ELSEVIER

Coordination Chemistry Reviews
185–186 (1999) 669–687

COORDINATION CHEMISTRY REVIEWS

Exploring the reactivity of the isolated iron-molybdenum cofactor of nitrogenase

B.E. Smith *, M.C. Durrant, S.A. Fairhurst, C.A. Gormal, K.L.C. Grönberg, R.A. Henderson, S.K. Ibrahim, T. Le Gall, C.J. Pickett

Nitrogen Fixation Laboratory, John Innes Centre, Colney, Norwich NR4 7UH, UK

Contents

Abstract

There is strong evidence that the iron-molybdenum cofactor (FeMoco) of nitrogenase forms part of the enzyme's active site. FeMoco, a $MoFe_7S_9$·homocitrate cluster, can be extracted intact from the enzyme into *N*-methylformamide solution but is reported to be inactive in substrate reduction unless powerful reductants are used and then only acetylene and cyclopropene reductions have been observed. The literature on the catalytic and substrate binding reactivities of extracted FeMoco is reviewed and new data on electrocatalytic hydrogen evolution presented. A comparison of the ligand binding properties of FeMoco from the wild-type and a $NifV^-$ mutant enzyme, which has citrate in place of

* Corresponding author. Tel.: +44-1603-456900; fax: +44-1603-455030.
E-mail address: barry.smith@bbsrc.ac.uk (B.E. Smith)

PII: S0010-8545(99)00017-X

R-homocitrate, is presented. These data are interpreted in terms of their significance for enzyme turnover and of the obligate requirement for *R*-homocitrate for dinitrogen reduction.

Keywords: Nitrogen fixation; Metal-sulfur clusters; Electrocatalysis; Enzyme mechanism

1. Introduction

The biological fixation of dinitrogen from the atmosphere to form ammonia, to be used by microbes and their symbiotic plant partners for growth, is of major importance in world agriculture and the biosphere. The process is mediated by the microbial enzyme nitrogenase, the most common form of which contains molybdenum and iron. This enzyme consists of two metalloproteins the iron protein (Fe protein) and the molybdenum iron protein (MoFe protein). The Fe protein passes electrons to the MoFe protein in a MgATP-hydrolysing reaction (Fig. 1). The MoFe protein contains the enzyme's substrate binding and reducing site and is capable of reducing a number of other substrates as well as dinitrogen. For example the enzyme can reduce cyanide to methane, ammonia, and some methylamine; azide to dinitrogen plus ammonia; acetylene to ethylene and, in the absence of other substrates, protons to dihydrogen. Dinitrogen reduction with concomitant dihydrogen evolution requires eight electrons and eight protons, see Eq. (1), and is apparently carried out by eight sequential, one electron transfers from the Fe protein to MoFe protein. When sodium dithionite is used as reductant in vitro the Fe protein and MoFe protein have to dissociate after each electron transfer and this dissociation is the rate determining step in enzyme turnover [1].

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i \quad (1)$$

The MoFe protein has been isolated from a number of organisms and is an $\alpha_2\beta_2$ tetramer containing two unique metallo-sulfur clusters. These are the, Fe_8S_7, P clusters and the, $MoFe_7S_9$. homocitrate, FeMoco centres. Neither of these clusters have been synthesised chemically nor have they been observed elsewhere in biology [2,3].

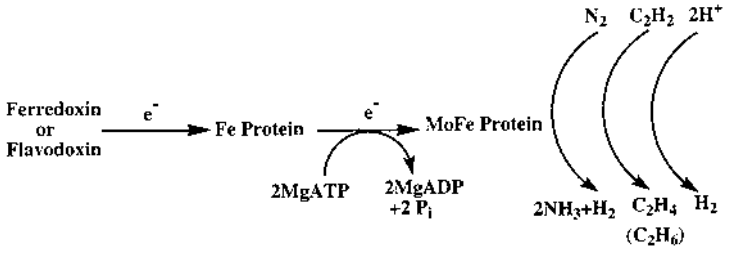

Fig. 1. Electron transfer path for molybdenum nitrogenase.

The FeMoco can be extracted from the protein into an organic solvent, usually *N*-methyl formamide (NMF) and used to activate the extracts of mutants which are unable to synthesise FeMoco [4]. The biosynthesis of FeMoco is extremely complex [5] and utilises at least 6 gene products. However FeMoco can be extracted intact from the protein and thus is available to study as an inorganic complex.

The 3-dimensional crystal structure of the MoFe protein of nitrogenase was first reported in 1992 [6] however the structures of FeMoco and the P clusters were not defined fully until somewhat later [7–9]. The structure of FeMoco is now agreed to be that shown in Fig. 2 with stoichiometry $MoFe_7S_9$. homocitrate. The molybdenum atom is six coordinate with three molybdenum-sulfur bonds, two to the hydroxyl and a carboxyl group from homocitrate and one to histidine from the protein polypeptide. It can be regarded as a $MoFe_3S_3$ partial cluster which is bonded to an Fe_4S_3 cluster by three bridging sulfide ions. The terminal iron atom in the cluster is tetrahedral and bound to the polypeptide by a cysteine residue. However the other six iron atoms are apparently all three-coordinate being bonded to only three sulfur atoms each. Nevertheless the metal–metal distances between these iron atoms are such that there is probably considerable metal–metal bonding. Theoretical calculations are consistent with this view [10].

There is considerable evidence that FeMoco constitutes at least part of the substrate binding and reducing site of the enzyme. The first convincing evidence for this hypothesis came from a study of a *nifV* mutant of *Klebsiella pneumoniae* [11]. We now know that the product of the *nifV* gene is a homocitrate synthase that provides the homocitrate for FeMoco biosynthesis [12]. In *Klebsiella pneumoniae* the enzyme from the *nifV* mutant contains citrate [13] in place of homocitrate and exhibits an altered phenotype compared with the wild type being very poor at fixing nitrogen but fully capable of reducing acetylene [14]. Furthermore the dihydrogen evolution ability is inhibited by carbon monoxide whereas, with the wild type enzyme, carbon monoxide although inhibiting reduction of other substrates does not inhibit dihydrogen evolution. When the FeMoco was isolated from the MoFe protein from the *nifV* mutant and combined with the polypeptides of the MoFe protein from mutants unable to synthesise FeMoco the phenotype of the *nifV* mutant was transferred with the FeMoco [11]. This provided very convincing evidence that FeMoco defined the substrate reducing properties of the enzyme.

Since that time numerous other studies have investigated the effects of substituting homocitrate with other analogues which once again have affected substrate reducing properties [5]. Furthermore site-directed mutagenesis of residues in the environment of FeMoco, but not bonded covalently to it, have altered substrate specificity (Fig. 2(b)) [15]. This is in marked contrast to site-directed mutagenesis of residues in the environs of the P clusters which sometimes either removes or decreases overall activity but does not affect the substrate specificity. The above combined evidence implies strongly that FeMoco is, or at least forms part of, the substrate reducing site although clearly some residues in its environment are also important for activity.

As noted above several amino acid neighbours of FeMoco (Fig. 2(b)) are critical to its activity. The Arg96Gln mutant grows slowly diazotrophically but the

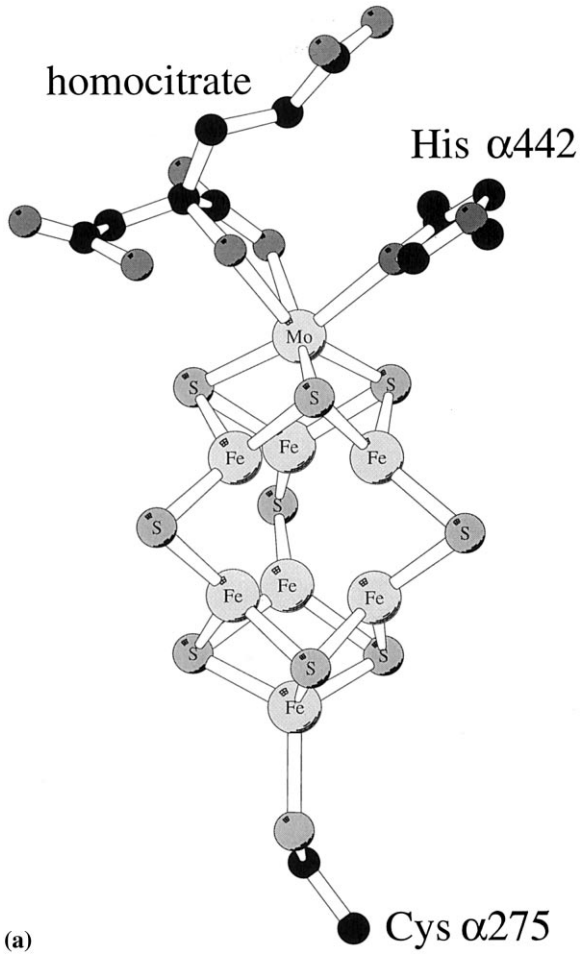

Fig. 2. Structure of the iron-molybdenum cofactor (FeMoco). (a) Showing only FeMoco and the amino-acid residues ligating it to the α subunit of the MoFe protein. (b) As (a) but showing also other important amino-acids in the FeMoco environment.

Arg359Gln, Phe381Arg or Arg277His mutants are all inactive in dinitrogen reduction but active in other substrate reductions [16,17]. A Gln191Lys mutant is also unable to reduce dinitrogen but can reduce acetylene to ethylene and also to ethane, furthermore carbon monoxide inhibited dihydrogen evolution with this mutant [18,19].

Probably the most interesting mutant discovered thus far is His195Gln which cannot reduce dinitrogen but dinitrogen can inhibit acetylene or proton reduction, i.e. the dinitrogen can bind but is not reduced [18,20]. The 3-dimensional structure of the MoFe protein implies a hydrogen bond between the nitrogen of His195 to one of the central sulfur atoms of the cluster and substitution by Gln could retain this hydrogen bond whereas other substitutions would not.

All of the above site-directed mutagenesis experiments emphasise the importance of the environment to the activity of FeMoco within the enzyme with hydrogen bonding probably playing an important role. Experiments with extracted FeMoco have not yet made it capable of reducing dinitrogen. In this article we shall review the literature on reactions of extracted FeMoco, relate them to our recent advances

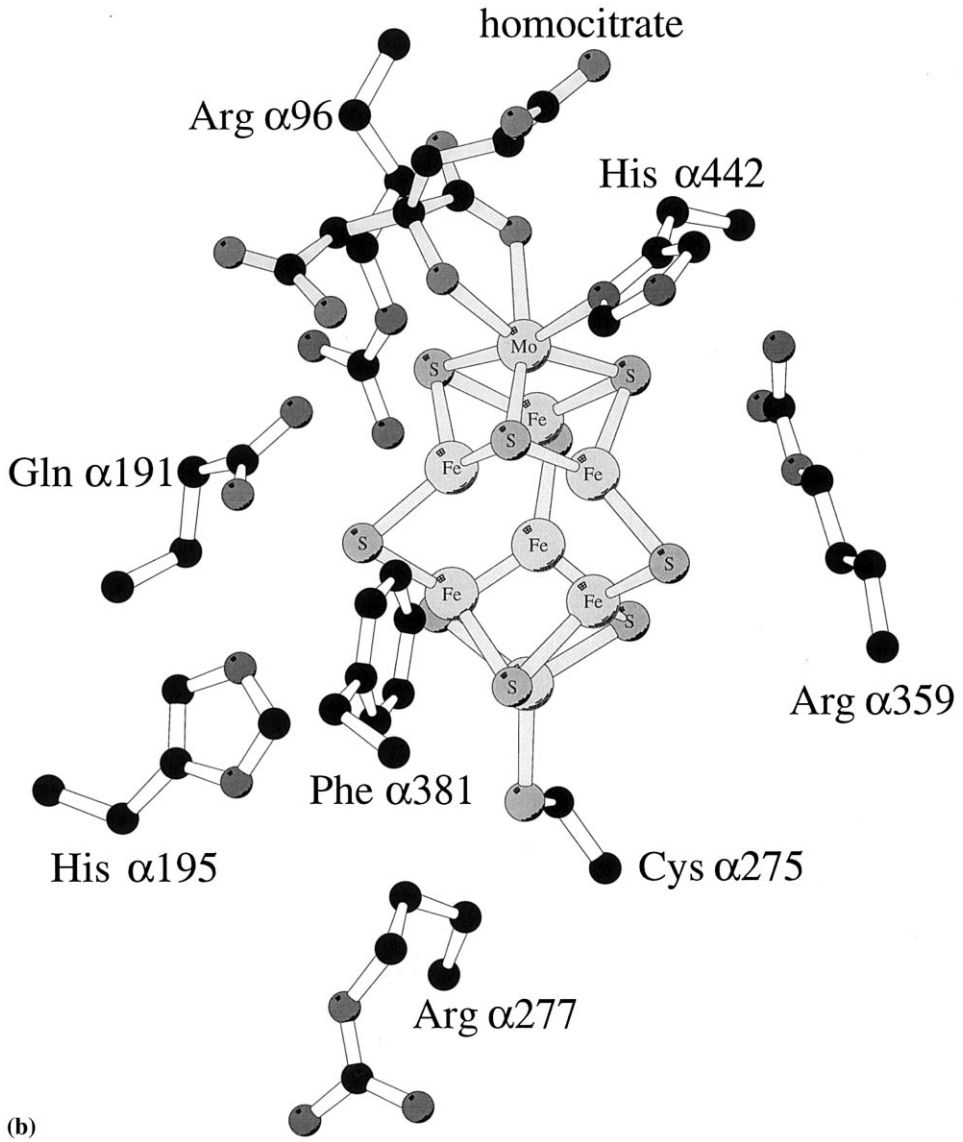

Fig. 2. (*Continued*)

in this area and indicate the importance of these findings to our understanding of the enzyme activity. Much of the early literature on the reactions of isolated FeMoco was analysed by Burgess [21] and therefore this current review will only summarize those data and place emphasis on more recent results.

2. Ligand binding to FeMoco

FeMoco is bound to the protein via a histidine to the molybdenum atom and a cysteine to the tetrahedral iron atom (Fig. 2) and when extracted these coordination positions are occupied presumably by other ligands. FeMoco solutions often contain a large number of chemical species, e.g. Na^+,HPO_4^{2-},$S_2O_4^{2-}$ and its oxidation products, H_2O and normally *N*-methylformamide (NMF) as the solvent. NMF is not the only solvent that can be used with FeMoco but most studies have used NMF solutions so we shall consider only data obtained with this solvent.

There is evidence [22] from sulfur K-edge X-ray absorption studies that FeMoco binds thiosulfate, a degradation product of dithionite, fairly tightly since it is not removed by anaerobic, dithionite-free gel exclusion chromotography. However, this ligand cannot be important for the integrity or stability of FeMoco since it is possible to isolate it from the protein in the absence of dithionite and its oxidation products [23].

Dithionite is unstable in the presence of concentrated solutions of FeMoco and on prolonged incubation the FeMoco becomes oxidized although without losing activity. It seems probable that in NMF solutions the NMF anion is bound to the tetrahedral iron atom in place of cysteine and NMF to the molybdenum site in place of histidine. Infrared spectra of FeMoco solutions are consistent with this assumption [24].

2.1. Thiol binding

It was demonstrated early on that thiols bind to FeMoco and cause a sharpening of its $S = 3/2$ EPR spectrum so that it becomes more like that observed in the protein (Fig. 3) [25]. This interaction has been quantified to show that one thiol binds per molybdenum atom i.e. per FeMoco molecule [24].

It is reasonable to assume that thiols bind at the same site as cysteine in the protein i.e. to the tetrahedral iron atom. Strong evidence supporting this hypothesis comes from studies on binding of seleno-phenol to FeMoco. Seleno-phenol causes a decrease in the line width at half-height of the lines in the EPR signal of FeMoco, which is complete at a ratio of one PhSeH per FeMoco [27]. Selenium EXAFS demonstrates that it binds to an iron atom [27,28] and iron EXAFS on the phenyl selenol complex showed one selenium atom binding per FeMoco molecule [27]. The iron-selenium distance of 2.36 Å from the iron K-edge data and of 2.38 Å from the selenium K-edge data are consistent with the selenol being bound in a non-bridging mode by iron on FeMoco [27]. This observation together with the stoichiometry of the reaction and the known structure of FeMoco in the protein (Fig. 2(a)), where

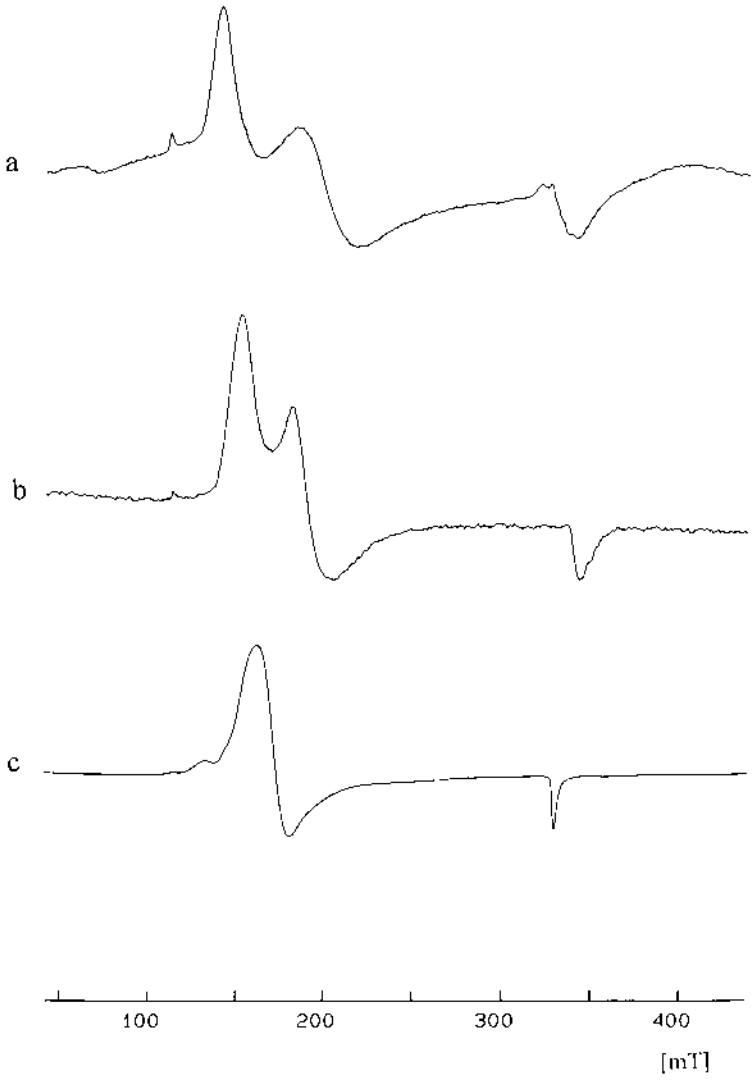

Fig. 3. Electron paramagnetic resonance (EPR) spectra of FeMoco. (a) FeMoco as isolated in NMF in the presence of dithionite. (b) As (a) with added thiophenol. (c) As (a) with added cyanide.

the terminal iron atom is bound by cysteine, are all consistent with thiols binding at the terminal, tetrahedral iron atom.

Binding of thiols has also been investigated at ambient temperatures utilising ^{19}F-NMR studies with p-$CF_3C_6H_4S^-$ as the reporter ligand [29]. The data obtained demonstrated that the FeMoco-thiolate complex was not a product of an irreversible reaction but that the complex is in dynamic equilibrium with free FeMoco and thiolate. The magnetic-circular-dichroism (MCD) spectra of FeMoco and its

thiol aducts were remarkably invariant above 450 nm but at wavelengths below 450 nm there was evidence of ligand to cluster charge transfer bands (30). In particular, on binding thiophenol, a band at 360 nm in free FeMoco was red-shifted to 387 nm with an increase in intensity.

2.2. Substrate binding

There have been many studies on the binding of nitrogenase substrates to FeMoco however few have given positive results. Most success has been achieved by studying cyanide and methyl isocyanide binding.

Cyanide binding was first reported [31] from this laboratory as a major perturbation of the EPR spectrum of isolated FeMoco (see Fig. 3(c)). Quantitation of this binding phenomenon was difficult; the binding curve could not be fitted with a single binding constant nor with a model in which two cyanide ions bound with similar binding constants. However a Job plot of the data implied binding of 1.5 cyanide ions per molecule of FeMoco [30]. These data could imply that FeMoco was present in solutions as a dimer which bound three cyanide ions, alternatively the analysis could be in error. We are inclined to the view that the data imply tight binding of one cyanide ion with weaker binding of another. The Job plot analysis assumes identical binding constants for the cyanide at the two sites and therefore cannot reflect this situation.

There is evidence from EXAFS studies of cyanide binding to molybdenum in FeMoco [32]. ^{19}F-NMR using p-$CF_3C_6H_4S^-$ as reporter ligand has provided evidence of cyanide and methyl isocyanide binding to isolated FeMoco cofactor [33]. Since this experiment monitors binding of these ligands to the thiol complex and, as we have indicated above, the thiol binds almost certainly to the tetrahedral iron atom, then the cyanide and methyl isocyanide ligands undoubtedly bind elsewhere on FeMoco although the authors report a parallel molybdenum K-edge EXAFS study which indicates that the methyl isocyanide does not bind to molybdenum. In these experiments no evidence for binding of carbon monoxide, dinitrogen, acetylene, or azide was observed. Other X-ray absorption spectroscopic studies did not find evidence of binding of cyanate or thiocyanate ions from either iron or molybdenum K-edge spectra [27].

2.3. A kinetic approach

Recently, a new approach to identifying substrate binding sites on isolated FeMoco has been developed [34]. This method is based on the reaction between thiophenolate and FeMoco which is monitored by stopped-flow spectrophotometry. An exponential increase in absorbance is observed at $\lambda \sim 450$ nm and the rate of reaction is independent of the concentration of thiolate. These observations are consistent with a mechanism involving rate limiting dissociation of a ligand, presumably the NMF anion, from FeMoco and its subsequent replacement with thiophenolate. If the reaction is carried out in the presence of other compounds which interact with FeMoco then the binding of these compounds will affect the

electron distribution within FeMoco and thus the strength of the FeMoco-NMF bond. The strength of this bond will in turn affect the dissociation rate of the NMF and consequently the rate of reaction with thiophenolate. Furthermore, the extent of the perturbation is an indicator of the propinquity of the substrate binding site to the dissociating bond. Thus this technique can be used not only to study which ligands bind FeMoco but also to indicate their binding site. So far this technique has only been applied to FeMoco in its dithionite reduced $S = 3/2$ state. Acetylene or carbon monoxide had no affect on the rate of reaction with thiophenolate and thus it was deduced that they did not bind to the cofactor in this state. However azide, tertiary-butyl isocyanide, cyanide, imidazole and protons all perturbed the rate of reaction with thiophenolate.

The reaction with cyanide was the most complex. First it inhibited the rate of reaction of FeMoco with thiophenolate and secondly the rate equation contained two terms, one with a first order dependence on, and the other independent of, thiophenolate concentration. These observations are consistent with cyanide being bound to the tetrahedral iron atom and being displaced by thiophenolate via parallel dissociative and associative pathways. On addition of excess cyanide the rate of the dissociative pathway increased while that of the associative pathway decreased, both by small factors. These effects indicate binding of an additional cyanide remote from the tetrahedral iron and probably at molybdenum, with the presence of this cyanide being transmitted through the cluster. Thus these data implied that cyanide could combine with both the tetrahedral iron atom and the molybdenum atom. The latter is consistent with the EXAFS [33] and ^{19}F-NMR [29] studies and the possibility of two sites with different binding constants is consistent with the EPR data [30]. The EPR data also demonstrated competition between cyanide and thiophenolate for FeMoco (presumably at the tetrahedral iron atom) with thiophenolate being favoured at equimolar concentrations.

In summary, cyanide seems to be capable of binding at both the tetrahedral iron atom and at the molybdenum atom of isolated cofactor. Thiophenolate binds only at the iron atom and can, at equimolar concentrations, displace cyanide.

The rate of thiophenolate substitution was only slightly perturbed by azide or tertiary-butyl isocyanide [34] consistent with these species binding at the molybdenum end of cofactor. Azide accelerates and tertiary-butyl isocyanide inhibits the rate of reaction with thiophenolate presumably as a consequence of the relative electron-releasing and -withdrawing properties of these molecules.

Our earlier studies on synthetic iron-sulfur based clusters [35] showed that the binding of protons increased the substitution reactivity of the clusters. The pK_as associated with these protonations were remarkably insensitive to the structure, charge and terminal ligands of the clusters and therefore it was proposed that protonation occurred at the bridging sulfur atoms. Electrochemical and EPR studies [48] have shown that both the oxidized and semi-reduced states of FeMoco can be protonated. Protonation of FeMoco by the weak acid $[NHEt_3]BPh_4$ resulted in an increased rate of its reaction with thiophenolate [34] and so, by analogy with the simpler iron-sulfur clusters, this was interpreted as indicating protonation of bridging sulfur.

The effect of imidazole binding is considered below.

In summary, this kinetic technique indicates that various nitrogenase substrates exhibit selectivity for different sites on FeMoco in the $S = 3/2$ spin state and that there are at least three sites at which substrates can combine, (a) the molybdenum atom (or a site at this end of the cluster) at which anionic and neutral substrates bind, (b) the tetrahedral iron atom at which cyanide and thiophenolate prefer to bind, and (c) the bridging sulfur atoms where protons bind.

3. Substrate reductions and electron transfer reactions of FeMoco

A number of groups have investigated direct reduction of nitrogenase substrates by isolated FeMoco. Shah et al. [36] first reported acetylene reduction by FeMoco using sodium borohydride as the reductant. They found an activity for acetylene reduction to ethylene of ca. 8% of that observed with the enzyme. However the significance of their results is questionable since the activity was independent of exposure to oxygen which would destroy FeMoco. Furthermore the reaction mixture was largely aqueous and FeMoco is unstable in such an environment. McKenna et al. [37] reported similar data and also investigated the reduction of cyclopropene by isolated FeMoco. Cyclopropene is reduced by the enzyme to a mixture of cyclopropane and propene, however only propene was formed by isolated FeMoco with borohydride as the reductant. In later work McKenna et al. [38] demonstrated that the reconstitution activity of FeMoco was strongly dependent on the level of water in the reaction mixture with borohydride as the reductant. However, by working in the absence of water, they were able to demonstrate some acetylene reduction to ethylene by the FeMoco/borohydride system in *N*-methylformamide with retention of FeMoco activity. These data show that isolated FeMoco combined with a very strong reductant is an active catalyst for acetylene reduction but this activity is common to a number of metal complexes and thus is perhaps not surprising. The activity of water-degraded FeMoco was higher that that of the original [38].

More recently, Shilov et al. [39] have been investigating the reactivity of isolated FeMoco using zinc amalgam as the reductant, dimethylformamide as the solvent and thiophenol or citric acid as the source of protons. Catalytic reduction of acetylene to ethylene was observed but unfortunately no activity measurements on FeMoco after the reaction were reported and therefore it is not possible to be sure that the FeMoco was not degraded in this system. Very recently [40], these authors have also reported investigations of the catalytic reduction of acetylene and dinitrogen by FeMoco and other polymolybdenum clusters using sodium, zinc or europium amalgams as reductants. With acetylene as the substrate, reactivity increased in the order zinc < europium < sodium amalgam i.e. with the decrease (more negative) in the redox potential of the reductant. Catalytic reduction of N_2 occurred only with sodium and europium amalgams and only with the synthetic polymolybdenum complexes. No catalytic reduction of N_2 by FeMoco was observed. The activity of FeMoco after these reactions was not reported.

3.1. Redox behaviour of FeMoco

There is an early report [26] that both *o*-phenanthroline and EDTA interact with FeMoco to abolish its $S = 3/2$ EPR signal. The EPR signal was restored by addition of ferrous iron or zinc ion respectively, indicating that the ligands bound to FeMoco and altered its redox potential thus abolishing its EPR signal but that the reactions could be reversed by complexing the ligands with metal ions.

More systematic investigations of the redox behaviour of FeMoco were reported initially by Schultz et al. [41] who used both quantitative titrations and electrochemistry to investigate FeMoco's redox behaviour. Using direct electrochemical reduction at a glassy carbon electrode in NMF solution two reduction waves were observed by cyclic voltammetry for oxidized FeMoco at -0.32 and -1.00 V versus NHE. The authors interpreted these reduction waves as corresponding to the reactions shown in Eq. (2). Reaction at the higher potential corresponded to the equilibrium between oxidized and semi-reduced FeMoco where the semi-reduced form corresponded to the $S = 3/2$ spin state. Titrations with sodium dithionite as reductant and potassium ferricyanide as oxidant and later [42] using controlled potential coulometry confirmed that this was a one electron redox reaction. Although the more negative reduction (Eq. (2)) was thought to correspond to the formation of more reduced FeMoco, and in a bulk electrolysis experiment about 14 electrons/FeMoco were consumed, no substrate reduction product was detected although it was suggested that protons might be being reduced [49].

$$(\text{FeMoco})_{\text{ox}} \leftrightarrow (\text{FeMoco})_{\text{sred}} \leftrightarrow (\text{FeMoco})_{\text{red}} \quad (2)$$

Recently [43], we have re-investigated the electrochemical behaviour of FeMoco in NMF. In common with Schultz et al. [41] we observed two redox waves at -0.32 V and at close to -1.0 V relative to NHE. These potentials are modified slightly by binding thiols to FeMoco. If a relatively acidic thiol, pentafluorothiophenol, is used then the behaviour seen in Fig. 4 is observed. With a ca. 30-fold excess of thiol over FeMoco a normal cyclic voltammogram corresponding to a redox process at -0.28 V relative to NHE is observed. However as the level of thiol is increased to 60- and then 75-fold excess the cyclic voltammogram changes and a catalytic process is observed. We conclude that the thiol is acting both as a ligand and as an acid and is providing protons for the catalytic evolution of H_2. To test this hypothesis we conducted a coulometric experiment in a sealed vessel using a vitreous carbon working electrode, a platinum secondary electrode and a reference electrode and sampled the gas space above the NMF solution of FeMoco containing pentafluorothiophenol. At least 85% of the electrons passed were recovered as H_2 as measured by gas chromatography. Thus we have shown that isolated FeMoco can be induced to evolve H_2 at the relatively high potential of -0.28V.

Reduction of $(\text{FeMoco})_{\text{ox}}$ to the semireduced state with concomitant protonation must allow further electron transfer thus accessing a $(\text{FeMoco})_{\text{red}}$ state at high potential. Presumably, attack of a proton on this species would liberate H_2 and regenerate $(\text{FeMoco})_{\text{ox}}$. However it is possible that lower (more reduced) redox

states could be accessed on protonation/reduction-the mechanism of this process is being examined further. Importantly, at fast cyclic voltammetry scan rates, the catalysis step is circumvented and the system approaches reversibility. This confirms the role of the $(FeMoco)_{ox/sred}$ system in the turnover *and* that the integrity of the cluster is maintained.

Clearly, by coupling electron and proton addition, access to more reduced states of FeMoco is achieved at high potential and this allows electrocatalytic H_2 evolution. There are distinct parallels with the proposed mechanism of H_2 evolution by the enzyme [1].

4. Relevance to the enzyme function

The above studies have demonstrated that it is possible to emulate at least some of the enzyme's reactivities, specifically acetylene and proton reduction, with the isolated co-factor. Furthermore the kinetic binding studies described above have indicated potential binding sites for substrates on FeMoco although the results must be treated with care because FeMoco when isolated has additional potential

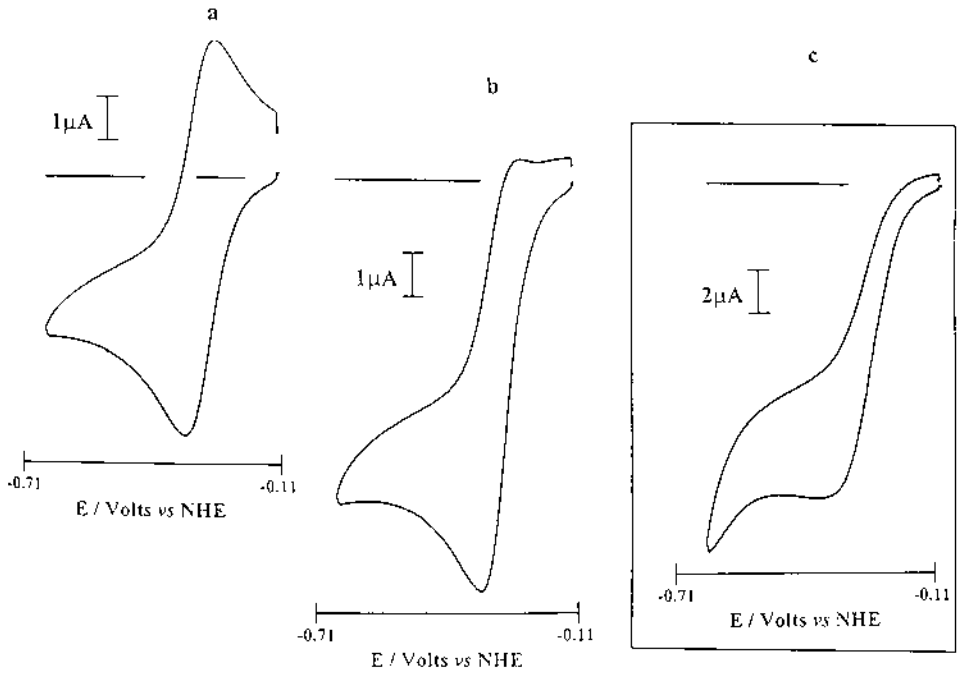

Fig. 4. Cyclic voltammetry of FeMoco in NMF. The voltammograms were recorded at 50 mV s^{-1} at a vitreous carbon electrode at room temperature (a) Ca. 1 mM FeMoco with 30 mM C_6F_5SH. (b) As (a) but with 60 mM C_6F_5SH. (c) As (a) but with 75 mM C_6F_5SH. Note that the ordinate scale is twice that in (a) and (b). The activity of the FeMoco was unaffected during the bulk electrolysis experiment.

binding sites, these binding sites being occupied by ligands when bound to the protein. However the studies do show that different species have very different affinities for different parts of the cofactor.

Very recently [44], we have been able to extend our kinetic binding studies to provide further insight into the functioning of FeMoco within the enzyme. As noted above *R*-homocitrate is an essential ligand for FeMoco and when it is replaced by citrate as in the *nifV* mutant the resultant enzyme is a poor nitrogen-fixer. We have isolated the FeMoco from the MoFe protein from a *nifV* mutant of *Klebsiella pneumoniae* and compared its reactivity with thiophenolate with that of the cofactor from the wild type enzyme. We found no distinction between the reactivities of the two types of cofactor when bound to cyanide, azide or the proton (see for example Fig. 5). However when imidazole was bound, the kinetics of the reactions of thiophenolate with the two cofactors were very different (see Fig. 6). The reaction of thiophenolate with the wild type cofactor shows a first order dependence on the concentration of cofactor and a non linear dependence on $[PhS^-]/[PhSH]$. This behaviour was rationalised [34] in terms of the ability of thiophenolate to act both as a ligand and as a base. As the base concentration increases the imidazole becomes deprotonated and this in turn affects the electron density within the FeMoco and the rate of reaction with thiophenolate. Both types of cofactor show this first order dependence on the concentration of cofactor and non-linear dependence on $[PhS^-]/[PhSH]$ but with wild type cofactor the rate decreases as $[PhS^-]/[PhSH]$ increases whereas with the $NifV^-$ cofactor the rate increases. Clearly therefore, when imidazole is bound to the cofactors, their reactivities are sensitive to whether citrate or homocitrate are bound despite the apparently identical mode of binding to molybdenum of these hydroxy acids.

The only difference between these two cofactors is the existence of an extra CH_2 group in the pendant carboxyl arm of *R*-homocitrate compared with citrate. Molecular mechanics calculations show that this pendant $CH_2CH_2CO_2$ arm of *R*-homicitrate is sufficiently long and flexible that it can form a hydrogen bond with the NH group of the imidazole ligand. This would impose imidazolate character on the ligand, which in turn will perturb electron distribution within the cluster core and the lability of the iron–NMF bond. However, the pendant arm of citrate, CH_2CO_2, in the $NifV^-$ cofactor is shorter and cannot interact with the imidazole ligand.

It is notable from the data in Fig. 6 that the reactivity of the $NifV^-$ cofactor in the presence of high levels of base (PhS^-) is very similar to that of the wild type cofactor at low concentrations of base. We believe that this rate reflects the reactivity of the cluster with imidazolate bound. The decreasing reactivity of the wild type cofactor as base increases (Fig. 6) may reflect adverse interactions between imidazolate and the negative charge on the pendant carboxyl of *R*-homocitrate which either inhibits interaction of imidazolate with the cluster by preventing overlap with the appropriate orbitals on the molybdenum atom or possibly by forcing dissociation of the imidazolate from the molybdenum atom.

Are these observations relevant to the enzymic behaviour?

We believe that they are and offer the following hypothesis:

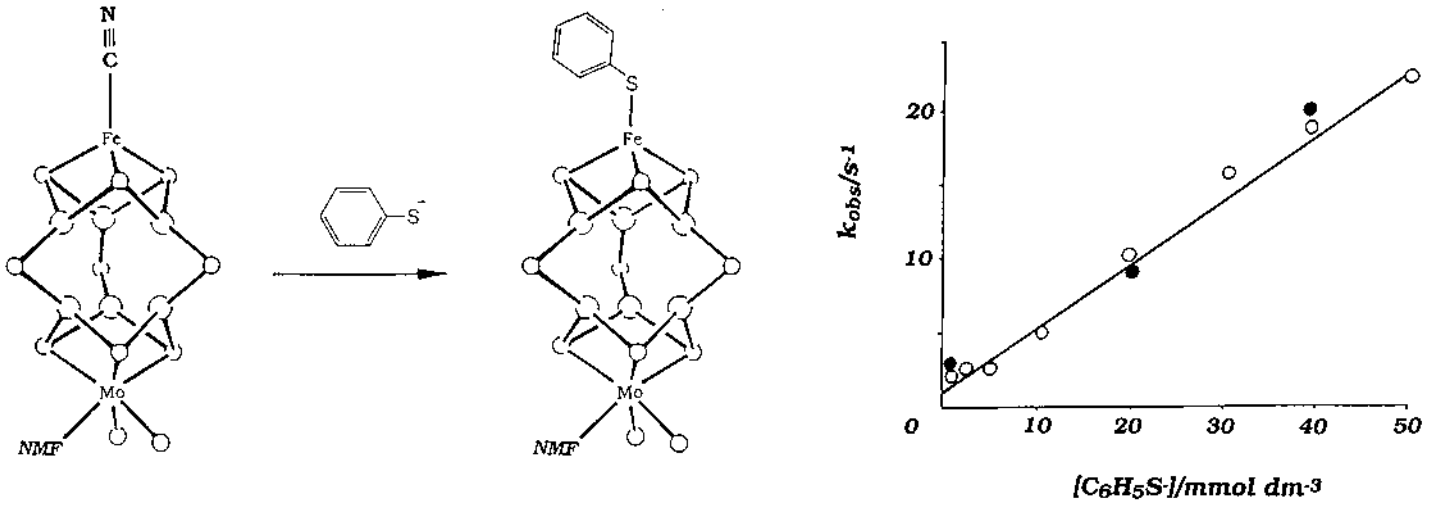

Fig. 5. Comparison of the kinetics of the reactions between thiophenol and wild-type (O) and NifV$^-$ FeMoco (●) in the presence of cyanide.

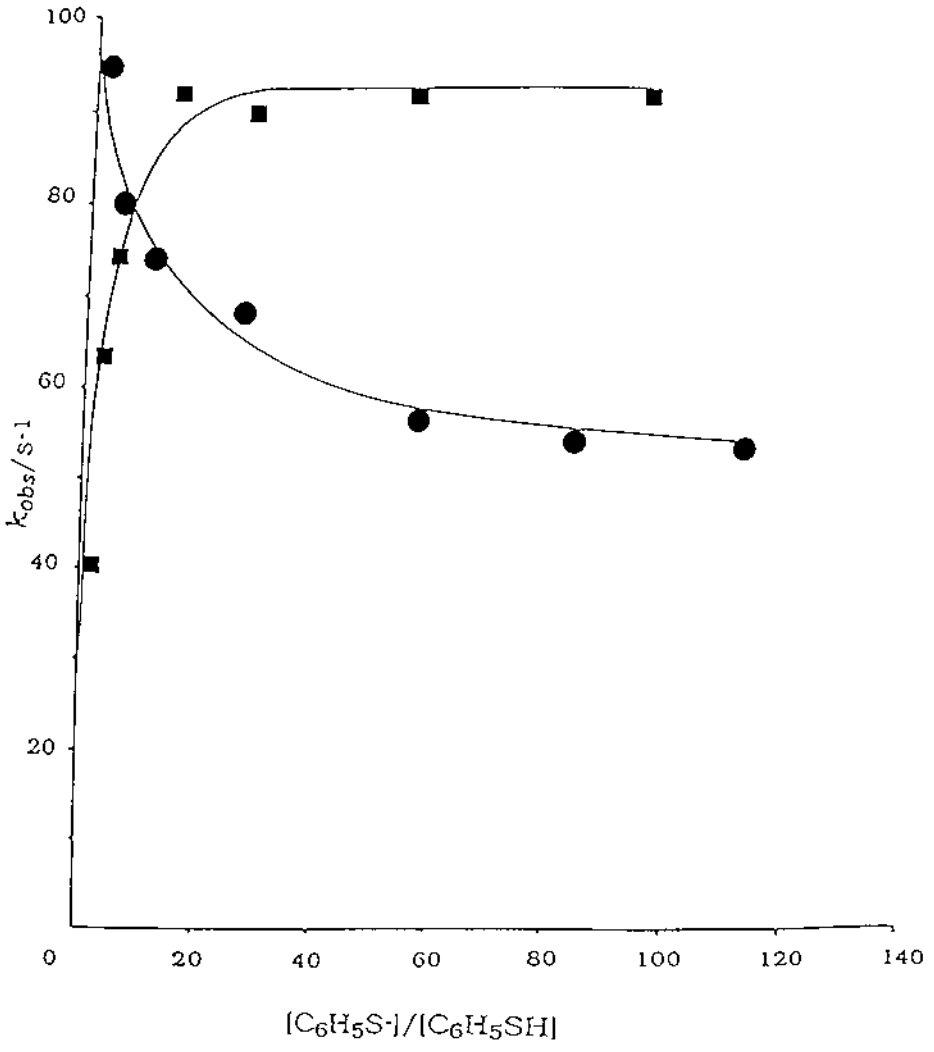

Fig. 6. Comparison of the $[PhS^-]/[PhSH]$ dependences of the rate of the reaction between thiophenol and wild-type (●) and $NifV^-$ FeMoco (■) in the presence of imidazole (1 mM) [imidazole]/[FeMoco] = 10.

We have examined the 3-dimensional crystallographic structure of the MoFe protein in its resting state and observe that it is not possible to form a hydrogen bond between the pendant carboxyl group of homocitrate and the nitrogen atom of histidine bound to the molybdenum atom of FeMoco. The hydrogen bond observed with the isolated cofactor required rotation of the imidazole ligand on molybdenum. However, earlier work [45] with model complexes with carboxyl ligands bound to molybdenum, indicated that during turnover the molybdenum atom, by reduction and protonation, could be activated for binding dinitrogen by

dissociation of the carboxyl group, i.e. that the homocitrate ligand could become mono-dentate during nitrogenase turnover [46] (see Fig. 7). With molecular modelling we have been able to show [44] that mono-dentate *R*-homocitrate is able to establish a hydrogen bond between the pendant carboxyl group and the NH group of His442 without rotation of the histidine ligand.

Assuming this we note that during rearrangement of the homocitrate a hydrogen bond previously between the homocitrate carboxyl group and a backbone residue would be broken but could be replaced by another at a nearby residue. There would be some distortion of the interaction between the other homocitrate carboxyl group and the important residue Gln191 but once again the hydrogen bond would be maintained. Therefore the proposed rearrangement of the mono-dentate *R*-homocitrate could be achieved without loss of overall stabilisation energy.

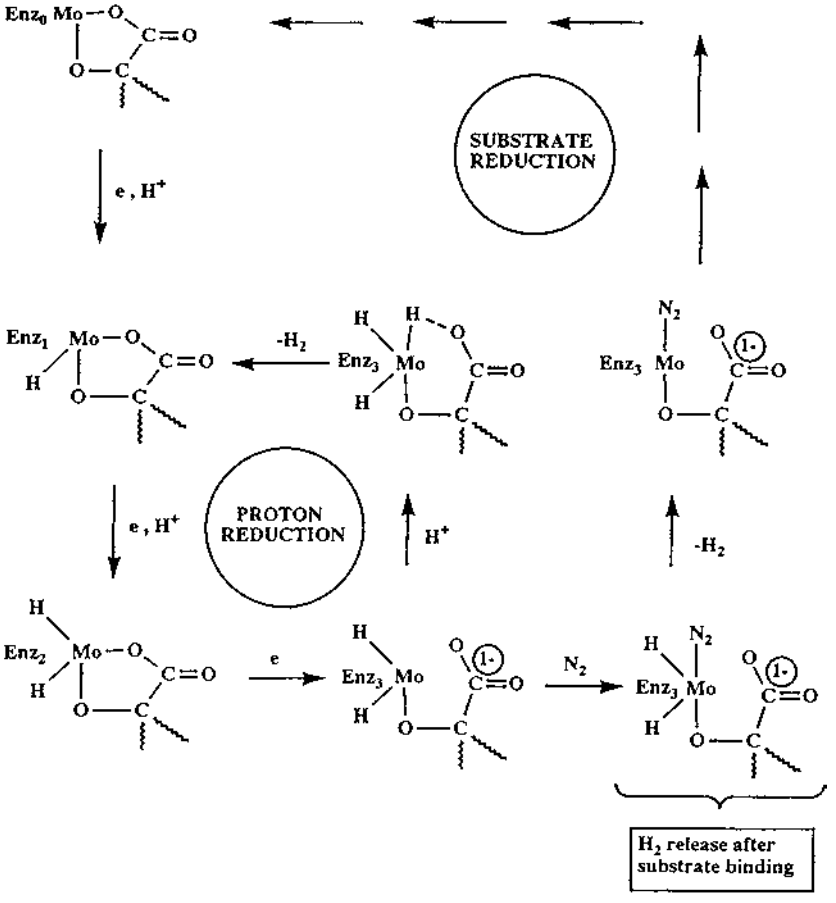

Fig. 7. Proposed mechanism of nitrogenase substrate reduction via dissociation of the carboxylate group of homocitrate from the molybdenum atom of FeMoco. The mechanism is based on the observed behaviour of chemical model molybdenum carboxylate complexes [45].

If homocitrate were to become mono-dentate a vacant site would be opened up on the molybdenum which would be suitable for binding dinitrogen [46] (Fig. 7). We have shown that forming a hydrogen bond between homocitrate and the imidazole ligand on molybdenum effectively releases electron density into the cluster. Studies on structurally defined dinitrogen complexes [47] have shown that the binding of dinitrogen to a metal site and the ability of that dinitrogen ligand to be protonated are favoured by electron-rich sites. Thus the above process would favour dinitrogen binding and its reduction. The 3-dimensional crystal structure of the MoFe protein shows that the molybdenum end of the FeMoco is surrounded by water which would provide the protons required for dinitrogen reduction.

We therefore propose that during enzyme turnover electrons are passed to FeMoco and induce the carboxyl group of *R*-homocitrate to dissociate from molybdenum. The mono-dentate homocitrate then hydrogen bonds to Hisα442, which increases the electron richness of FeMoco particularly at the molybdenum site and allows binding of dinitrogen. Further electron transfer to FeMoco followed by protonation would result in the formation of ammonia.

The above hypothesis provides a very satisfying explanation of the absolute requirement for *R*-homocitrate as a ligand to the molybdenum atom for dinitrogen reduction by nitrogenase. Close structural analogues of *R*-homocitrate bound to the molybdenum atom allow reduction of acetylene or protons but not dinitrogen. In model complex chemistry the electron richness of the FeMoco at a metal site can be varied by altering the ligands. This possibility is not available to the enzyme and so it modulates the activity of the FeMoco cluster through subtle hydrogen bonding interactions.

Acknowledgements

The authors gratefully acknowledge support from John Innes Foundation and the Biotechnology and Biological Sciences Research Council and the help of Dr D.M. Lawson and Suzy Mayer in preparing Figs. 2(a and b).

References

[1] B.K. Burgess, D.J. Lowe, Chem. Rev. 96 (1996) 2983.
[2] J.B. Howard, D.C. Rees, Chem. Rev. 96 (1996) 2965.
[3] D.J. Evans, R.A. Henderson, B.E. Smith, in: J. Reedijk, E. Bouwman (Eds.), Bioinorganic Catalysis, 2nd edition, Marcel Dekker, New York, 1999, pp. 153–208.
[4] V.K. Shah, W. Brill, Proc. Natl. Acad. Sci. USA 74 (1977) 3249.
[5] R.M. Allen, R. Chatterjee, M.S. Madden, P.W. Ludden, V.K. Shah, Crit. Rev. Biotech. 14 (1994) 225.
[6] J. Kim, D.C. Rees, Science 257 (1992) 1677.
[7] J.T. Bolin, N. Campobasso, S.W. Muchmore, T.V. Morgan, L.E. Mortenson, in: E.I. Stiefel, D. Coucouvanis, W.E. Newton (Eds.), Molybdenum Enzymes, Cofactors and Model Systems, ACS Symposium Series 535, American Chemical Society, Washington, DC, 1993, pp. 186–195.

[8] J.W. Peters, M.H.B. Stowell, S.M. Soltis, M.G. Finnegan, M.K. Johnson, D.C. Rees, Biochemistry 36 (1997) 1181.
[9] S.M. Mayer, D.M. Lawson, C.A. Gormal, S.M. Roe, B.E. Smith, submitted for publication.
[10] I.J. Dance, Chem. Soc. Chem. Commun. (1997) 165.
[11] T.R. Hawkes, P.A. McLean, B.E. Smith, Biochem J. 217 (1984) 317.
[12] L. Zheng, R.H. White, D.R. Dean, J. Bacteriol. 179 (1997) 5963.
[13] J. Liang, M. Maden, V.K. Shah, R.H. Burris, Biochemistry 29 (1990) 8577.
[14] P.A. McLean, R.A. Dixon, Nature 292 (1981) 655.
[15] F.K. Yousafzai, M. Buck, B.E. Smith, Biochem. J. 318 (1996) 111.
[16] W.E. Newton, D.R. Dean, in: E.I. Stiefel, D. Coucouvanis, W.E. Newton (Eds.), Molybdenum Enzymes, Cofactors and Model Systems, ACS Symposium Series 535, American Chemical Society, Washington DC, 1993, pp. 216–230.
[17] J. Shen, D.R. Dean, W.E. Newton, Biochemistry 36 (1997) 4884.
[18] D.J. Scott, H.D. May, W.E. Newton, K.E. Brigle, D.R. Dean, Nature 343 (1990) 188.
[19] D.J. Scott, D.R. Dean, W.E. Newton, J. Biol. Chem. 267 (1997) 20002.
[20] C.-H. Kim, W.E. Newton, D.R. Dean, Biochemistry 34 (1995) 2798.
[21] B.K. Burgess, Chem. Rev. 90 (1990) 1377 and references therein.
[22] B. Hedman, P. Frank, S.F. Gheller, A.L. Roe, W.E. Newton, K.O. Hodgson, J. Am. Chem. Soc. 110 (1988) 3798.
[23] S.-S. Yang, W.-H. Pan, G.D. Friesen, B.K. Burgess, J.L. Corbin, E.I. Stiefel, W.E. Newton, J. Biol. Chem. 257 (1982) 8042.
[24] M.A. Walters, S.K. Chapman, W.H. Orme-Johnson, Polyhedron 5 (1986) 561.
[25] J. Rawlings, V.K. Shah, J.R. Chisnell, W.J. Brill, R. Zimmermann, E. Münck, W.H. Orme-Johnson, J. Biol. Chem. 253 (1978) 1001.
[26] B.K. Burgess, E.I. Stiefel, W.E. Newton, J. Biol. Chem. 255 (1980) 353.
[27] I. Harvey, R.W. Strange, R. Schneider, C.A. Gormal, C.D. Garner, S.S. Hasnain, R.L. Richards, B.E. Smith, Inorg. Chim. Acta 275 (1998) 150.
[28] S.D. Conradson, B.K. Burgess, W.E. Newton, A. Di Cicco, A. Filipponi, Z.Y. Wu, C.R. Natoli, B. Hedman, K.O. Hodgson, Proc. Natl. Acad. Sci. USA 91 (1994) 1290.
[29] S.D. Conradson, B.K. Burgess, R.H. Holm, J. Biol. Chem. 263 (1988) 13743.
[30] A.J. M. Richards, D.J. Lowe, R.L. Richards, A.J. Thomson, B.E. Smith, Biochem. J. 297 (1994) 373.
[31] B.E. Smith, P.E. Bishop, R.A. Dixon, R.R. Eady, W.A. Filler, D.J. Lowe, A.J.M. Richards, A.J. Thomson, R.N.F. Thorneley, J.R. Postgate, in: H.J. Evans, P.J. Bottomley, W.E. Newton (Eds.), Nitrogen Fixation Research Progress, Nijhoff, Dordrecht, 1985, pp. 597–603.
[32] H.I. Liu, A. Filipponi, N. Gavini, B.K. Burgess, B. Hedman, A. Di Cicco, C.R. Natoli, K.O. Hodgson, J. Am. Chem. Soc. 116 (1994) 2418.
[33] S.D. Conradson, B.K. Burgess, S.A. Vaughn, A.L. Roe, B. Hedman, K.O. Hodgson, R.H. Holm, J. Biol. Chem. 264 (1989) 15967.
[34] K.L.C. Grönberg, C.A. Gormal, B.E. Smith, R.A. Henderson, J. Chem. Soc. Chem. Commun. (1997) 713.
[35] K.L.C. Grönberg, R.A. Henderson, J. Chem. Soc. Dalton Trans. (1996) 3667 and references therein.
[36] V.K. Shah, J.R. Chisnell, W.J. Brill, Biochem. Biophys. Res. Commun. 81 (1978) 232.
[37] C.E. McKenna, J.B. Jones, H. Eran, C.W. Huang, Nature 280 (1979) 611.
[38] C.E. McKenna, P.J. Stephens, H. Eran, G.-M. Luo, F.X. Zhang, M. Ding, H.T. Nguyen, in: C. Veeger, W.E. Newton (Eds.), Advances in Nitrogen Fixation Research, Nifhoff, Junk, 1984, pp. 115–122.
[39] T.A. Bazhenova, M.A. Bazhenova, S.A. Mironova, G.N. Petrova, A.K. Shilova, N.I. Shuvalova, A.E. Shilov, Inorg. Chim. Acta 270 (1998) 221.
[40] T.A. Bazhenova, M.A. Bazhenova, G.N. Petrova, A.K. Shilova, A.E. Shilov, Russ, Chem. Bull. 47 (1998) 861.
[41] F.A. Schultz, S.F. Gheller, B.K. Burgess, S. Lough, W.E. Newton, J. Am. Chem. Soc. 107 (1985) 5364.

[42] F.A. Schultz, S.F. Gheller, W.E. Newton, Biochem. Biophys. Res. Commun. 152 (1988) 629.
[43] T. Le Gall, S.K. Ibrahim, C.A. Gormal, S.A. Fairhurst, B.E. Smith, C.J. Pickett, unpublished work.
[44] K.L.C. Grönberg, C.A. Gormal, M.C. Durrant, B.E. Smith, R.A. Henderson, J. Am. Chem. Soc. 120 (1998) 10613.
[45] D.L. Hughes, S.K. Ibrahim, C.J. Pickett, G. Querne, A. Laouenan, J. Talarmin, A. Queiros, A. Fonseca, Polyhedron 13 (1994) 3341.
[46] C.J. Pickett, J. Bioinorg. Chem. 1 (1996) 601.
[47] R.L. Richards, Coord. Chem. Rev. 154 (1996) 83.
[48] W.E. Newton, S.F. Gheller, B.J. Feldman, W.R. Dunham, A. Schultz, J. Biol. Chem. 264 (1989) 1924.
[49] F.A. Schultz, S.F. Gheller, B.J. Feldman, W.E. Newton, in: H. Bothe, F.J de-Bruijn, W.E. Newton (Eds.), Nitrogen Fixation: Hundred Years After, Gustav Fischer, Stuttgart, 1988, pp. 121–126.