002) Nitrogenase MoFe-protein at 1.16 Å resolution: a central ligand in the FeMo-cofactor. *Science* **297**, 1654–1655
- Lee, H. I., Thrasher, K. S., Dean, D. R., Newton, W. E. and Hoffman, B. M. (1998) ¹⁴N electron spin-echo envelope modulation of the S = 3/2 spin system of the *Azotobacter vinelandii* nitrogenase iron-molybdenum cofactor. *Biochemistry* **37**, 13370–13378
- Lowe, D. J. and Thorneley, R. N. F. (1984) The mechanism of *Klebsiella pneumoniae* nitrogenase action: the determination of rate constants required for the simulation of the kinetics of N₂ and H₂ evolution. *Biochem. J.* **224**, 895–901
- Georgiadis, M. M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J. and Rees, D. C. (1992) Crystallographic structure of the nitrogenase iron protein from *Azotobacter vinelandii*. *Science* **257**, 1653–1659
- Kim, J. and Rees, D. C. (1992) Structural models for the metal centers in the nitrogenase molybdenum-iron protein. *Science* **257**, 1677–1682
- Schindelin, H., Kisker, C., Schlessman, J. L., Howard, J. B. and Rees, D. C. (1997) Structure of ADP × AlF₄⁻-stabilized nitrogenase complex and its implications for signal transduction. *Nature (London)* **387**, 370–376
- Mayer, S. M., Lawson, D. M., Gormal, C. A., Roe, S. M. and Smith, B. E. (1999) New insights into structure-function relationships in nitrogenase: a 1.6 Å resolution X-ray crystallographic study of *Klebsiella pneumoniae* MoFe-protein. *J. Mol. Biol.* **292**, 871–891
- Dos Santos, P. C., Igarashi, R. Y., Lee, H. I., Hoffman, B. M., Seefeldt, L. C. and Dean, D. R. (2005) Substrate Interactions with the nitrogenase active site. *Acc. Chem. Res.* **38**, 208–214
- Barney, B. M., Laryukhin, M., Igarashi, R. Y., Lee, H. I., Dos Santos, P. C., Yang, T.-C., Hoffman, B. M., Dean, D. R. and Seefeldt, L. C. (2005) Trapping a hydrazine intermediate on the nitrogenase active site. *Biochemistry* **44**, 8030–8037
- Barney, B. M., Yang, T.-C., Igarashi, R. Y., Dos Santos, P. C., Laryukhin, M., Lee, H. I., Hoffman, B. M., Dean, D. R. and Seefeldt, L. C. (2005) Intermediates trapped during nitrogenase reduction of N≡N, CH₃-N≡NH, and H₂N-NH₂. *J. Am. Chem. Soc.* **127**, 14960–14961
- Durrant, M. C. (2002) An atomic level mechanism for molybdenum nitrogenase. Part 1. Reduction of dinitrogen. *Biochemistry* **41**, 13934–13945
- Kastner, J., Hemmen, S. and Blochl, P. E. (2005) Activation and protonation of dinitrogen at the FeMo cofactor of nitrogenase. *J. Chem. Phys.* **123**, 074306
- Huniar, U., Ahlrichs, R. and Coucouvanis, D. (2004) Density functional theory calculations and exploration of a possible mechanism of N₂ reduction by nitrogenase. *J. Am. Chem. Soc.* **126**, 2588–2601
- Dance, I. (2004) The mechanism of nitrogenase: computed details of the site and geometry of binding of alkyne and alkene substrates and intermediates. *J. Am. Chem. Soc.* **126**, 11852–11863
- Dance, I. (2005) The hydrogen chemistry of the FeMo-co active site of nitrogenase. *J. Am. Chem. Soc.* **127**, 10925–10942
- Durrant, M. C. (2004) An atomic level model for the interactions of molybdenum nitrogenase with carbon monoxide, acetylene, and ethylene. *Biochemistry* **43**, 6030–6042
- Hawkes, T. R., McLean, P. A. and Smith, B. E. (1984) Nitrogenase from *nifV* mutants of *Klebsiella pneumoniae* contains an altered form of the iron-molybdenum cofactor. *Biochem. J.* **217**, 317–321

- 20 Newton, W. E., Vichitphan, K. and Fisher, K. (2002) Substrate reduction and CO susceptibility of the Δ NifV and α -Q191K MoFe-proteins of *Azotobacter vinelandii* nitrogenase. In *Nitrogen Fixation: Global Perspectives* (Finan, T., O'Brian, M. R., Layzell, D. B., Vessey, J. K. and Newton, W. E., eds.), p. 370, CABI Publishing, Wallingford.
- 21 Imperial, J., Hoover, T. R., Madden, M. S., Ludden, P. W. and Shah, V. K. (1989) Substrate reduction properties of dinitrogenase activated *in vitro* are dependent upon the presence of homocitrate or its analogues during iron–molybdenum cofactor synthesis. *Biochemistry* **28**, 7796–7799.
- 22 Pickett, C. J. (1996) The Chatt cycle and the mechanism of enzymic reduction of molecular nitrogen. *J. Biol. Inorg. Chem.* **1**, 601–606.
- 23 Grönberg, K. L., Gormal, C. A., Durrant, M. C., Smith, B. E. and Henderson, R. A. (1998) Why R-homocitrate is essential to the reactivity of FeMo-cofactor of nitrogenase: studies on NifV[−] extracted FeMo-cofactor. *J. Am. Chem. Soc.* **120**, 10613–10621.
- 24 Durrant, M. C. (2001) Controlled protonation of iron–molybdenum cofactor by nitrogenase: a structural and theoretical analysis. *Biochem. J.* **355**, 569–576.
- 25 Durrant, M. C. (2002) An atomic-level mechanism for molybdenum nitrogenase. Part 2. Proton reduction, inhibition of dinitrogen reduction by dihydrogen, and the HD formation reaction. *Biochemistry* **41**, 13946–13955.
- 26 Hales, B. J. and Case, E. E. (1987) Nitrogen fixation by *Azotobacter vinelandii* in tungsten-containing medium. *J. Biol. Chem.* **262**, 16205–16211.
- 27 Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51–59.
- 28 Shen, J., Dean, D. R. and Newton, W. E. (1997) Evidence for multiple substrate-reduction sites and distinct inhibitor-binding sites from an altered *Azotobacter vinelandii* nitrogenase MoFe-protein. *Biochemistry* **36**, 4884–4894.
- 29 Strandberg, G. W. and Wilson, P. W. (1968) Formation of the nitrogen-fixing enzyme system in *Azotobacter vinelandii*. *Can. J. Microbiol.* **14**, 25–32.
- 30 Kim, C.-H., Newton, W. E. and Dean, D. R. (1995) Role of the MoFe-protein α -subunit histidine-195 residue in FeMo-cofactor binding and nitrogenase catalysis. *Biochemistry* **34**, 2798–2808.
- 31 Dilworth, M. J., Eldridge, M. E. and Eady, R. R. (1992) Correction for creatine interference with the direct indophenol measurement of NH₃ in steady-state nitrogenase assays. *Anal. Biochem.* **207**, 6–10.
- 32 Dilworth, M. J. and Fisher, K. (1998) Elimination of creatine interference with the indophenol measurement of NH₃ produced during nitrogenase assays. *Anal. Biochem.* **256**, 242–244.
- 33 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.
- 34 Fisher, K., Newton, W. E. and Lowe, D. J. (2001) Electron paramagnetic resonance analysis of different *Azotobacter vinelandii* nitrogenase MoFe-protein conformations during enzyme turnover: evidence for $S = 3/2$ spin states from reduced MoFe-protein intermediates. *Biochemistry* **40**, 3333–3339.
- 35 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685.
- 36 Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N. and Bourne, P. E. (2000) The Protein Data Bank. *Nucleic Acids Res.* **28**, 235–242.
- 37 Maskos, Z., Fisher, K., Sorlie, M., Newton, W. E. and Hales, B. J. (2005) Variant MoFe-proteins of *Azotobacter vinelandii*: effects of carbon monoxide on electron paramagnetic resonance spectra generated during enzyme turnover. *J. Biol. Inorg. Chem.* **10**, 394–406.
- 38 Hwang, J. C. and Burris, R. H. (1972) Nitrogenase-catalyzed reactions. *Biochim. Biophys. Acta* **283**, 339–350.
- 39 Edgar, R. C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797.
- 40 Scott, D. J., Dean, D. R. and Newton, W. E. (1992) Nitrogenase catalysed ethane production and CO-sensitive hydrogen evolution from MoFe-proteins having amino acid substitutions in an α -subunit FeMo cofactor-binding domain. *J. Biol. Chem.* **267**, 20002–20010.
- 41 Dos Santos, P. C., Dean, D. R., Hu, Y. and Ribbe, M. W. (2004) Formation and insertion of the nitrogenase iron–molybdenum cofactor. *Chem. Rev.* **104**, 1159–1173.
- 42 Christiansen, J., Cash, V. L., Seefeldt, L. C. and Dean, D. R. (2000) Isolation and characterization of an acetylene-resistant nitrogenase. *J. Biol. Chem.* **275**, 11459–11464.
- 43 Moore, V. G., Tittsworth, R. C. and Hales, B. J. (1994) Construction and characterization of hybrid component 1 from V-nitrogenase containing FeMo cofactor. *J. Am. Chem. Soc.* **116**, 12101–12102.
- 44 Smith, B. E., Eady, R. R., Lowe, D. J. and Gormal, C. A. (1988) The vanadium–iron protein of vanadium nitrogenase from *Azotobacter croococcum* contains an iron–vanadium cofactor. *Biochem. J.* **250**, 299–302.
- 45 Farrugia, L. J. (1997) ORTEP-3 for Windows – a version of ORTEP-III with a Graphical User Interface (GUI). *J. Appl. Crystallogr.* **30**, 565.

Received 16 January 2006/24 March 2006; accepted 27 March 2006

Published as BJ Immediate Publication 27 March 2006, doi:10.1042/BJ20060102