The Iron–Molybdenum Cofactor of Nitrogenase

BARBARA K. BURGESS

Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717

Received July 18, 1990 (Revised Manuscript Received September 20, 1990)

Contents

Barbara K. Burgess was born in Cleveland and is a graduate of Purdue University (B.S., Ph.D.). She served on the research staff at the Charles F. Kettering Research Laboratory in Yellow Springs, OH. Since 1984 she has been at the University of California Irvine where she is currently a Professor of Molecular Biology and Biochemistry. Her research interests are in the area of bioinorganic chemistry, with emphasis on the structural organization, function, and reactivity of the protein-bound [FeS] and [MoFeS] clusters in nitrogenase and ferredoxin. She has two sons, Tristan and Erin.

I. Introduction

Nitrogenase is the catalytic component of the biological nitrogen-fixation system which reduces atmospheric N_2 to ammonia. As shown in Figure 1 the enzyme is actually composed of two separately purified proteins called the iron protein (Fe protein) and the molybdenum–iron protein (MoFe protein). The Fe protein is a M_r 60 000 dimer of identical subunits and is encoded by the *nif*H gene. It contains a single $[Fe_4S_4]$ cluster bridged between the subunits and it functions as a specific one-electron donor to the MoFe protein. No other protein or artificial electron donor has ever successfully replaced the Fe protein in this capacity. This unique property of the Fe protein can be attributed, at least in part, to the fact that it binds two molecules of MgATP and that the hydrolysis of that MgATP is tightly coupled to electron transfer from the

0009-2665/90/0790-1377$09.50/0

TABLE 1. **Modifications to Original Acid-Treated Method of FeMo-Cofactor Isolation**

method	reagents used for: unfolding and precipitation	precipitate washing	FeMo-cofactor extraction (base)	ref
1a. citrate/phosphate	0.1 M citrate 0.2 M Na_2HPO_4	DMF	NMF (Na_2HPO_4)	1
b. citrate/phosphate, pH 2	citrate to pH 2 Na_2HPO_4 to pH 5	DMF (Na_2HPO_4)	NMF	5
2. HCl/NaOH	0.1 M HCl 0.1 M NaOH	DMF	NMF (NaOH)	6
3. acidic NMF	0.1 M citrate 0.2 M Na_2HPO_4	acidic NMF	NMF (Na_2HPO_4)	6
4. formamide	0.1 M citrate 0.2 M Na_2HPO_4	DMF	$HCONH_2$ (Na_2HPO_4)	6
5. phosphate	0.33 M H_3PO_4 0.20 M Na_2HPO_4	DMF	NMF (Na_2HPO_4)	6
6. $S_2O_4^{2-}$ free[a]	0.1 M HCl 0.1 M NaOH	DMF	NMF (NaOH)	6
7. DMF	0.1 M citrate 0.2 M Na_2HPO_4	DMF	DMF ($[Et_4N]_2[S_2O_4]$); ($[Et_4N][OH]$) (2 mM)	13
8. MeCN	0.1 M citrate 0.2 M Na_2HPO_4	DMF	MeCN ($[Et_4N]_2[S_2O_4]$); ($[Et_4N][OH]$) (50 mM)	13
9. DMSO	DMSO	DMF	NMF (Na_2HPO_4)	5
10. pyrrolidinone	0.1 M citrate 0.2 M Na_2HPO_4	DMF	pyrrolidinone	7

[a] Contains no $S_2O_4^{2-}$. All other solvents are 1.2 mM in $Na_2S_2O_4$.

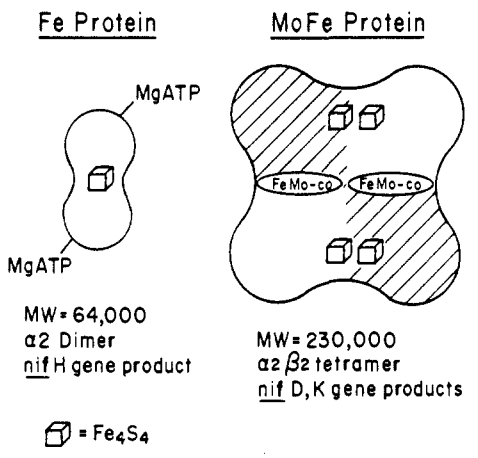


Figure 1. Schematic representation of Mo nitrogenase showing the two component proteins and their metal centers. The $[Fe_4S_4]$-type clusters in the MoFe protein (called P-clusters) are unusual relative to other protein-bound $[Fe_4S_4]$ clusters.

Fe protein to the MoFe protein during turnover. The MoFe protein contains the actual site of N_2 reduction. It is a 2α (*nif*D-encoded) and 2β (*nif*K-encoded) tetramer, M_r approximately 220 000 containing two Mo, 32 ± 3 Fe, and a similar number of S^{2-} atoms per molecule. These metal atoms are believed to be arranged into six discrete metal centers, four unusual $[Fe_4S_4]$-type clusters (called P-clusters) and two iron–molybdenum cofactor (FeMo-cofactor) centers. The structure of the FeMo-cofactor is not known, but it is believed to contain the substrate-reducing site of nitrogenase.

In 1977 a major breakthrough occurred in the nitrogen-fixation field with the successful isolation of FeMo-cofactor from the MoFe protein.[1] This review covers what has been learned since then about the structural organization, function, and biosynthesis of this metal cluster.

II. Isolation and Identification

A. Isolation Methods

1. Isolation Methods Which Use Acid Treatment

The original isolation of FeMo-cofactor in 1977 involved acid treatment of purified MoFe protein followed by extraction of the resulting protein pellet with NMF.[1] Because both the MoFe protein and FeMo-cofactor are oxygen sensitive, the procedure was carried out under anaerobic conditions. Although this general acid-treatment procedure has not changed significantly since 1977, each experimental step has been studied in detail and modified when appropriate. Specific modifications to each step are summarized in Table 1 and are described in the following section.

a. Starting Material. The starting material for FeMo-cofactor isolation is the MoFe protein of nitrogenase. While the MoFe protein is usually highly purified,[1] FeMo-cofactor has been successfully isolated from fairly crude protein preparations.[2,3] FeMo-cofactor has been isolated with MoFe proteins obtained from *Azotobacter vinelandii*, *Clostridium pasteurianum*, *Klebsiella pneumoniae*, *Bacillus polymyxa*, and *Rhodospirillum rubrum* and appears to be identical regardless of bacterial source.[1] The MoFe protein preparations used as starting material in different laboratories may vary in concentration and in the type and pH of the buffer used. For example, a standard *A. vinelandii* MoFe protein preparation would be approximately 15–50 mg/mL in 0.025 M Tris-HCl, pH 7.4, 0.25 M in NaCl, and 1.2 mM in $Na_2S_2O_4$,[1,4] whereas *K. pneumoniae* MoFe protein is generally purified in 0.25 M Tris-HCl, pH 8.7, 0.1 mg/mL in dithiothreitol, and 1.0 M in $Na_2S_2O_4$.[5]

b. Acid Treatment. The purpose of the acid-treatment step is to unfold the MoFe protein. This step also results in the destruction of the P-clusters contained within that protein. It is essential that the acid-

treatment step be carried out in an ice bath. Where necessary, and prior to the addition of acid, the protein is diluted with H_2O (containing 1.2 mM $Na_2S_2O_4$) to a concentration of about 5 mg/mL.[1,4] In the original isolation method the protein was acidified by adding a citric acid solution to a final concentration of 15 mM.[1] The pH of that solution was later estimated to be 2.8 in a study of the effects of pH on yields of FeMo-cofactor.[5] That study also revealed that the optimum pH for the acid treatment step was 2.0–2.2 with yields decreasing steeply below pH 2.0. H_3PO_4 and HCl have been shown to be as effective as citric acid in FeMo-cofactor isolation.[6] Thus, it is the pH and not the type of acid that is important. The acid solutions are made 1.2 mM in $Na_2S_2O_4$ *immediately* prior to their addition to the protein because $Na_2S_2O_4$ decomposes in the acid to give a cloudy, white solution within 1–2 min after its addition.

Published procedures for FeMo-cofactor isolation generally involve addition of a fixed amount of acid.[1,4-6] Since the amount of buffer present varies from preparation to preparation, it is more convenient to monitor the pH continuously during titration to pH 2.2. The MoFe protein undergoes two phase transitions during this titration. At the starting pH of 7.4 the MoFe protein is dark brown and in solution. As the pH is lowered to the region of the isoelectric point (pH 5.6–5.0) the protein precipitates to give a cloudy, gray-brown solution. When the pH is further decreased, the protein goes back into solution. At pH 2.2 the solution is green-brown and the color is much lighter than that of the starting material. The color change observed during the acid treatment step is believed to be due to the decomposition of the P-clusters of the MoFe protein to give H_2S and presumably ferrous citrate.[1] Like the P-clusters, FeMo-cofactor contains acid-labile sulfide (vide infra); however, FeMo-cofactor is not decomposed during the acid-treatment step. Since isolated FeMo-cofactor decomposes rapidly under similar conditions, the protein must somehow protect FeMo-cofactor from acid. This protection is not absolute: the MoFe protein solutions remain at pH 2.0–2.8 for only 2–3 min;[1,4-6] longer exposure to acid does result in the decomposition of the FeMo-cofactor.

Following the 2–3 min acid-treatment step, the pH of the solution is raised by addition of base until the isoelectric point of pH 5.0–5.5 is reached and the protein precipitates to give a cloudy, gray-brown solution. Although Na_2HPO_4 is the most commonly used base, NaOH is equally effective[1,4-6] and it is likely that other bases would work as well. As with the addition of acid, the base solutions are made 1.2 mM in $Na_2S_2O_4$ immediately prior to their addition to the protein.[1,4-6] In the original isolation the solution remained at pH 5.0 for 25 min prior to centrifugation and no reports have appeared in which this time period was varied.[1] FeMo-cofactor bound to denatured protein is thus stable in aqueous solution at pH 5.0 for fairly long periods of time. Following the 25-min incubation at pH 5.0–5.5, the protein is centrifuged. The original isolation method reported a centrifugation speed of 8000*g*.[1] However, this speed results in a gray, claylike pellet which does not yield significant FeMo-cofactor on subsequent extraction with NMF, and lower speeds of 120–1500*g* are now used by many investigators.[5-7] Following centrifugation the colorless supernatant solution is discarded. This solution contains about half of the iron originally associated with the MoFe protein. This iron is believed to arise from the decomposition of the P-clusters.[1]

c. Washing with DMF. Because isolated FeMo-cofactor is unstable in aqueous solutions, the protein pellet is washed at 4 °C two times with freshly distilled DMF.[1] Washing consists of resuspending the protein pellet in DMF for as brief a time as possible, usually for approximately 5 s, followed by centrifugation.[1,4-7] Again the original method reported a centrifugation speed of 8000*g*,[1] which does not yield significant FeMo-cofactor upon subsequent extraction with NMF. Consequently, lower speeds are used by many investigators.[5-7] The DMF is made 1.2 mM in $Na_2S_2O_4$ immediately prior to its use even though $Na_2S_2O_4$ has low solubility in DMF. The DMF that is added to the protein is cloudy due to dithionite precipitation. However, the DMF supernatant formed after centrifugation is clear, indicating that dithionite (or its decomposition products) remains with the protein pellet, where it may be solubilized by subsequent NMF extraction.

Varying reports have appeared concerning FeMo-cofactor extraction into DMF during this step ranging from no extraction[1] to extraction of about 10% of the total FeMo-cofactor.[5] If no extraction occurs the supernatant is colorless whereas FeMo-cofactor extraction leads to a brown supernatant. In our hands one centrifuge tube out of eight presumably identical ones in a single prep may be brown while the others remain colorless. Although the reason for this is unknown, the amount of FeMo-cofactor extracted at this step can be reduced by keeping the temperature at 4 °C throughout and by minimizing the time the protein pellet spends in contact with DMF. No reports have appeared on the complete characterization of FeMo-cofactor extracted during the DMF wash step. The pellet at the end of this step is a much darker brown color than the aqueous pellet following the acid-treatment step.

d. Extraction in NMF. The final step in FeMo-cofactor isolation is the extraction of the DMF-washed pellet with freshly distilled NMF.[1] This step involves vigorous vortexing of the NMF-suspended protein pellet for 5 min, followed by centrifugation, and may be carried out successfully at room temperature at 4 °C.[4-7] FeMo-cofactor is in the supernatant solution which is removed so that the pellet can be extracted a second time with NMF. Following the second (or in some cases third[1]) extraction and centrifugation steps the light gray to white protein pellet is discarded and the supernatant solutions are pooled. As with all steps in FeMo-cofactor isolation the NMF is made 1.2 mM in $Na_2S_2O_4$ immediately prior to use. The dithionite is added as an aqueous solution and thus the NMF contains about 1% H_2O. Dithionite is soluble at this concentration in NMF.

The original isolation method used vacuum-distilled NMF from Aldrich which was 5 mM in Na_2HPO_4.[1] Subsequently it was discovered that NMF treated in the same manner but purchased from Eastman Organic Chemicals was not effective in FeMo-cofactor extraction.[6] Further, Aldrich NMF which had been used once for FeMo-cofactor extraction and was later redistilled

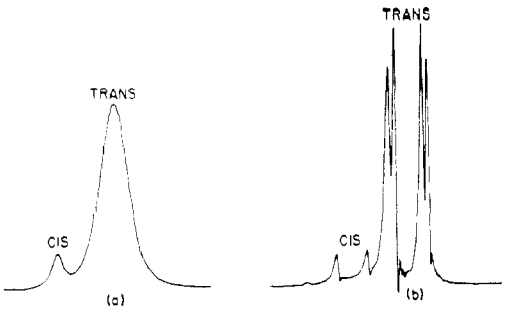


Figure 2. ^1H nuclear magnetic resonance spectra in the methyl region of neat NMF in the presence of base (a) and acid (b).[6] Form a is effective in FeMo-cofactor extraction while form b is not.

was also ineffective in FeMo-cofactor isolation.[6] These puzzling observations led to an investigation of the ^1H NMR spectral properties of these different samples of NMF.[6] As shown in Figure 2 the ^1H NMR spectrum of NMF which extracts FeMo-cofactor differs markedly from that of NMF which does not extract cofactor. Effective NMF shows two broad singlets for the methyl protons which are assigned to the trans (A) and cis (B)

isomers (Figure 2a).[6] In contrast, NMF which does not extract FeMo-cofactor shows a well-resolved doublet for the methyl protons of the cis isomer and a sharp doublet of doublets for the trans isomer (Figure 2b).

The doublet of doublets in the trans isomer arises from coupling of the methyl protons to both the N–*H* and C–*H* protons. In the cis isomer, the coupling to the C–*H* proton is very small and the doublet arises because only the N–*H* coupling is being observed. Under sufficiently basic conditions the proton on the nitrogen undergoes exchange, giving rise to the broadening of the methyl peaks in both isomers. Prior reports, unrelated to FeMo-cofactor, had shown that NMF plus a trace amount of base gave the spectrum shown in Figure 2a, while the addition of acid gave the spectrum shown in Figure 2b.[8,9] This led Yang et al. to investigate the apparent pH of the different samples of NMF.[6]

The apparent pH of NMF which is effective in FeMo-cofactor isolation is generally higher than 8 when measured after 1:5 dilution with water. The apparent pH of the ineffective NMF similarly diluted is generally lower than 5.[6] It thus appears that the ability of NMF to extract FeMo-cofactor depends on its "acidity" or "basicity". Ineffective (acidic) NMF can be converted to effective NMF simply by treatment with base. Sodium hydroxide has been used in this treatment because dibasic sodium phosphate is not sufficiently soluble in NMF to serve this function.[6] However if too much base is added, the protein pellet will also dissolve in NMF. In order to routinely obtain NMF of the proper "basicity" for FeMo-cofactor extraction, regardless of source, the NMF can be stirred overnight with sodium bicarbonate followed by filtration and distillation.[10] NMF treated in this way, without further addition of any base, never fails to extract FeMo-cofactor.

NMF solutions of FeMo-cofactor were originally reported to be brown.[1] In our hands the solutions always appear to be green-brown and others have reported them to be green.[3,7] Occasionally during the DMF or NMF steps the protein pellet and solution turn pink. This can usually be traced to oxygen contamination and these tubes are discarded.

e. Concentration. A major problem in working with FeMo-cofactor is that the final NMF solution contains only about 50 μM FeMo-cofactor. Because FeMo-cofactor is in NMF, it cannot be concentrated using usual biochemical techniques. In spite of the low vapor pressure and high boiling point of NMF, a vacuum distillation method has been developed for FeMo-cofactor concentration.[4] In this method FeMo-cofactor is placed with a stir bar in a 200-mL round-bottom flask which is connected to a 500-mL round-bottom liquid-N_2 trap using a short glass tube with 24/40 joints. The trap is connected to a high-vacuum line (10^{-7} atm) through a glass stopcock adaptor. During concentration, the flask containing FeMo-cofactor is placed in a water bath at room temperature or 30 °C.[3] FeMo-cofactor can be concentrated 10-fold in 1 h by using this method.[4] The highest FeMo-cofactor concentrations that have been reported by using this method are of the order of 1–2 mM.[11] It is not clear why higher concentrations have not been obtained. One reason may be the limited amount of material available. An additional problem is that the NMF solutions contain contaminating protein and high concentrations of salts. These species, which are present in much larger quantities than FeMo-cofactor, precipitate during concentration.

Another very different procedure for concentration involves absorbing FeMo-cofactor onto an anion-exchange column.[10] For this method a column of DEAE-cellulose in NMF is prepared. The DEAE-cellulose is in the acetate and not the chloride form. After washing the column with $Na_2S_2O_4$ in NMF, dilute FeMo-cofactor is loaded onto the column. Following further washing of the column with NMF the FeMo-cofactor is eluted as a single intense green-brown band with 0.1 M $[Et_4N]Br$ in NMF.[10] In this way FeMo-cofactor has been concentrated to about 1 mM.

2. Isolation in NMF without Acid Treatment

Although acid treatment is still used by many laboratories for the initial steps in FeMo-cofactor isolation, it is not essential. An early report demonstrated that FeMo-cofactor could be extracted from protein pellets produced by precipitation with DMSO, $(NH_4)_2SO_4$, or by treatment at pH 5.0 directly.[5] Very recently a method for FeMo-cofactor isolation has been developed which eliminates the need for the acid-treatment step and greatly simplifies the isolation process.[12] In this procedure the MoFe protein in buffer is first adsorbed onto DEAE-cellulose. The DEAE-cellulose-bound protein can then be unfolded in place by washing the column with NMF, DMF, or a 9:1 DMF/NMF mixture, stirring the column material, and allowing it to stand for a few minutes in the presence of the denaturing agent. This procedure uses $[(Bu_4N)]_2[S_2O_4]$ in place of $Na_2S_2O_4$. FeMo-cofactor is eluted by washing the column with 0.2–0.5 M $[Et_4N]Br$ or $[Et_4N]Cl$ in NMF. The denatured MoFe protein remains bound to the column throughout this procedure.

3. Isolation in Other Solvents

NMF is not the only solvent that has been used to successfully extract FeMo-cofactor. Base-treated $HCONH_2$,[6,7] pyrrolidinone,[7] base-treated DMF containing 5 mM $[Et_4N]_2[S_2O_4]$,[13] and base-treated CH_3CN containing 5 mM $[Et_4N]_2[S_2O_4]$[13] have all been reported to be effective in FeMo-cofactor extraction from acid-denatured pellets. Attempts to take NMF-isolated FeMo-cofactor solutions to dryness and then redissolve the resulting tacky, brown precipitate in solvents other than NMF have met with more limited success. One report suggested that FeMo-cofactor loses significant activity when taken to dryness even when redissolved in NMF.[13] However, others have recently reported successfully dissolving "dried" FeMo-cofactor in CH_3CN, CH_3COCH_3, CH_2Cl_2, THF, and C_6H_6.[14] One advantage of this method may be that higher concentrations of FeMo-cofactor can be obtained and concentrations as high as 10 mM have been reported for FeMo-cofactor redissolved in DMF or CH_3CN.[14]

Recently Wink et al. have discovered that the key to obtaining FeMo-cofactor solutions in solvents other than NMF appears to be the presence of a suitable counterion.[14] They demonstrated that when DEAE-cellulose-bound MoFe protein was first unfolded with DMF, FeMo-cofactor could subsequently be eluted from the column in a variety of solvents provided the appropriate counterion was present. Solvents used include the following: DMF/0.1 M $[Bu_4N]Cl$ or $[Bu_4N]Br$, CH_3CN/0.1 M $[Bu_4N]Cl$, acetone/0.1 M $[Bu_4N]Cl$, CH_2Cl_2/0.1 M $[Bu_4N]Cl$, THF/0.1 M Bu_4NBr, and benzene/0.1 M $[Bu_4N]Br$.

The successful isolation of FeMo-cofactor in solvents other than NMF and at concentrations greater than 1–2 mM should greatly facilitate attempts to crystallize FeMo-cofactor, which have so far been unsuccessful. The ability of one solvent versus another to extract FeMo-cofactor from the MoFe protein also gives information about possible ligands to FeMo-cofactor which is more appropriately discussed in section V.B.3.

4. Yields

Yields of FeMo-cofactor are defined as the percentage of the Mo present originally in the MoFe protein that is recovered in NMF. The original isolation method gave a total yield of 87% for three repeated NMF extractions of the same protein pellet.[1] Smith reported much lower yields using the same method although he did obtain similar yields by decreasing the pH during the acid-treatment step.[5] Yields of 60–70% were obtained for a single NMF wash using the same procedure, increasing to a 75–85% total yield upon a second extraction.[4,6] For the method which uses HCl/NaOH instead of citrate/phosphate, lower yields of 35% for a first wash were originally reported.[6] These yields are increased to 75–85% for a total of two washes by continuous titration to pH 2.2 during the acid treatment instead of batch addition of HCl without monitoring the pH. Low yields were also reported for a preparation in which $Na_2S_2O_4$ was excluded from each step.[6]

For extraction of acid-denatured protein pellets with different solvents, base-treated $HCONH_2$ has been reported to give yields equivalent to NMF[6] or much lower than NMF.[2] This apparent discrepancy might be due to the relative "basicity" of the $HCONH_2$ used in the two procedures. Pyrrolidinone was reported to give yields equivalent to those obtained with NMF.[7] Variable yields were reported for extraction in DMF containing 5 mM $[Et_4N]_2[S_2O_4]$ and 2.0 mM $[Et_4N][OH]$.[13] When FeMo-cofactor was isolated in NMF from denatured, DEAE-cellulose-bound MoFe protein, yields were comparable (ca. > 70%) to those of the acid-treatment methods.[12] If solvents other than NMF were used to extract FeMo-cofactor from the column, yields were variable.[14] CH_3CN and DMF gave yields comparable to NMF while THF and C_6H_6 gave the lowest yields, possibly due to the lower solubility of the counterions in those solvents.

It should be noted that even a 100% yield is not very much FeMo-cofactor when compared to the quantities of synthetic inorganic clusters that are routinely synthesized. In our hands, 1 kg (~500 L) of *A. vinelandii* cells yields ~1 g of purified MoFe protein which in turn yields about 6–7 mg of FeMo-cofactor.[4] This limitation in the amount of material available is one reason that FeMo-cofactor is not yet adequately characterized.

5. Purification

FeMo-cofactor solutions isolated by the original method[1] potentially contain a variety of chemical species including Tris, Cl^-, Na^+, HPO_4^{2-}, citrate, $S_2O_4^{2-}$ (and its oxidation products), DMF, NMF, water, residual protein, and adventitious iron and/or molybdenum. Two general approaches have been used to minimize the number of chemical species contaminating FeMo-cofactor solutions. One approach is to change the isolation method and the other is to add a purification step following isolation and concentration.

Recent reports have appeared describing new isolation procedures which used partially purified MoFe protein as starting material.[3,12-15] In one case a thorough analysis of the resulting FeMo-cofactor solution revealed that only about one-seventh of the total Mo present was FeMo-cofactor-associated.[3] The rest was associated with an unidentified contaminating species. Thus, to maximize the purity of FeMo-cofactor solutions it is probably advisable to maximize the purity of the MoFe protein starting material as well. A number of other modifications of the original FeMo-cofactor isolation procedure have been aimed at maximizing the purity of the product. For example, it is possible to eliminate tris and chloride from FeMo-cofactor preparations by dialyzing the starting material against water or some other buffer.[6] Although this procedure may be advisable if the FeMo-cofactor is to be used for compositional analysis, it is sufficiently tedious that it is rarely carried out in practice. Citrate and phosphate have also been eliminated by substituting HCl and NaOH, which at least eliminates one potential organic contaminant. This procedure is used routinely.[6] Although dithionite can be eliminated from the entire procedure, this greatly increases the difficulty of handling the FeMo-cofactor and is generally not done.[6] Only one report has appeared which eliminated the DMF wash step, replacing it with acidic NMF.[6] No thorough examination of the purity of FeMo-cofactor isolated in solvents other than NMF has been published. DMF is expected to yield a purer product at least with respect to dithionite contamination because of its low solubility in that solvent. CH_3CN, on the

other hand, may give FeMo-cofactor solutions with much higher levels of protein contamination.[13]

As indicated above only minor improvements on the purity of FeMo-cofactor are routinely obtained by variation of the isolation method. A more common approach is to purify FeMo-cofactor after it has been isolated in NMF. For example, during concentration by vacuum distillation a large amount of white precipitate is formed which can be removed by simple filtration. This precipitate contains contaminating protein and salts.[6] Some investigators have used gel filtration with Sephadex G-100 or G-25 in NMF to remove contaminating protein, salts, and excess Fe^{2+} from FeMo-cofactor solutions.[1,5,6] This method, however, may do more than simply remove contaminating species. Thus, NMF which has passed through a Sephadex G-100 column sometimes forms a gel when it is heated for subsequent distillation, indicating that some of the resin has dissolved in NMF.[6]

A more promising column technique is the anion-exchange method described in section II.A.1.e. This technique reportedly yields material which is much cleaner by the criterion of electrochemical analysis.[10] However, because FeMo-cofactor is eluted by $[Et_4N]$-Br[10] from the acetate form of an anion-exchange resin, this procedure introduces at least one and possibly two additional chemical species. A similar anion-exchange method has been developed to remove adventitious iron from FeMo-cofactor solutions.[12,14,17] In this procedure the cofactor is adsorbed onto a DEAE-cellulose column that has been equilibrated with DMF. The column is then washed with 2,2'-bipyridyl in DMF (to remove loosely associated Fe^{2+}) and the cofactor is eluted with DMF in high salt.[14] A major limitation of all DEAE-cellulose methods is that the final solutions contain high concentrations of salt, which for many purposes must be removed subsequently by gel filtration. Another method designed to remove adventitious Fe^{2+} used the chelator 1,10-phenanthroline to complex Fe^{2+} followed by treatment with a cation-exchange resin in order to remove the $[Fe(o\text{-phen})_3]^{2+}$ complex.[6] Unfortunately, FeMo-cofactor is not stable indefinitely in the presence of the residual 1,10-phenanthroline, so this method is not used routinely.[6]

B. Assay for Biological Activity

The most important criterion for the identification of FeMo-cofactor is its biological activity. To date this criterion has not been met by any of the numerous synthetic Mo–Fe–S complexes that are currently available.[18] The assay has two steps which are represented schematically in Figure 3.

1. Activation of MoFe Protein

The first step in the FeMo-cofactor assay is the addition of isolated FeMo-cofactor in NMF to a solution which contains an FeMo-cofactor-deficient MoFe protein.[1] Because the protein is missing the cofactor, it does not have any nitrogenase activity. The source of the FeMo-cofactor-deficient MoFe protein is usually a mutant bacterial strain of *A. vinelandii* or *K. pneumoniae* which is blocked at some step in FeMo-cofactor biosynthesis (section VII). The group who described the original isolation of FeMo-cofactor used an *A. vinelandii* strain designated University of Wisconsin-45

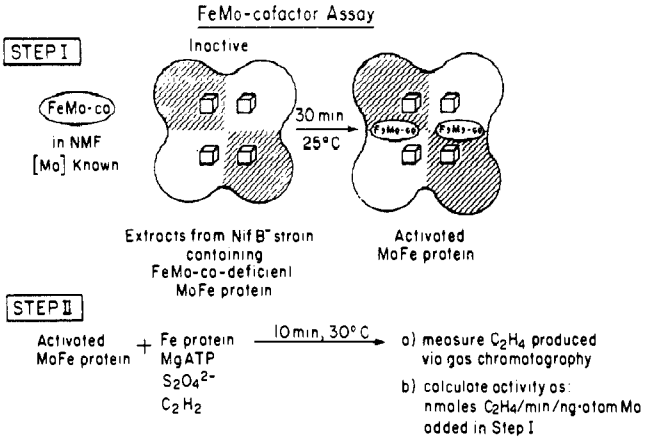


Figure 3. Schematic representation of the two portions of the biological activity assay for FeMo-cofactor.

(i.e., UW45).[1] This mutant was originally isolated by *N*-nitrosoguanidine mutagenesis[19] and has more recently been shown to have a defect in the FeMo-cofactor biosynthetic gene which is designated *nif*B.[20,21] A number of *K. pneumoniae* mutants with defects in the *nif*B gene (e.g. UN109,[1] UNF 1718)[22] are also commonly used for FeMo-cofactor assays. Occasionally *A. vinelandii* or *K. pneumoniae* strains with defects in other FeMo-cofactor biosynthetic genes are also used.[23]

The growth of FeMo-cofactor-deficient strains is complicated by the fact that they do not fix N_2 and hence cannot be grown under N_2-fixing conditions.[19] They are therefore first grown in the presence of a fixed-nitrogen source (e.g. ammonia). Unfortunately, under these conditions the cells do not synthesize the FeMo-cofactor-deficient MoFe protein. To derepress the synthesis of the desired protein the ammonia-grown cells are switched to nitrogen-free media. This can be accomplished either by harvesting the cells and resuspending them in fresh nitrogen-free media[24] or by letting the culture exhaust the initial ammonia supply.[4] The culture remains in the nitrogen-free medium for 3 h. During that time period the cells synthesize the MoFe protein polypeptides and presumably assemble them with P-clusters into an FeMo-cofactor-deficient MoFe protein which can then be used for FeMo-cofactor assays.

The FeMo-cofactor-deficient MoFe protein is generally not purified prior to the assay.[1] Rather, the cells are simply broken open and centrifuged briefly to remove cell debris. The resulting cell-free extract contains hundreds of proteins in addition to the FeMo-cofactor-deficient MoFe protein. It is not known to what extent, if any, these other proteins participate in the activation process.

For a typical assay equal aliquots of extracts containing the FeMo-cofactor-deficient MoFe protein are incubated with varying amounts of isolated FeMo-cofactor. Generally about 6 mg of extract protein would be mixed with 0–10 nmol of FeMo-cofactor.[1] The proportion of NMF is kept below 1% because NMF inhibits reconstitution.[4,5] This can be accomplished by diluting the FeMo-cofactor with buffer immediately before its addition to the protein.[4] After the FeMo-cofactor is added to the extracts containing the FeMo-cofactor-deficient MoFe protein, the mixture is incubated at room temperature for 30 min.[1] During this period it is assumed that a normal MoFe holoprotein

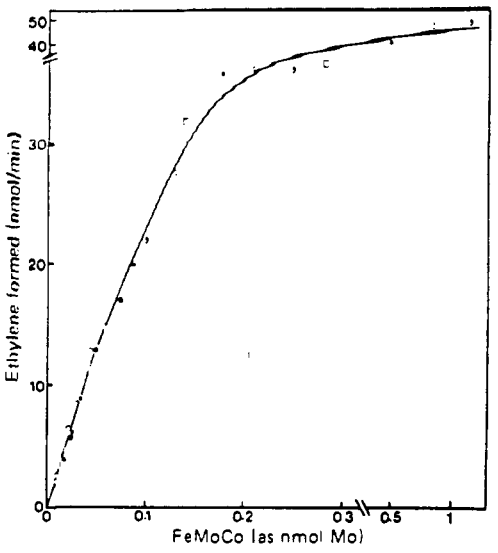


Figure 4. Ability of FeMo-cofactor to activate the inactive FeMo-cofactor-deficient MoFe protein present in extracts of the *A. vinelandii* Nif B⁻ strain, UW45.[1] The FeMo-cofactor was isolated from a variety of MoFe proteins purified from *C. pasteurianum* (○); *K. pneumoniae* (*); *B. polymyxa* (□); and *R. rubrum* (●).

is assembled as shown in Figure 3a. The time required to assemble the MoFe holoprotein, however, is much shorter than the 30 min which is generally used.[25]

2. Assay of the Activated MoFe Holoprotein

The second step in the FeMo-cofactor assay is to measure the ability of the activated MoFe holoprotein to reduce C_2H_2 to C_2H_4 as shown in Figure 3b.[1] The C_2H_2 reduction assay requires the Fe protein, MgATP, and $Na_2S_2O_4$ in addition to the activated MoFe protein. The assay is generally performed by adding the activated cell-free extracts to a reaction mixture containing purified Fe protein, MgATP, an ATP generating system, dithionite, and C_2H_2 followed by monitoring the appearance of C_2H_4.[1] Typical data from this type of assay are shown in Figure 4, which is a plot of nanomoles of C_2H_4 formed per minute versus the number of nanogram-atoms of Mo present as FeMo-cofactor. The amount of active MoFe holoprotein formed increases linearly with increased addition of FeMo-cofactor until a saturation point is reached (Figure 4). At this point all of the available FeMo-cofactor-deficient MoFe protein has been activated with FeMo-cofactor. The specific activity of the FeMo-cofactor is taken from the linear portion of the graph and is reported in terms of nanomoles of C_2H_4 formed per min per nanogram-atom of Mo.[1] Problems in quantifying this assay have been considered in detail.[5,26,27] The theoretical maximum for the specific activity of FeMo-cofactor is about 275 nmol of C_2H_2 min^{-1} (ng-atom of Mo)$^{-1}$ based on both the measured and the calculated maximum specific activities of the MoFe protein.[26] The unreasonably high activity of 430 nmol C_2H_4 min^{-1} (ng-atom of Mo)$^{-1}$ was originally reported for *A. vinelandii* FeMo-cofactor,[1] but this value was later revised to 275 by the same group.[28] Most investigators report values in the 200–275 range.[5-7,3-16,28]

Biological activity is the best criterion for the identification of FeMo-cofactor and the best test of its integrity. Therefore, it is important to consider situations where low activities might be obtained for reasons unrelated to the condition of the FeMo-cofactor. Such a situation arises if there is some species present in the FeMo-cofactor solution which binds to the FeMo-cofactor-deficient MoFe protein, preventing FeMo-cofactor from binding. For example, in addition to oxygen-degraded FeMo-cofactor, a subcomponent of FeMo-cofactor called the MoFe cluster,[29] MoS_4^{2-}, or the compounds $[(C_4H_5)_4P]_2MoS_4Fe(SC_6H_5)_2$ and $[(C_4H_5)_4P]_2MoS_4FeCl_2$ (which contain a MoS_4^{2-} core) all inhibit the activation portion of the FeMo-cofactor assay.[30] If partially purified MoFe protein is used as starting material for FeMo-cofactor isolation, the solutions contain a contaminating Mo species, of unknown origin, which also inhibits the first portion of the assay.[3] Because of their similarity to portions of FeMo-cofactor, these species are likely to bind specifically to the FeMo-cofactor site of the FeMo-cofactor-deficient MoFe protein. Thus, the ability of inorganic FeMo-cofactor model complexes to inhibit the reconstitution assay may be one measure of their similarity to FeMo-cofactor or portions of FeMo-cofactor. To date, no complete study of the inhibitory effects of model complexes has been undertaken.

Other chemical species might inhibit the activation portion of the assay nonspecifically by unfolding the protein or by binding to the protein at a site remote from the FeMo-cofactor site. NMF appears to be one such species as it interferes both with the activation and with the activity-measurement portions of the assays.[27] Thus, for any experiment where the addition of a solvent or chemical species to FeMo-cofactor leads to a decrease in activity it is important to demonstrate whether or not the reagent is affecting the FeMo-cofactor-deficient protein before concluding that the FeMo-cofactor itself has been damaged by the reagent. Finally, it is theoretically possible that the FeMo-cofactor could be modified by addition of a tight-binding ligand such that the FeMo-cofactor-deficient MoFe protein could not compete successfully.

III. Chemical Composition

A. Inorganic Composition

1. Molybdenum

During FeMo-cofactor isolation the Mo originally present in the MoFe protein is extracted into NMF. The amount of Mo present is generally quantified by using atomic absorption spectroscopy at 313 nm[31] with a graphite furnace or by a toluene-3,4-dithiol colorimetric method.[6,32] In either case the sample is digested overnight at 100 °C in acid prior to performing the assay. Evidence that the Mo is present as part of a cluster containing Fe and S atoms first came from Mo XAS experiments[33] which are discussed in section VI.E.

2. Iron

During FeMo-cofactor isolation approximately half of the iron originally associated with the MoFe protein is extracted into NMF.[1] Quantitation of Fe present is straightforward, involving acid digestion of the sample followed by either atomic absorption spectroscopy at 248.3 nm[31] with a graphite furnace or by colorimetric methods using bathophenanthroline.[34,35] However, because the MoFe protein contains P-clusters in ad-

TABLE 2. Analytical Values of Fe:Mo Ratio for Various FeMo-Cofactor Preparations

isolation method	concn	Fe:Mo	ref
original[1]	no	7.9	1
original[1]	no	8.2	31
citrate/phosphate, pH 2.0[5]	no	10.0	5
DMSO[5]	no	8.0	5
original[1]	vacuum distillation	6.9 ± 0.1	4
variations on original[6]	no	7.6 ± 0.4	6
HCl/NaOH[6]	DEAE	6.3 ± 0.5	10
DEAE/2,2'-bipyridyl wash[12]	DEAE	5.5 ± 0.5	17
DEAE/2,2'-bipyridyl in DMF[12]	DEAE	5.2 to 7.5	12
HCl/NaOH[6]	vacuum distillation	6.8 to 7.1	74
HCl/NaOH[6]	DEAE	6.1 ± 0.1	108
pyrrolidinone[7]	no	7	7

dition to FeMo-cofactor, it is possible that not all of the Fe extracted into NMF is FeMo-cofactor associated.

Table 2 gives representative analytical data for FeMo-cofactor preparations from a number of laboratories and reports the values in terms of a ratio of Fe to Mo. The original report of FeMo-cofactor isolation gave a value of 8Fe:1Mo.[1] Using the same method other laboratories reported both higher (10Fe:1Mo)[5] and lower (7Fe:1Mo)[4] values. Specific activities were similar for all preparations. The higher values were attributed to insufficient washing of the protein pellet, leading to the presence of adventitious Fe in the final NMF solution.[5] This argument has also been used to explain the observation that the first wash of the protein pellet gives ratios of Fe to Mo higher than those obtained for subsequent washes.[6]

Three arguments have been offered in favor of the 8Fe:1Mo stoichiometry. First, this ratio is obtained routinely by a number of groups (Table 2). Second, the elution profile of FeMo-cofactor from a size-exclusion, Sephadex G-100 column gave an 8Fe:1Mo ratio for all fractions which contained FeMo-cofactor.[1] Third, an Fe- and Mo-containing cluster has been isolated from the MoFe protein by acidified methyl ethyl ketone extraction, which has a lower ratio of 6Fe:1Mo.[29] Although this cluster, when transferred to NMF, has the same spectral properties as FeMo-cofactor, it has no biological activity in the *A. vinelandii* UW45 assay.[29] This has led to the suggestion that the two additional Fe atoms per Mo found in FeMo-cofactor solutions were an integral component of FeMo-cofactor and were required for biological activity.[29]

Alternative explanations have been offered for all three of the arguments listed above. First, lower ratios of 6–7Fe:1Mo have been reported by a number of groups (Table 1) and are generally obtained for more concentrated and purified samples.[6,10,17] These samples also have high specific activity. Second, a number of small molecules (e.g. Cl^-, HPO_4^{2-}) do comigrate with FeMo-cofactor on Sephadex G-100 and it is thus possible that adventitious Fe also comigrates.[6] Third, it is possible that the FeMo-cluster originally isolated in acidified methyl ethyl ketone which, when transferred to NMF, has the spectral properties of FeMo-cofactor[37] represents the true inorganic composition of FeMo-cofactor. Its inability to activate the FeMo-cofactor-deficient MoFe protein could then be explained if it were missing an essential organic component (section III.B.).

There is little doubt that FeMo-cofactor solutions do contain some adventitious Fe. The problem is in trying to determine if the adventitious Fe arises from the

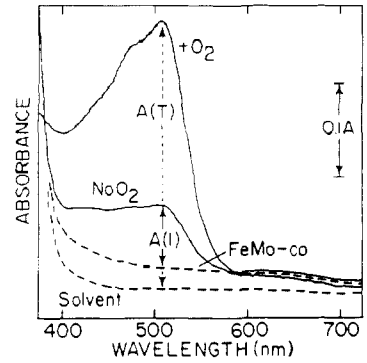


Figure 5. Visible spectral changes for the interactions of 1,10-phenanthroline with FeMo-cofactor.[6] 1,10-phenanthroline was added anaerobically to a FeMo-cofactor solution. A(I) is $[Fe(phen)_3]^{2+}$ formed immediately and is believed to represent adventitous iron. A(T) represented the total $[Fe(phen)_3]^{2+}$ formed after air oxidation. FeMo-cofactor associated iron is calculated from [A(T)] – [A(I)].

P-clusters originally in the MoFe protein or if it arises from decomposition of FeMo-cofactor after its isolation. To help resolve this issue methods have been developed to identify, quantitate, and remove adventitious Fe from FeMo-cofactor solutions. Attempts to remove Fe directly by binding it to a cation-exchange resin were unfortunately unsuccessful, indicating that the adventitious Fe present in FeMo-cofactor solutions is complexed in some way to give it a net neutral or negative charge.[6]

Figure 5 shows a simple assay that has been developed for quantifying the amount of adventitious Fe present in FeMo-cofactor solutions.[6] If 1,10-phenanthroline (or 2,2'-bipyridyl) is added to an FeMo cofactor solution, a variable percentage of Fe reacts immediately with the chelator without affecting the FeMo-cofactor reconstitution activity. If the FeMo-cofactor solution is then exposed to O_2, all reconstitution activity is lost and all of the Fe present in the solution reacts with the chelator (Figure 5).[6] Using this method Yang et al. have shown that there is an inverse correlation between the amount of easily complexed Fe in an FeMo-cofactor solution and the specific activity of the FeMo-cofactor. These results demonstrate that most of the easily complexed, adventitious Fe arises not from the P-clusters originally in the MoFe protein, but rather from the decomposition of some of the FeMo-cofactor in solution. Thus, unfortunately, this method does not resolve the question of whether 8Fe:1Mo or 6–7Fe:1Mo is the correct composition for FeMo-cofactor. The authors do point out that the solutions with the lowest percentage of easily complexed Fe and the highest specific activities give the lower ratio of 7Fe:1Mo.

To date the best analytical argument in favor of the lower 6–7Fe:1Mo ratio comes from recent reports of preparations that have been purified by anion-exchange chromatography. One report gave a ratio of 6.3 ± 0.5Fe:1Mo for an average of eight purified samples which contained less than 15% adventitious Fe by the 1,10-phenanthroline assay described above.[10] These samples had high specific activities and did not contain electrochemically active contaminating species. The other report gave a ratio of 5.5 ± 0.5Fe:1Mo for samples of high activity which had been treated with 2,2'-bipyridyl on an anion-exchange column to remove adventitious Fe.[17] Because the starting material for these

preparations was crystalline MoFe protein, which presumably did not contain any adventitious Mo, it is difficult to see how preparations with normal activity could have such low Fe:Mo ratios if the true ratio for active FeMo-cofactor is 8Fe:1Mo. Although the analytical data described above do not give a definitive Fe composition for FeMo-cofactor, they do point to a minimum stoichiometry of 6Fe:1Mo.

3. Sulfide

FeMo-cofactor was originally reported to contain six S^{2-} atoms per Mo.[1] This measurement was made on an "essentially dithionite-free" sample by using a standard methylene blue formation assay.[36] This general method was later criticized, however, because it failed to accurately measure the sulfide content in various [MoFeS] model compounds.[37] A new method was suggested and a subsequent report by a different group compared it directly to the methylene blue assay,[36,38] using FeMo-cofactor samples that had been isolated in the complete absence of dithionite.[6] Although these samples gave 3.9 ± 0.3 S^{2-} per Mo regardless of which method was used, in that study, both methods gave low values for model complexes of known S^{2-} content.

Because of the controversy surrounding these chemical methods and their inability to measure, accurately, the sulfide content of model compounds, other techniques were developed in attempts to determine the sulfur content of FeMo-cofactor. The simplest involved growing cells on radioactive $^{35}SO_4^{2-}$, purifying the MoFe protein, isolating and purifying its FeMo-cofactor, and measuring its ^{35}S content. The method measures total S and not just acid-labile S^{2-}. One group reported four S atoms per Mo using this method[39] although, it was later reported that this value was not reproducible and varied from 3.7 to 12.5 S per Mo.[26] The major problem with the technique was that it required an accurate knowledge of the quantity of ^{35}S, relative to total S in the sample. This problem was overcome by Nelson et al., who used proteins of known S composition as internal standards and arrived at a ratio of 8.7 ± 1 of non amino acid sulfur per Mo in FeMo-cofactor.[31] A very similar value of 8.82 ± 0.55S:1Mo was later obtained in a different way by inserting [^{35}S]FeMo-cofactor into purified FeMo-cofactor-deficient MoFe protein and then purifying and characterizing the resulting MoFe holoprotein.[26] Thus, there now appears to be agreement that the minimum stoichiometry is about 8–9S:1Mo. Because there is no evidence for organic S in isolated FeMo-cofactor, all endogenous S is presumed to be acid-labile S^{2-}.

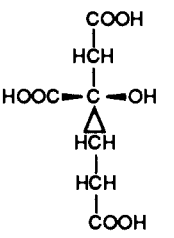
B. Organic Composition

Shortly after FeMo-cofactor was originally isolated attempts were made to establish whether or not it contained an endogenous organic component. Attention first focused on the possibility of a peptide component, but extensive independent investigations demonstrated that active, purified FeMo-cofactor solutions do not contain amino acids.[5,6,40] Attempts to identify sugars in isolated FeMo-cofactor also produced negative results[6] and an early report that lipoic acid and coenzyme A were part of FeMo-cofactor[41] was later shown to be erroneous.[6] These early experiments eliminated some obvious possibilities without providing definitive evidence either for or against an organic component.

The realization that FeMo-cofactor does contain an endogenous organic component has come slowly through a combined genetic/biochemical approach. The story began in 1981 with the characterization of mutant strains of *K. pneumoniae* which were defective in their ability to fix N_2 but were able to reduce C_2H_2.[42] This phenotype had previously been shown to be associated with a defect in the *nif*V gene[43,44] (see section VII for discussion of *nif* genes). The inability of Nif V⁻ strains to fix N_2 was first shown to be due to an alteration in the MoFe protein[42,45,46] and was later traced further to the FeMo-cofactor site of that protein.[47] Thus, when FeMo-cofactor isolated from purified Nif V⁻ MoFe protein was used to activate the FeMo-cofactor-deficient MoFe protein synthesized Nif B⁻ strains the resulting MoFe holoprotein was unable to fix N_2 but could reduce C_2H_2.[47] It was therefore of great importance to discover what structural feature of the Nif V⁻ FeMo-cofactor was responsible for its inability to fix N_2.

One approach to this problem was to isolate FeMo-cofactor from purified Nif V⁻ MoFe protein and subject it to all of the analytical procedures and spectroscopic probes (section IV) that had previously been used to characterize wild type FeMo-cofactor. It was thus established that (1) the Fe:Mo ratio was identical in wild type and Nif V⁻ FeMo-cofactor,[47] (2) the electron paramagnetic resonance (EPR) spectral properties were unchanged,[26] (3) the number and distances of Fe and S atoms observed by XAS to be close to the Mo were unchanged,[48,49] and (4) the low-temperature magnetic circular dichroism (MCD) spectral properties were unchanged.[26] This lack of evidence for a clear difference in the metal core of Nif V⁻ FeMo cofactor, when compared to the wild type, led Smith et al. to suggest that the difference might be due to some unknown endogenous organic component.[26] To date the only spectroscopic difference that has been demonstrated between the Nif V⁻ and wild type FeMo-cofactors is a change in the ^{95}Mo electron nuclear double resonance (ENDOR) spectra which shows that the Mo site is perturbed in the mutant cofactor.[49] Those data were interpreted to indicate that the Nif V⁻ FeMo-cofactor differs by addition, subtraction, or replacement of a non-sulfur ligand weakly bound at or near Mo[49] and were consistent with the suggestion[26] that the differences might be due to an alteration in an unknown organic component.

Proof that FeMo-cofactor has an organic component was finally obtained via the biochemical detective work of Hoover et al.[50–52] In 1986 they discovered that a low molecular weight factor was needed for FeMo-cofactor biosynthesis which was present in wild type cells but absent in Nif V⁻ cells.[50] This factor was later purified and identified as homocitric acid ((R)-2-hydroxy-1,2,4-



(*R*)-homocitric acid

butanetricarboxylic acid) with ^1H and ^{13}C nuclear magnetic resonance (NMR) and positive-ion fast atom bombardment (FAB) mass spectroscopy.[53]

Because Nif V$^-$ cells do not synthesize homocitrate, the *nif*V gene was proposed to code for a homocitrate synthetase.[53] Although confirmation of this proposal must await purification of the *nif*V gene product, it has been demonstrated that the addition of homocitrate to the media of Nif V$^-$ cells of *K. pneumoniae* during nitrogenase synthesis leads to the production of normal MoFe protein.[52] Presumably Nif V$^-$ cells should also be able to grow at wild-type rates under N_2-fixing conditions if homocitrate is added to the medium, although this experiment has not been reported.

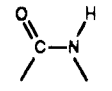
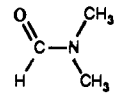
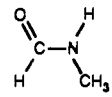
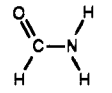
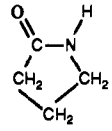
The specific function of homocitrate in FeMo-cofactor biosynthesis is currently unknown. The fact that it is excreted into the medium of N_2-fixing *K. pneumoniae* suggests that it may have a role in metal chelation, uptake, and/or initial processing.[51] Whatever its role in these early steps in FeMo-cofactor biosynthesis, it is now clear that homocitrate is also incorporated into the final FeMo-cofactor product to give a ratio of one homocitrate per Mo.[53] Thus, homocitrate has been released from purified MoFe protein and identified by ^1H NMR spectroscopy.[53] Homocitrate has not yet been isolated from purified FeMo-cofactor solutions. However, when ^3H-labeled homocitrate was added to an in vitro FeMo-cofactor biosynthesis system, the ^3H label remained with the FeMo-cofactor during its isolation from the resulting ^3H-labeled MoFe protein.[53] Taken together with the observation that the inability of the Nif V$^-$ MoFe protein to fix N_2 was due to a defect in its FeMo-cofactor,[47] these data provide very strong evidence that homocitrate is an endogenous organic component of FeMo-cofactor.

The observation that the MoFe protein isolated from Nif V$^-$ cells was still able to reduce C_2H_2 at reasonable rates led to the suggestion that it may have some other organic molecule (e.g. citrate) incorporated into its FeMo-cofactor in place of homocitrate.[51,53] Support for this idea came from the in vitro biosynthesis of FeMo-cofactor using organic acids other than homocitrate.[54,55] A number of these analogues (e.g. citrate, (*R*)-citramalate, *cis*-aconitate) were able to support the synthesis of MoFe proteins which could not reduce N_2 but which still had the C_2H_2-reducing ability of the Nif V$^-$ MoFe protein. Of those analogues, only citrate is likely to be present in high enough quantities in vivo to support the synthesis of an aberrant FeMo-cofactor and a preliminary report indicates that citrate has been isolated from Nif V$^-$ MoFe protein.[55]

C. Exogenous Ligands to Isolated FeMo-Cofactor

FeMo-cofactor solutions often contain a large number of chemical species (e.g. Cl^-, Na^+, citrate, HPO_4^{2-}, $S_2O_4^{2-}$ and its oxidation products, and NMF) in addition to the Mo, Fe, S^{2-}, and homocitrate that make up the FeMo-cofactor core cluster. One or more of these species are likely to serve as exogenous ligands to the metal atoms in isolated FeMo-cofactor, replacing the ligands that were originally supplied by the MoFe protein. Just as the chemical species present depend upon the isolation method used, the ligands may vary from preparation to preparation. This possibility was recognized early on by Smith et al., who showed that the apparent molecular weight of FeMo-cofactor varied from 800 to 1500, depending upon the isolation method.[5] That molecular weight range is slightly higher than the 900–1000 range calculated for a 1Mo:6–8Fe:8–9S:1homocitrate core, which is also consistent with the presence of exogenous ligands.

TABLE 3. Solvents That Have Been Used To Extract FeMo-Cofactor from Acid Denatured Protein Pellets without Addition of $[Et_4N]_2[S_2O_4]$[7]

		required for extraction
DMF		does *not* extract
NMF		extracts well
formamide		extracts poorly
pyrrolidinone		extracts well

At least some of the exogenous ligands are likely to be anionic because isolated FeMo-cofactor has been demonstrated to bind strongly to anion-exchange resins and to migrate toward the anode during anaerobic electrophoresis.[6] The 1Mo:6–8Fe:8–9S core is undoubtedly positively charged and one homocitrate ligand is unlikely to produce an FeMo-cofactor species with an overall negative charge. Early experiments indicated that NMF was a likely ligand because 10 nmol of the NMF hydrolysis product, methylamine, per nmol of FeMo-cofactor, remained in samples that had been evaporated to dryness.[6] Because only basic NMF was able to extract FeMo-cofactor, it was further suggested that the ligation was through nitrogen and required a dissociable proton.[6,7] This was supported by FT-IR data showing an intense band at 1600 cm^{-1} characteristic of N-deprotonated amide ligands.[7] As shown in Table 3, the observation that pyrrolidinone, formamide, and NMF could extract FeMo-cofactor from acid-denatured protein pellets, while DMF could not, further supported the idea that NMF was behaving as an anionic ligand.[6,7]

The requirement for a N-deprotonated amide ligand was first questioned by Lough et al., who suggested that the inability of a solvent to extract FeMo-cofactor might be related to the lack of solubility of $Na_2S_2O_4$ in that solvent.[13] Using DMF-soluble $[Et_4N]_2[S_2O_4]$ they were able to extract good yields of active FeMo-cofactor in DMF, demonstrating that a dissociable proton was not required. The probable function of $S_2O_4^{2-}$ in that system was recently revealed by Hedman et al. who used sulfur K and molybdenum L edge XAS to examine samples of FeMo-cofactor that had been isolated in the presence of $S_2O_4^{2-}$ and its oxidation products.[56] The data clearly showed the presence of an oxidized sulfur species in FeMo-cofactor with the spectral features of a thiosulfate ligand. Thus, it seems most likely that $S_2O_3^{2-}$, derived from the disproportionation of $S_2O_4^{2-}$

into $S_2O_3^{2-}$ and HSO_3^-, is a ligand to isolated FeMo-cofactor.[56] The same study showed that Cl^- was not a ligand for FeMo-cofactor preparations that had been isolated in the presence of both HCl and $S_2O_4^{2-}$. It should be noted that $S_2O_4^{2-}$ is not required for FeMo-cofactor isolation.[6] Thus, anionic species other than $S_2O_3^{2-}$ must be able to serve as ligands to isolated FeMo-cofactor, if necessary.

That there is no absolute requirement for an amide solvent to extract FeMo-cofactor was further demonstrated by Wink et al., who produced active samples in a variety of solvents including DMF, MeOH, CH_3CN, acetate, THF, and CH_2Cl_2.[14] They showed that the solubility of FeMo-cofactor in these solvents was controlled by the presence of a suitable counterion. Thus, FeMo-cofactor was readily soluble in basic NMF when Na^+ and K^+ were the only available counterions while in less polar solvents tetrabutylammonium counterions had to be added in order to solubilize FeMo-cofactor. That study did not address directly the issue of exogenous ligands to FeMo-cofactor. However, it did point out that although FeMo-cofactor could be solubilized in a variety of solvents after extraction into NMF only a few of these could be used to release FeMo-cofactor from the protein initially. Thus, those solvents (e.g. DMF, THF) may actually displace protein ligands and be bound to FeMo-cofactor.[14]

IV. Physical Properties

Based on the above considerations, the minimum composition of isolated FeMo-cofactor is likely to be 1Mo:6Fe:8S:homocitrate:1$(S_2O_3^{2-})_n$ with probably additional ligation by solvent. This section discusses what is known about the organization of the metal atoms relative to each other and to the ligands. This information has come primarily from spectroscopic studies which compared the properties of isolated FeMo-cofactor to those of the $S = {}^3/_2$ center (M-center) of the MoFe protein and to inorganic model complexes of known structure.[18]

A. Optical Spectroscopy

The original report of FeMo-cofactor isolation, done in the presence of excess dithionite, showed a visible absorption spectrum which was featureless with gradually decreased absorbance in the 300–800-nm region. Since then UV/vis spectroscopy has not been used as a tool to characterize FeMo-cofactor, presumably because NMF prevents studies below 265 nm and the dithionite ion, which is generally present, absorbs maximally at 313 nm. This situation may soon change with the isolation of FeMo-cofactor in solvents other than NMF[13,14] and a recent report of successful removal of dithionite and other absorbing impurities by Sephadex G-25 chromatography.[16] The latter report, however, showed that the near-UV/vis spectra of purified FeMo-cofactor samples varied from preparation to preparation. The authors attributed this finding to FeMo-cofactor existing in a mixture of electronic states in solution which unfortunately greatly complicates the interpretation of optical data.

It has been reported that isolated FeMo-cofactor in NMF (which does not have protein ligands) does not exhibit any detectable CD.[57] This result may now be somewhat surprising in view of the fact that FeMo-cofactor appears to contain the chiral ligand (*R*)-homocitrate.

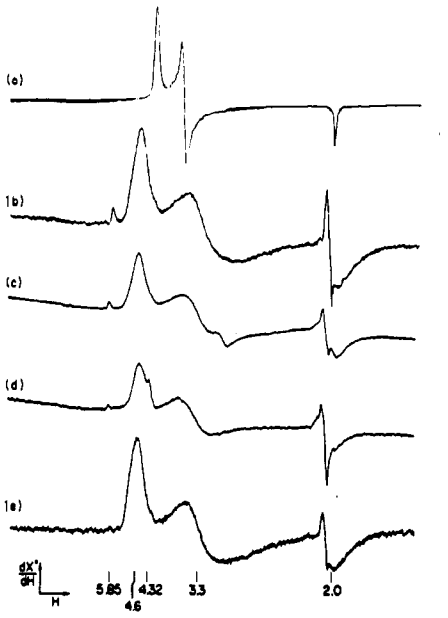


Figure 6. EPR spectra at 13 K of the *A. vinelandii* MoFe protein (a) and isolated FeMo-cofactor in NMR from *A. vinelandii* (b), *C. pasteurianum* (c), and *K. pneumoniae* (d).[58] Spectrum e represents the *A. vinelandii* FeMo-cofactor at 4 K.

B. EPR Spectroscopy

EPR spectra of isolated FeMo-cofactor samples from a variety of organisms were first reported in 1978 by Rawlings et al.[58] As shown in Figure 6 the EPR signal exhibited by isolated FeMo-cofactor in NMF[58] is similar to, although much broader than, the signal exhibited by the M-center of the MoFe protein.[59] Both signals are unique in biology, and are characteristic of $S = {}^3/_2$ centers. The demonstration of this signal provided the first evidence that the EPR active M center of the MoFe protein had been isolated as an intact entity in NMF and that the two species were very similar in structure. The integration of the signals for both the M-center and isolated FeMo-cofactor to 1.0 spin per Mo was also consistent with the presence of 1 Mo per FeMo-cofactor center.[58] The broadening of the EPR signal upon extraction into NMF was attributed to an unspecified change in ligation.[59] In addition to this broadening, there is a small absorption feature in the $g = 6.0$ region that is observed at 14 K for the isolated FeMo-cofactor but not for the protein (Figure 6). This feature, which disappears at 4 K, has been attributed to components of the $M_s = {}^3/_2$ excited state, of the $S = {}^3/_2$ ($M_s = {}^1/_2$ ground state) system.[58,60,61]

C. Mössbauer Spectroscopy

Mössbauer spectroscopy has played a major role in increasing our understanding of the structural organization of the FeMo-cofactor. Prior to the isolation of FeMo-cofactor, biophysical experiments had shown that the $S = {}^3/_2$ M-center of the MoFe protein was an Fe-containing complex.[62,101] Proof that the EPR signal of isolated FeMo-cofactor similarly arose from an Fe-containing cluster was obtained by Mössbauer experiments performed shortly after the initial isolation of FeMo-cofactor.[58] These experiments showed that the Mössbauer spectrum of isolated FeMo cofactor in NMF

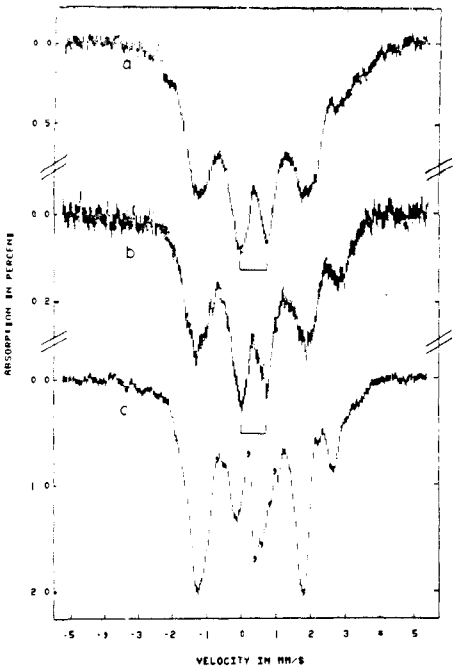


Figure 7. Mossbauer spectra at 4.2 K for *A. vinelandii* isolated FeMo-cofactor in NMF (a). Sample b is after addition of thiophenol to a, and c is for the M-center of the intact *C. pasteurianum* MoFe protein.[58]

is very similar to, but not identical with, that obtained for the $S = {}^3/_2$ M-center of the MoFe protein. The $S = {}^3/_2$ center in both species was also shown to contain five to seven, most probably six, Fe atoms, consistent with the lower values obtained from analytical studies.[58,62,63] Mössbauer data did not resolve each of the individual Fe atoms but did group them into two distinct classes. Taken together, the Mössbauer data demonstrated that at least the majority of Fe atoms in isolated FeMo-cofactor solutions were components of a novel spin-coupled complex which was responsible for the $S = {}^3/_2$ EPR signal. All of the Fe atoms associated with the $S = {}^3/_2$ center in isolated FeMo-cofactor were reported to have the same quadrupole splitting (ΔE_Q = 0.75 mm/s) and isomer shift ($S = \pm 0.37$).[58] These parameters were used to examine the valencies of the Fe atoms in the $S = {}^3/_2$ M-center by comparison with those from [FeS] proteins of known structures. One conclusion from that comparison was that the Fe sites of FeMo-cofactor do not appear to exhibit trapped valencies as seen in $[Fe_2S_2]$ ferredoxins, but rather appear to exhibit delocalized valencies as seen for $[Fe_4S_4]$ ferredoxins.

As shown in Figure 7, the differences between the Mössbauer spectra of the M-center and isolated FeMo-cofactor were primarily observed as a broadening of the absorption bands upon isolation in NMF. These differences may be due to a greater magnetic anisotropy of the Fe environment in FeMo-cofactor and also a greater heterogeneity of the molecules in the sample.[58] A small quadrupole doublet, marked by brackets in Figure 7, was attributed to a minor Fe contaminant or to the presence of some oxidized (S=O, vide infra) FeMo-cofactor in the sample.[58,60,62,63] The observed changes in the Mössbauer spectrum upon NMF extraction of the FeMo-cofactor from its protein environment have been interpreted in terms of increased symmetry for the extracted species.[63] This idea is supported by MCD experiments which clearly show

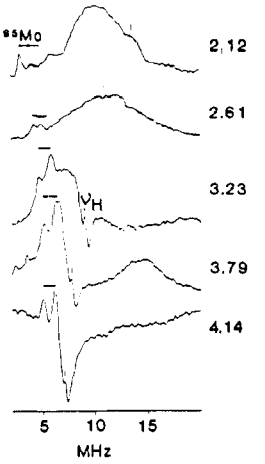


Figure 8. Background-subtracted Mo ENDOR of the MoFe protein from *C. pasteurianum* at listed g values corresponding to fields across the EPR absorption envelope.[68] The center (•) and splitting (|–|) of the ^{95}Mo m_I ($\pm {}^1/_2$) Larmor doublet is indicated in each spectrum; a vertical line corresponding to ν_H also is indicated.

much sharper features for the isolated FeMo-cofactor than for the M-center of the MoFe protein, consistent with the geometry of the cluster being more regular in the extracted state.[64] It is of course logical that a chiral protein ligand would have much more control over the geometry of the cluster and a far greater ability to induce asymmetry in the cluster than the much smaller ligands available in NMF solution. However, the argument raised by one group, that the narrow line widths of the quadrupole doublets in the Mössbauer spectrum of isolated FeMo-cofactor were indicative of all Fe atoms being in equivalent environments,[63] is clearly inconsistent with other spectroscopic data (e.g. XAS, vide infra).

D. ENDOR Spectroscopy

1. Mo ENDOR

The EPR and Mössbauer data described above demonstrated that the M-center of the MoFe protein and isolated FeMo-cofactor were structurally similar, that as isolated with $S_2O_4^{2-}$ both were metal clusters with total electronic spin of $S = {}^3/_2$, that in both cases about six Fe atoms were present in the $S = {}^3/_2$ centers, and that the unpaired spins were associated with Fe. Although the $S = {}^3/_2$ EPR signal of the MoFe protein does not broaden with enrichment by ^{95}Mo,[62] a small participation of Mo in the $S = {}^3/_2$ M-center was suggested early on by other EPR experiments.[65,66] These included brief reports of multiple-pulse EPR which showed that ^{96}Mo- and ^{95}Mo-containing MoFe proteins exhibited differences in echo decay.[66] However, the electronic integration of Mo into the M-center, $S = {}^3/_2$ system was first demonstrated directly by ^{95}Mo-ENDOR spectroscopy of the MoFe protein.[67,68] (ENDOR experiments have not been reported for isolated FeMo-cofactor.)

Figure 8 shows the ^{95}Mo ENDOR spectra of the MoFe protein, measured at a variety of magnetic fields corresponding to g values within the EPR absorption envelope.[68] The top and bottom spectra give single-crystal-like ENDOR because they are taken at the extreme edges of the EPR spectrum, near g'_z (top) and g'_y (bottom). The existence of the ^{95}Mo ENDOR signal

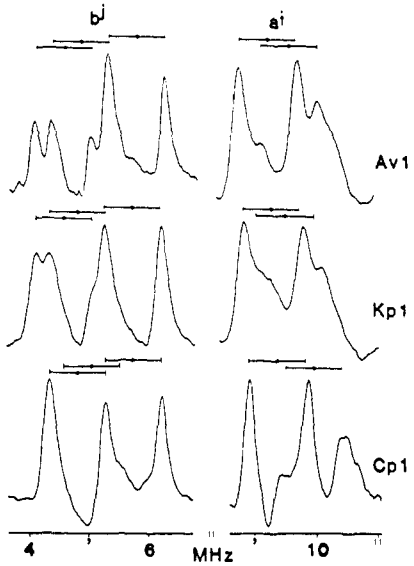


Figure 9. ^{57}Fe ENDOR of MoFe proteins from *A. vinelandii* (Avl), *K. pneumoniae* (Kpl), and *C. pasteurianum* (Cpl).[68] The assignment of five Larmor-split doublets is indicated, as are the frequencies corresponding to $A/2$ (O).

proves that Mo is integrated into the $S = {}^3/_2$ M-center of the MoFe protein.[67] The g'_z ^{95}Mo ENDOR signal is broad and consistent with a hyperfine coupling constant of $A_z^{Mo} = 8.1$ MHz. This value was used to simulate broadening of the EPR signal upon enrichment with ^{95}Mo, assuming one or two Mo atoms per M-center. The lack of broadening observed experimentally[62] was found to be consistent with one Mo atom per M-center and inconsistent with two.[67,68] The small value of the ^{95}Mo hyperfine coupling, relative to paramagnetic Mo complexes, was used to argue that the Mo should be classified as a diamagnetic, even-electron ion with an even formal valency and not as Mo(V) or Mo(III).[68]

Additional information has come from consideration of the ^{95}Mo quadrupole coupling parameters inferred from the ENDOR measurements. The quadrupole interaction strength, P_z^{Mo}, for the MoFe protein was found to be ca. 1.6 MHz, reflecting the asymmetric distribution of electrons in the vicinity of the Mo nucleus. These data were used to argue that the Mo atom in the M-center was in a highly asymmetric coordination environment.[68]

2. Fe ENDOR

^{57}Fe ENDOR for the M-center of the MoFe protein has been published at low[69] and high[68,70] resolution. Figure 9 gives the high-resolution data at g'_z clearly showing five Larmor-split doublets.[68] These data demonstrate that there are at least five distinct types of Fe sites in the $S = {}^3/_2$ M center, each of which appears to have different hyperfine tensor values. If each type of site contained a single Fe atom then these data would be in agreement with the lowest analytical values and the lower limit on the number of Fe atoms (five per Mo) observed by Mössbauer. However, the currently available ^{57}Fe ENDOR data do not eliminate the possibility of a sixth, seventh, or eighth Fe atom in the $S = {}^3/_2$ center if it has very small hyperfine coupling.[68]

E. X-ray Absorption Spectroscopy

In 1977, when the isolation of FeMo-cofactor was first reported, a promising new method called X-ray absorption spectroscopy (XAS) was being developed to study the local environment around a specific type of metal atom in a metalloprotein of unknown structure.[71] Following an XAS study of Mo complexes,[72] the technique was immediately applied to the FeMo-cofactor problem. Mo K edge X-ray absorption data have now been collected for both the MoFe protein and the isolated FeMo-cofactor in NMF.[11,33,73] Two general regions of the spectrum have been analyzed to give structural information about the Mo environment in FeMo-cofactor, the X-ray absorption edge and near edge (collectively referred to herein as XANES) and the extended X-ray absorption fine structure (EXAFS). Analysis of the XANES gives information about the oxidation level and coordination geometry of Mo, while analysis of the EXAFS gives information concerning the number and distances of atoms neighboring to Mo. Figure 10 gives a qualitative comparison of both regions of the spectrum for isolated FeMo-cofactor and the MoFe protein. These 1978 data led to the unambiguous conclusion that the Mo environments of both species were extremely similar in structure. Evaluation of these data has subsequently led to a detailed picture of the structural organization of the Mo environment in both species.

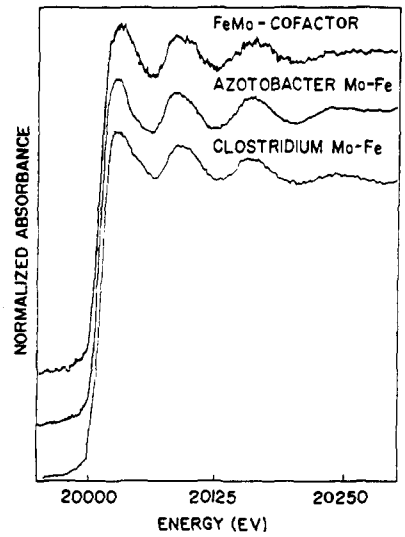


Figure 10. A comparison of the Mo K edge X-ray absorption components of lypolized *C. pasteurianum* MoFe protein (bottom), crystallized *A. vinelandii* MoFe protein (middle), and FeMo-cofactor extracted in NMF (top).[33]

1. Mo K XANES

In the initial analysis (1978) the Mo K XANES region of MoFe protein and FeMo-cofactor spectra were compared with the data collected for Mo complexes that were then available.[33,72] Many of those complexes contained terminal oxo linkages[74] which gave rise to a distinctive "preedge" feature.[72] This feature was clearly absent in the MoFe protein and FeMo-cofactor spectra, demonstrating that the Mo in these species did not have terminal oxo ligands. Quantitative analysis of the 1978 EXAFS data (vide infra) spurred the synthesis of numerous additional Mo–Fe–S complexes with oxygen and sulfur ligands in the first coordination sphere.[18] A detailed comparison of the XANES spectra of those complexes and FeMo-cofactor was subsequently reported.[75] That study grouped the model complexes according to the following categories: (I) those con-

TABLE 4. Average EXAFS Results on the Mo Sites in the MoFe Protein, FeMo-Cofactor, and FeMo-Cofactor + Thiophenol/Selenophenol[11]

	O shell		S shell		Fe shell	
	Mo–O dist, Å	no. of atoms	Mo–S dist, Å	no. of atoms	Mo–Fe dist, Å	no. of atoms
semireduced MoFe protein	2.12 ± 0.01	1.7	2.37 ± 0.01	4.5	2.68 ± 0.01	3.5
as-isolated FeMo-cofactor	2.10 ± 0.02	3.1	2.37 ± 0.02	3.1	2.70 ± 0.02	2.6
FeMo-cofactor + PhSH/PhSeH	2.11 ± 0.01	2.6	2.37 ± 0.01	4.0	2.70 ± 0.01	3.3

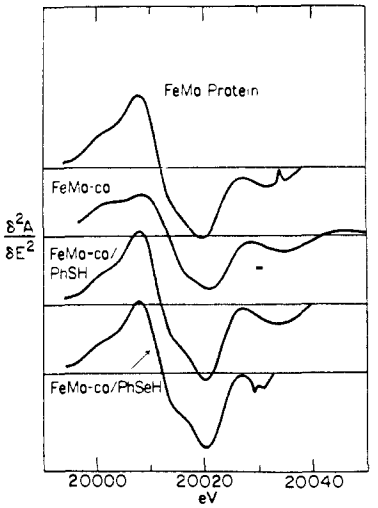


Figure 11. $\delta^2A/\delta E^2$ of the Mo K absorption edge of the dithionite-reduced MoFe protein of *C. pasteurianum*, isolated *A. vinelandii* FeMo-cofactor in NMF, FeMo-cofactor + thiophenol, and FeMo-cofactor + selenol.[74]

taining tetrahedral $[MoS_4]^{2-}$, (II) mononuclear complexes with the MoS_6 coordination unit, (III) complexes with MoS_3O_3 units with effective C_{3v} symmetry, (IV) those containing nearly isometric MoS_4O_2 coordination units of effective C_s symmetry, and (V) a single complex with a MoS_3O_2C coordination unit.

Figure 11 shows the second-derivative Mo K XANES spectra for the FeMo-cofactor and the MoFe protein.[74] These data demonstrated that in both species the Mo geometry and the first shell of the nearest neighbors to the Mo atoms were very similar, but not identical. These data were compared to those obtained for the five classes of model complexes discussed above.[74] The results unambiguously demonstrated that the coordination unit in FeMo cofactor is not tetrahedral $[MoS_4]^{2-}$ (category I) or MoS_6 (category II) and that the Mo environment is also significantly different from those of complexes in categories IV and V. As shown in Figure 11, the FeMo-cofactor spectra most closely resemble those obtained for the complexes in category III which have the MoS_3O_3 coordination unit with Mo–O bond lengths in the 2.1–2.2-Å range. The two complexes in that category are $(Et_4N)_3[MoFe_4S_4(SEt)_3$(catecholate)][76] and $(Et_4N)_3[Mo_2Fe_6S_8(SEt)_6(OMe)_3]$ (Figure 12).[77] Thus, a major conclusion from that study was that the first shell of nearest neighbors to the Mo atoms in FeMo-cofactor consists of significant numbers of both hard and soft ligands.

2. Mo K EXAFS

The original analysis of the Mo EXAFS region of the MoFe protein produced the first indication of the cluster nature of the Mo-containing complex in FeMo-cofactor by demonstrating that Fe and S atoms were nearest neighbor atoms to Mo, with sulfur being

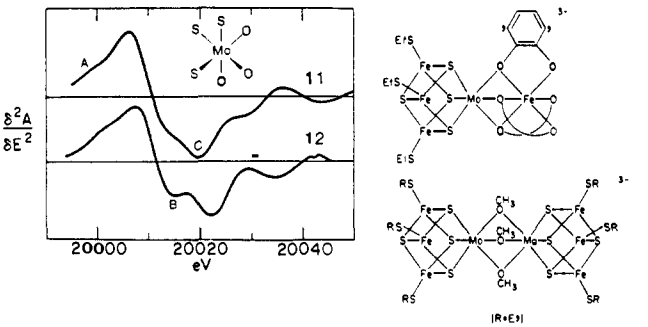


Figure 12. $\delta^2A/\delta E^2$ of the Mo K absorption edge[74] of $(Et_4N)_2[MoFe_4S_4(SEt)_3$(cat.)$_3]$[76] (top) and $(Et_4N)_3[Mo_2Fe_6S_8(SEt_4)_6(OMe)_3]$[77] (bottom). Corresponding structures are shown on the right. The similarity of the Mo sites in the complexes to the Mo site in FeMo-cofactor can be observed by comparing Figures 11 and 12.

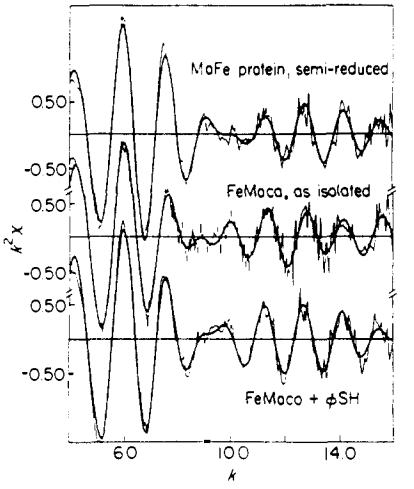


Figure 13. EXAFS data, k_2 weighted, of the MoFe protein (top), isolated FeMo-cofactor in NMF (center), and FeMo-cofactor after addition of thiophenol (bottom).[11] The light lines are actual data and the dark lines are derived by Fourier filtering.

at a sulfide-like bridge-bonding distance.[33] That study did not indicate the presence of soft (O or N) ligands and suggested that there might be an additional S present at longer distances.[33] However, that analysis was limited by the availability of suitable model compounds to extract appropriate parameters. For example, no Mo–Fe containing complexes had then been synthesized, and so an untested Mo–Fe parameter had to be used to model the real curve fits for the MoFe protein. In fact, it was those early EXAFS results[33] that initially stimulated the synthesis of the array of Mo–Fe–S model complexes that are currently available.[18] Those which most closely resembled the FeMo-cofactor Mo environment by the XANES criteria discussed above were subsequently used to analyze additional, higher resolution EXAFS data obtained for the MoFe protein and isolated FeMo-cofactor.[11]

Figure 13 shows the higher resolution EXAFS data, k^2 weighted, for the MoFe protein and isolated FeMo-

cofactor, again clearly demonstrating that the Mo sites for the two species are very similar, though not identical. Table 4 shows the quantitative analysis of those data. Consistent with the XANES data described above, these quantitative EXAFS data showed that the Mo atoms in both the MoFe protein and isolated FeMo-cofactor had at least two O(N) atoms as nearest neighbors which were not detected in the earlier EXAFS analysis.[33] The observed Mo–O(N) distances were consistent with anionic ligands and were too short for neutral solvent molecules. Within the protein, the ligands could be homocitrate, endogenous oxo or hydroxo bridges to Fe atoms, or amino acid residues. In isolated FeMo-cofactor they could be homocitrate, oxo, or hydroxo endogenous bridges to Fe atoms, or they could be available exogenous ligands (e.g. OH^-, $S_2O_3^{2-}$, HPO_4^{2-}, deprotonated NMF). The data in Table 4 also show that there are at least three S atoms nearest neighbors to Mo at 2.37 Å. The additional longer distance S shell suggested by earlier analysis[33] was not observed in the higher resolution data and may have been an artifact caused by using untested Mo–Fe parameters.[11]

There is no difference in the Mo–O(N) or Mo–S distances when the MoFe protein data are compared to the isolated FeMo-cofactor data. However, there is a difference in the number of those atoms, with the Mo atom in the MoFe protein having fewer O(N) ligands and more S ligands than the Mo atom in isolated FeMo-cofactor (Table 4). In both cases, however, the total number of atoms is at least six. In agreement with the XANES analysis, these results eliminated the tetrahedral geometry for Mo that had been suggested on the basis of interpretation of ^{95}Mo ENDOR experiments[67] and required a structure with geometries of significantly higher coordination number. This conclusion was also supported by Mo L edge studies.[56] Taken together, the Mo K and L edge XANES and EXAFS data require that in both the MoFe protein and in isolated FeMo cofactor the Mo atom is surrounded in the first coordination shell by a mixed S and O(N) ligation sphere with probable distorted-octahedral geometry. Combining this information with ^{95}Mo ENDOR data on the MoFe protein[66] and XAS analysis of suitable Mo–Fe–S model complex of known structure[56,74] leads to the additional conclusion that the most likely formal oxidation state for the Mo atom in both species is +4.[56]

In addition to the O(N) and S atoms located in the first coordination shell, the EXAFS analysis shows the presence of Fe atoms at ca. 2.7 Å with the Mo atom in the MoFe protein "seeing" one more Fe atom than in the isolated FeMo-cofactor[11,33] (Table 4). It has been argued that the most likely Mo:Fe numbers were three in isolated FeMo-cofactor and four in the MoFe protein.[33] In both cases the distances were consistent with the Fe being connected to the Mo via sulfide bridges. The change in the number of Fe atoms observed upon extraction of the FeMo-cofactor from the protein matrix suggests that the differences in the two species is not simply an exchange of protein (O, N, or S) ligands for exogenous (O, N, or S) ligands. The core structure also appears to change such that an Fe atom moves from its position 2.7 Å away from Mo to out of "sight" of the Mo atom.

TABLE 5. Average EXAFS Results on the Fe Sites in FeMo-Cofactor

		Antonio et al.[79]	Arber et al.[80]
Fe–S	dist, Å	2.25	2.20
	no. of atoms	3.4	3
Fe–Fe	dist, Å	2.66	2.64
	no. of atoms	2.3	2.2
Fe–Mo	dist, Å	2.76	2.7
	no. of atoms	0.4	0.8
Fe–O(N)	dist, Å	1.81	
	no. of atoms	1.2	0.0
Fe–Fe	dist, Å		3.68
	no. of atoms		1.3

3. Single-Crystal Mo EXAFS

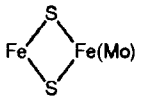
One of the major advantages of the XAS technique is that it can provide structural information for proteins that are only available in solution. One major disadvantage of the technique is that it only gives a radially averaged picture of the environment around the Mo atom. It does not give information about angles and therefore cannot be used to arrive at a complete three-dimensional structure of a complex like FeMo-cofactor. Fortunately, in recent years, single crystals of the MoFe protein have become available.[78] Although the complete structure of the MoFe protein has not yet been determined, Flank et al. recognized the potential to combine the available crystals with the XAS technique in order to obtain information about the geometry and orientation of the atoms within the FeMo-cofactor center of the MoFe protein. In their initial study they demonstrated that for different orientations, the Mo–Fe amplitude of the Mo K edge EXAFS changed by a factor of 2.5, whereas the Mo–S component varied by only ±15%. They found that the experimental data were not well-simulated by clusters with a linear arrangement of Fe–Mo–Fe atoms, whereas trinuclear clusters with a Fe–Mo–Fe angle between 50° and 130° gave satisfactory results.[73] Tetrahedral $MoFe_3$ and square-pyramidal $MoFe_4$ cluster symmetries were also consistent with their data. Thus, the $MoFe_3$ core-containing model complexes that appeared to correspond most closely to the Mo environment by the XANES criteria[74] were also consistent with the single-crystal data.

4. Fe XAS

It is possible to run Mo XAS experiments on the MoFe protein because the Mo atom in that species is in a unique environment. For Fe XAS, however, the situation becomes impossible because the MoFe protein contains ca. 14–15 Fe atoms per Mo, many of which are present in different environments. Fe XAS has been used to study the isolated FeMo-cofactor, which contains ca. six to eight Fe atoms per Mo.[79,80] Table 5 gives the quantitative Fe EXAFS data obtained by two different groups.[79,80] In agreement with the Mo EXAFS data (Table 4), which demonstrated that each Mo atom "sees" ca. three of the six to eight Fe atoms present in FeMo-cofactor, both Fe EXAFS studies revealed that each Fe atoms "sees", on average, 0.4–0.8 Mo atoms. The reported Fe–Mo distances were in one case consistent with the 2.70 Å from Mo EXAFS and, in the other case, somewhat longer at 2.76 Å.

Both studies revealed that on average each Fe atom "sees" ca. three S atoms at 2.2–2.25 Å. These data were

interpreted in terms of FeMo-cofactor containing rhombs of metal–sulfur atoms[79,80] as found in a variety of Fe–Mo–S clusters.[18] It is interesting to note that



only one type of Fe–S interaction was observed by Fe XAS, whereas three types of sulfur (sulfide, thiolate-like, and bound thiosulfate-like) were shown to be associated with FeMo-cofactor by sulfur K edge XAS studies.[56] (The possibility that some of the S atoms observed by Fe XAS were Cl^- atoms was also eliminated by the latter study.[56])

One controversial aspect of the Fe XAS experiments was that one group reported the presence of an average of 1.2 light (O, N) atoms per Fe at 1.81 Å,[79] whereas the other group could not find evidence for any O(N) ligands.[80] The latter group suggested that the O(N) atoms observed in some Fe XAS experiments were an artifact of having adventitious Fe present in the FeMo-cofactor solutions. Unfortunately, the group observing the (O, N) atoms did not report either the Fe:Mo ratio or the specific activity of their FeMo-cofactor samples[79] while the other group reported Fe:Mo ratios for all samples but some had low specific activities of 36–190 nmol of C_2H_2/min per ng-2 atom of Mo (versus ca. 250 for fully active samples).[80] Surprisingly the latter study did not see a correlation between the spectra and the specific activity of the sample, unless extraneous iron was present. In view of the recent data demonstrating that FeMo-cofactor contains homocitrate as a ligand, it is surprising that none of the Fe atoms "sees" any O and certainly worth repeating an Fe XAS study with material of higher specific activity and defined Fe:Mo ratio.

As shown in Table 5, both groups reported that each Fe atom also sees on average about two additional Fe atoms at 0.264–0.266 Å consistent with an average Fe–S–Fe (Mo) angle of ca. 74°. One group additionally reported that each Fe atom "sees" ca. one Fe at a much longer distance of 3.68 Å. This result is consistent with FeMo-cofactor having a more extended structure than has been observed by other techniques.

F. Summary

The spectroscopic data discussed above require that the metal cluster which gives rise to the $S = {}^3/_2$ EPR signal exhibited by the MoFe protein and isolated FeMo-cofactor contains one Mo with a minimum of five, and most probably six, Fe atoms. It is not necessary to suggest that FeMo-cofactor contains additional Fe in order to explain the bulk of the analytical data. If it does contain additional Fe atoms (up to a maximum of eight), however, they do not participate in the $S = {}^3/_2$ center. The three unpaired electrons appear to be delocalized among the Fe atoms with the Mo atom playing a minor role in the $S = {}^3/_2$ system.

The Mo atom is most likely in the +4 formal oxidation state. It is surrounded in the first coordination shell by a mixed S, O(N) ligation sphere having, most likely, a distorted-octahedral geometry. The O(N) ligands must be anionic (Mo–O(N) distance of 2.1 Å) and some could arise from homocitrate, which is present in the ratio of one homocitrate per Mo atom. The Fe atoms must exist in at least two different environments, those which "see" the Mo (about four for the MoFe protein and three for isolated FeMo-cofactor) and those which do not. The Fe atoms are most likely connected to the Mo atom and to each other via sulfide bridges using a total of 8–9 sulfides. The relevant distances are Mo–S 2.37 Å, Fe–S 2.2–2.5 Å, and Mo–Fe 2.7 Å, with an Fe–Mo–Fe angle between 50° and 130° and an average Fe–S–Fe(Mo) angle of 74°. Some of the Fe atoms also appear to "see" other Fe atoms at 3.68 Å. Isolated cofactor has an overall negative charge (magnitude unknown), which most likely arises due to some Fe atoms having bound thiosulfate as exogenous ligands. There is no evidence to indicate whether or not homocitrate coordinates directly to Fe. FeMo-cofactor appears to be in a highly asymmetric environment when it is bound to the protein, and there are at least five different Fe environments. When it is released from the protein it becomes more symmetrical but without major change in the magnetic properties of the $S = {}^3/_2$ center. The change observed upon extraction, however, may not be simply an exchange of protein ligands for small molecule ligands. The structure of the core also appears to change, resulting in the movement of an endogenous Fe atom (probably with its bridging sulfide) away from the Mo atom.

V. Chemical Reactivity

A. The FeMo-Cluster

Some of the spectroscopic techniques discussed above (e.g. EPR, ENDOR) only give information about the metal cluster that gives rise to the $S = {}^3/_2$ EPR signal, whereas other techniques (e.g. XAS, Mössbauer) can observe metal atoms whether or not they are associated with the $S = {}^3/_2$ system. The following question therefore arises: Are FeMo-cofactor and the $S = {}^3/_2$ center identical or is the latter a subset of the former? In 1981 an Fe- and Mo-containing subset of FeMo-cofactor (designated FeMo-cluster) was isolated by acidified methyl ethyl ketone extraction of the MoFe protein.[29] This species did contain the portion of FeMo-cofactor that gives rise to the $S = {}^3/_2$ EPR signal but was missing some portion of FeMo-cofactor that was necessary for activity. Thus, this FeMo-cluster exhibited the $S = {}^3/_2$ EPR signal when transferred to NMF, but could not activate the FeMo-cofactor-deficient MoFe protein. FeMo-cluster could inhibit FeMo-cofactor binding to the FeMo-cofactor-deficient MoFe protein, suggesting that it was bound to the same or a neighboring site. The FeMo-cluster has never been obtained directly from isolated FeMo-cofactor.

The FeMo-cluster was reported to contain six Fe atoms per Mo[29] by the same group that reported that the FeMo-cofactor contained eight Fe atoms per Mo,[1] thus leading them to suggest that the difference between the two species was the number of Fe atoms. However, as considered in detail in section III.A.2., it is equally plausible that the FeMo-cofactor solutions simply contained more adventitious Fe than the FeMo-cluster solutions. Certainly the one-step FeMo-cluster isolation procedure[29] involved much less handling than the original FeMo-cofactor isolation procedure.[1] Another possibility is that the FeMo-cluster contained the full metal component of FeMo-cofactor but was missing the organic component homocitrate

and thus did not have any activity. Elucidation of the differences between the $S = {}^3/_2$ center and FeMo-cofactor will have to await a more complete spectroscopic characterization of the FeMo-cluster.

B. Stability and Degradation Products

Like the MoFe protein, isolated FeMo-cofactor is rapidly inactivated by O_2. When exposed to O_2, concentrated green-brown solutions rapidly turn red and then yellow and eventually become colorless. The nature of the red product(s) has been examined both for the MoFe protein[81] and for isolated FeMo-cofactor.[82] Degradation of both species by O_2 was shown to elicit either the tetrathiomolybdate ion ($[MoS_4]^{2-}$) or the oxotrithiomolybdate ion ($[MoOS_3]^{2-}$), depending upon reaction conditions.[81,82] The Mo EXAFS data (Table 4) are consistent with there being three S atoms around Mo in isolated FeMo-cofactor and thus with $[MoOS_3]^{2-}$ being an oxidative decomposition product. This is the major product observed by simple addition of O_2 to FeMo-cofactor solutions.[82] For isolated FeMo-cofactor, $[MoS_4]^{2-}$ arises only after addition of MeOH, which precipitates dithionite out of solution. The formation of $[MoS_4]^{2-}$ from the putative MoS_3 core has been rationalized by the suggestion that the fourth S atom arises from free sulfide produced from oxidative decomposition of the non-Mo portion of FeMo-cofactor in the absence of dithionite and the presence of O_2.[82]

In addition to looking for Mo-containing FeMo-cofactor fragments, some decomposition experiments have attempted to release recognizable iron–sulfur clusters from FeMo-cofactor.[58,83] These attempts failed to elicit any such clusters,[58,83] providing strong evidence against the suggestion[84] that FeMo-cofactor contains $[Fe_4S_4]$ clusters. Other arguments against the presence of recognizable [FeS] clusters in FeMo-cofactor include the observation that unlike [FeS] clusters, FeMo-cofactor can be easily isolated without the use of mercaptides.[6]

In addition to its O_2 sensitivity, isolated FeMo-cofactor is decomposed slowly (~25% per hour) in anaerobic aqueous solution.[1] Not surprisingly, FeMo-cofactor is also decomposed by the mercury reagent sodium mersalyl.[1,58]

C. Ligand-Exchange Reactions

When bound to the MoFe protein, the FeMo-cofactor is expected to have some open coordination sites for reaction with substrate. When the FeMo-cofactor is isolated from the protein environment, additional open coordination sites should be generated as protein ligands are removed. As discussed in section III.C. some or all of these open coordination sites may be occupied either by solvent or by exogenous ligands (e.g. thiosulfate). This section considers how at least some of these ligands can be subsequently exchanged with complexing reagents without effecting the activity of isolated FeMo-cofactor.

1. Chelators

The reaction of isolated FeMo-cofactor in NMF with the Fe-chelating reagents EDTA, 1,10-phenanthroline, and 2,2′-bipyridyl has been investigated by monitoring the $S = {}^3/_2$ EPR signal. The addition of these chelators

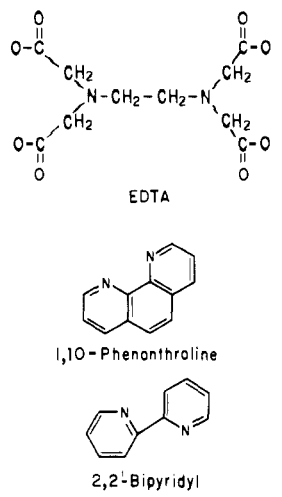


Figure 14. Structures of chelators that have been used in FeMo-cofactor complexation experiments.

(Figure 14) had some unusual effects upon FeMo-cofactor. For example, the addition of a ca. 40-fold excess of EDTA to a basic NMF solution of FeMo-cofactor resulted in the complete disappearance of the $S = {}^3/_2$ EPR signal.[85] This treatment had no effect on the specific activity of the FeMo-cofactor and 100% of the $S = {}^3/_2$ EPR signal was recovered by addition of a 2-fold excess of Zn^{2+} over EDTA.[6] A qualitatively identical result was obtained by addition of 1,10-phenanthroline followed by Fe^{2+} to reverse the reaction.[6] These results have been interpreted in terms of weak binding of either reagent to FeMo-cofactor to form specific complexes, suggesting that at least some of the metal atoms in FeMo-cofactor have a labile coordination sphere.

The disappearance of the $S = {}^3/_2$ EPR signal could most easily be explained either by chelator-induced formation of FeMo-cofactor dimers (or higher aggregates) with resultant spin–spin coupling or as an oxidation–reduction reaction.[6] (Both oxidized and fully reduced forms of FeMo-cofactor are expected to be EPR silent; vide infra.) Oxidation of FeMo-cofactor, however, seems unlikely because the experiments were carried out in the presence of excess dithionite. One possibility, at least for the 1,10-phenanthroline reaction, is that the neutral ligand 1,10-phenanthroline replaces an anionic ligand on FeMo-cofactor, decreasing its overall charge and raising its reduction potential, so that it can be reduced by the dithionite in the samples to an EPR-silent state.

It is puzzling that 2,2′-bipyridyl has no effect on the FeMo-cofactor EPR signal, given its similarity to 1,10-phenanthroline.[6] This could be due to a subtle structural problem or to an unrecognized experimental problem (e.g., weak binding, lowered solubility of 2,2′-bipyridyl in NMF). It is also puzzling that unlike synthetic [FeS] clusters, which are rapidly destroyed by chelating reagents, the activity of FeMo-cofactor is unaffected by these ligand-exchange reactions.

2. Reaction with Thiolate Ligands

In 1978, Rawlings et al. reported that the addition of thiophenol to isolated FeMo-cofactor in NMF caused its EPR signal to sharpen, making it look more like the signal exhibited by the M-center of the MoFe protein.[58] This reaction is illustrated in Figure 15. Quantitative analysis of this reaction, as measured by EPR spec-

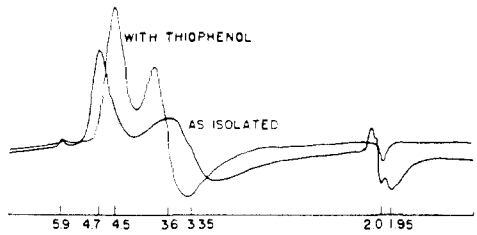


Figure 15. EPR spectral change which occurs on addition of thiophenol to FeMo-cofactor in NMF.[58] *g* values change from 4.6, 3.35, and 2.0 to 4.5, 3.5, and 2.0.

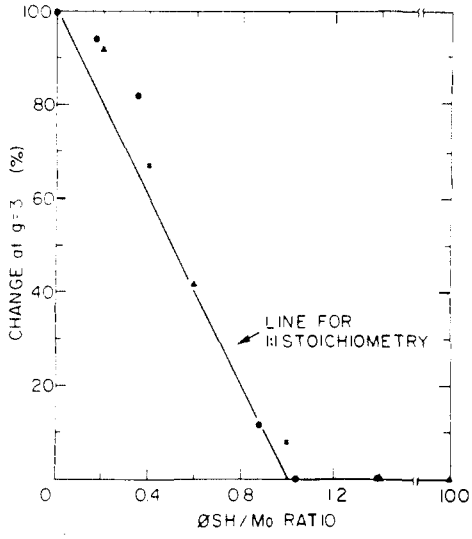


Figure 16. Titration of the EPR signal of FeMo-cofactor with thiophenol.[85]

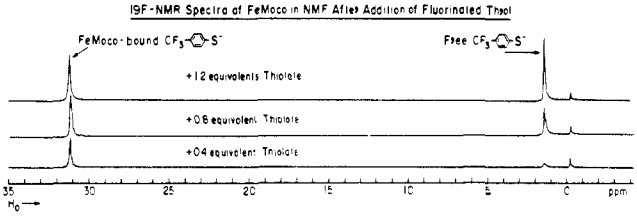


Figure 17. ^{19}F NMR spectra of mixtures of *p*-$CF_3C_6H_4S^-$ and dithionite-reduced FeMo-cofactor with indicated ratios of thiolate to FeMo-cofactor.[86b]

troscopy, showed that it was complete upon the addition of one thiophenol per Mo (Figure 16).[85] Thus, thiophenol appears to bind to a single site on FeMo-cofactor. The existence of a single, thiolate binding site was also confirmed by ^{19}F NMR using *p*-$CF_3C_6H_4S^-$ as the reporter ligand.[86] As shown in Figure 17, the addition of *p*-$CF_3C_6H_4S^-$ to dithionite-reduced FeMo-cofactor in NMF gave rise to only two resonances, one magnetically shifted and arising from the FeMo-cofactor–thiolate complex and the other from free thiolate. However, in contrast to the EPR analysis, there was significant free thiolate present in the ^{19}F NMR spectra even with less than stoichiometric addition (Figure 17a). A more thorough investigation of this phenomenon, which included magnetization transfer experiments (Figure 17b), demonstrated that at room temperature the FeMo-cofactor–thiolate complex is not the product of an irreversible reaction.[86] Rather, the complex is in dynamic equilibrium with the free FeMo-cofactor and free thiolate. The room-temperature reversibility of the thiolate reaction is also consistent with the observation that thiolate-treated FeMo-cofactor has the same activity as untreated FeMo-cofactor.[58] Presumably the bulky thiolate ligand is removed when the FeMo-cofactor binds to the FeMo-cofactor-deficient MoFe protein to form active holoprotein.

The data described above demonstrate that thiolate binds specifically and reversibly to a single site on FeMo-cofactor. By comparing the ^{19}F NMR spectra of *p*-$C_6H_4CF_3S^-$ derivatives of synthetic Fe–S and Fe–Mo–S complexes with similar derivatives of FeMo-cofactor, it was concluded that an Fe atom is the probable thiolate binding site.[86] This conclusion was also supported by the observation that the Mo K XANES and EXAFS spectra of FeMo-cofactor treated with thiophenol and selenol were identical.[11,74] If the thiolate ligand were on Mo, then the spectra should have been quite different. Proof that the thiolate binding site is on Fe and not on Mo has come from EXAFS data collected at the Se edge with FeMo-cofactor treated with selenophenol. These data show that each Se atom "sees" two types of atoms in the first coordination shell, C at 1.92 Å and Fe at 2.42 Å.[87,88] The Se does not "see" Mo. Thus, any model for FeMo-cofactor must include a unique Fe site for thiolate binding on a complex that contains six to eight Fe atoms. It is not known how many Fe atoms are involved in that site.

When thiolate binds to isolated FeMo-cofactor in NMF its structure changes. As indicated above, that structural change has been observed as a sharpening of the $S = {}^3/_2$ EPR signal. The effect of thiolate addition then is to produce a species that more closely resembles the $S = {}^3/_2$ M-center of the MoFe protein.[58] This effect has also been observed by Mo K XANES and EXAFS measurements.[11,74] As shown in Figure 11, the XANES spectrum of FeMo-cofactor in the presence of thiophenol (or selenol) is far more similar to the spectrum of the MoFe protein then to that of untreated FeMo-cofactor.[74] The quantitative EXAFS data shown in Table 4 also demonstrate that the structural changes induced by thiolate binding to isolated FeMo-cofactor yielded a species that more closely resembled the Mo site of the MoFe protein. Thus, as discussed in section III, the removal of FeMo-cofactor from the protein appears to cause a change in the core structure such that an Fe atom and a S atom move "out of sight" of the Mo atom. The addition of thiophenol to isolated FeMo-cofactor reverses this process (Table 4). It is important to note that the FeMo-cofactor–thiolate complex is still not identical with the FeMo-cofactor site of the MoFe protein; its EPR signal remains much broader than the protein signal.[58] In addition, the number of O(N) ligands on Mo increases when FeMo-cofactor is isolated from the protein, but does not appear to decrease significantly when thiophenol is subsequently added (Table 4).

3. *Putative Protein Ligands*

a. Cysteine. The reactivity of FeMo-cofactor with a thiolate ligand strongly suggests that an Fe site in FeMo-cofactor is attached to the protein via a single cysteine ligand. Recently, site-directed mutagenesis studies have been directed at trying to identify that cysteine ligand within the MoFe protein. Currently available information, based in part on sequence comparisons, strongly supports the assignment of α-subunit cysteine residue 275 as a FeMo-cofactor ligand.[89-92] Thus, the removal of the putative thiolate ligand at

position 275 by site-directed mutation of that cysteine residue to an alanine resulted in an inactive MoFe protein[90-92] and the accumulation of an active FeMo-cofactor species with the same EPR spectral properties as isolated FeMo-cofactor.[92]

The possibility of a second cysteine ligand seems remote but cannot be eliminated at this time. For example, the isolated FeMo-cofactor does have one less S and one additional O (or N) atom attached to Mo when compared to the MoFe protein (Table 4). Although this could be due to the removal of a cysteine ligand, such a possibility seems unlikely because the data clearly show that added thiolate does not bind to the Mo center in isolated FeMo-cofactor.[11,74,87,88] ENDOR data have also been used to argue against multiple cysteine ligands.[67,68,93] Unlike the situation for cysteine-bound [FeS] clusters, the addition of thiolate ligands does not appear to facilitate the removal of the FeMo-cofactor from the protein matrix, an observation which also argues against the presence of multiple cysteine ligands.

b. Histidine. Although the majority of biological [FeS] clusters are attached to their protein partners via cysteine residues, deprotonated N, histidine linkages have also been demonstrated in some cases.[94] As described in section II, the availability of deprotonated N ligands also appears to facilitate the removal of FeMo-cofactor from the protein matrix.[6,7] Because EXAFS cannot distinguish O from N, one or more of the low Z ligands to Mo observed for the MoFe protein could also arise from histidine. Recently, direct evidence for N ligation to FeMo-cofactor in the MoFe protein has been obtained by comparing electron spin echo data for the MoFe protein and isolated FeMo-cofactor.[94] The electron spin echo modulation spectrum of the protein was shown to contain lines characteristic of nuclear quadrupole transitions for nitrogen coordinated to a paramagnetic metal center. These frequencies were clearly absent from the spectrum of isolated FeMo-cofactor, leading the authors to suggest that the FeMo-cofactor is coordinated to the protein through at least one nitrogen ligand.[94] More recent preliminary reports have provided strong evidence that the nitrogen ligand is on Mo.[95]

A possible identity for that nitrogen ligand has recently been suggested by site-directed mutagenesis studies.[96] The site directed mutation of MoFe protein α-subunit histidine residue 195 to an asparagine resulted in the inability of the protein to fix N_2, an alteration in other substrate-reducing properties, and a decrease and change in both line shape and g values for the $S = {}^3/_2$ MoFe protein EPR signal.[96]

VI. Redox Properties

A. In the Oxidizing Direction

When the MoFe protein is in the presence of excess $Na_2S_2O_4$ its M-center exhibits an $S = {}^3/_2$ EPR signal.[62,97] When redox-active dyes with midpoint potentials of ca. 0 to −100 mV vs SHE are added to the protein, the $S = {}^3/_2$ EPR signal disappears. Thus, the M-center of the MoFe protein can be oxidized to an EPR-silent state in a reaction that is reversible by addition of reductant.[98-102] As illustrated in Figure 18, the stoichiometry of this reaction is one electron per $S = {}^3/_2$ M center.

Redox States of the M-center of the MoFe Protein

MoFe(ox)	+1e- / -1e-	MoFe(s-r)	+ne- / -ne-	MoFe(red)
S = 0		S = 0		S = integer
EPR-silent		EPR-active		EPR-silent

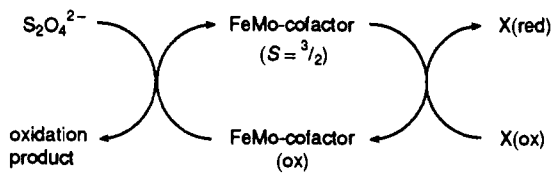
Redox States of Isolated FeMo-cofactor[10]

FeMo-co(ox)	+1e- / -1e-	FeMo-co(s-r)	+1e- / -1e-	FeMo-co(red)
S = ?		S = 3/2		S = ?
EPR-silent		EPR-active		EPR = ?

Figure 18. Known oxidation states of the M-center of the MoFe protein and the corresponding oxidation states of isolated FeMo-cofactor. Note: The MoFe (semireduced) to MoFe (reduced) transition only occurs upon addition of the reduced Fe protein and MgATP. MoFe (reduced) may actually be a mixture of species that have been reduced by one or more electrons.

MCD data collected on the MoFe protein have shown that the EPR-silent, one-electron-oxidation product, has a diamagnetic ground state.[103] Therefore, the removal of one electron from the M-center causes the remaining unpaired electrons to become paired to yield an $S = 0$ product. The reduction potential of this oxidation reaction appears to vary greatly (−260 to 0 mV) with species and reaction conditions.[104]

As shown in Figure 18 this general oxidation reaction has also been demonstrated for isolated FeMo-cofactor in NMF. Thus, the addition of methylene blue to dithionite-reduced FeMo-cofactor caused the $S = {}^3/_2$ EPR signal to disappear.[85] This reaction was reversed by addition of excess dithionite without any change in the specific activity of the FeMo-cofactor sample. Subsequently, it was discovered that FeMo-cofactor isolated and stored in the presence of excess dithionite underwent a similar oxidation reaction spontaneously.[10] Thus, FeMo-cofactor in NMF that started out in the $S = {}^3/_2$ state "self-oxidized" completely to its EPR-silent state, quickly under anaerobic conditions at room temperature and more slowly when stored under dry ice.[10,86] Again there was no change in specific activity. A possible mechanism for this "self-oxidation reaction" has been offered, and is illustrated below, where $X_{(ox)}$ is an unknown material in the FeMo-cofactor NMF preparation which is capable of oxidizing FeMo-cofactor from its $S = {}^3/_2$ to an EPR-silent state:



This scheme is consistent with the observation that stored samples that contained EPR-silent but active FeMo-cofactor no longer contained any $S_2O_4^{2-}$.[10] It should be noted that, in contrast, samples of $S_2O_4^{2-}$ in NMF stored in the absence of FeMo-cofactor are stable.

The reversible oxidation of the $S = {}^3/_2$ state of FeMo-cofactor to an EPR-silent state has been studied electrochemically in order to establish the stoichiometry and reduction potential of the transition. During these studies it was established that the FeMo-cofactor exchanged electrons directly with an electrode.[10] The

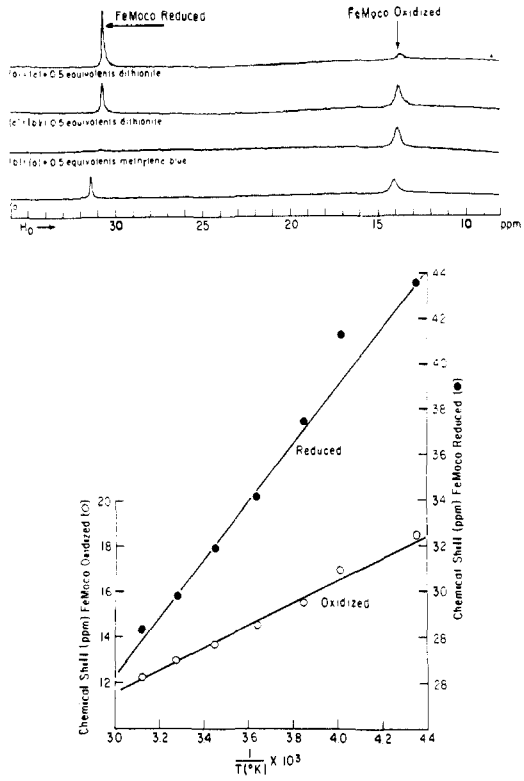


Figure 19. (Top) ^{19}F NMR spectra of a 2:1 mixture of *p*-$CF_3C_6H_4S^-$ and FeMo-cofactor as it comes from the storage vial (a), after 0.5 electron equivalent oxidation by methylene blue (b), after rereduction with 0.5 equivalent of dithionite (c), and after further reduction by an additional 0.5 equivalent of dithionite (d). (Bottom) Temperature dependence of ^{19}F shifts of oxidized and reduced FeMo-cofactor–*p*-$(CF_3)C_6H_4S^-$ complexes over the range of −60 to 30 °C.[86b]

stoichiometry was demonstrated to be one electron per FeMo-cofactor by (i) chemical titration of FeMo-cofactor$_{(ox)}$ with $S_2O_4^{2-}$ with concomitant EPR monitoring, (ii) comparison of the magnitude of the voltammetric peak currents with that of standard one-electron-transfer reagents, and (iii) controlled-potential coulometry in both the oxidizing and reducing directions.[10,87,105,106] Thus, the transition is analogous to the oxidation of the M-center of the MoFe protein. The FeMo-cofactor$_{(ox)}$ to $S = {}^3/_2$ transition was shown by cyclic voltammetry to be quasi-reversible with a formal reduction potential of −0.32 V vs SHE.[10] This potential is consistent with the range reported for the same transition in the MoFe protein,[104] allowing for shifts due to transfer from an aqueous protein matrix to NMF.[10] Mo XANES experiments, carried out at 4 °C on one-electron-oxidized FeMo-cofactor, demonstrate that the oxidation state of the Mo atom does not change during this transition.[56]

If the EPR-silent isolated FeMo-cofactor$_{(ox)}$ is analogous to the same state of the M-center of the MoFe protein then it should have an $S = 0$ ground state.[103] Mössbauer data collected on FeMo-cofactor$_{(ox)}$ in the temperature range 4.2–125 K were consistent with that assignment.[63] However, the ^{19}F chemical shift for the EPR silent, oxidized FeMo-cofactor *p*-$CF_3C_6H_4S^-$ complex was shown to be substantially isotropically shifted from its diamagnetic position near 1.5 ppm (Figure 19a).[86] The paramagnetism of this oxidized species was confirmed as shown in Figure 19b by temperature-dependence studies.[86] The apparent discrepancy between the $S = 0$ ground state observed for both the oxidized

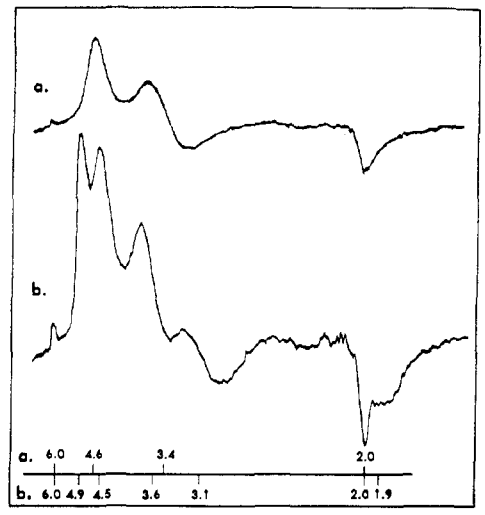


Figure 20. EPR spectra of FeMo-cofactor in NMF after dilution into alkaline solvent (a) or acidic solvent (b).[108]

M-center by low temperature MCD[103] and for the oxidized FeMo-cofactor by Mössbauer[63] and the paramagnetic behavior of the oxidized FeMo-cofactor–thiolate complex has yet to be resolved. At present the most likely explanation is that the oxidized FeMo-cofactor has a low-lying $S = 1$ (or other S = integer) state which is not populated at the cryogenic temperatures of the EPR (40–10 K), MCD (4 K), or Mössbauer (4–125 K) measurements, but is populated at the higher temperatures (240–320 K) used for the NMR experiment.[86]

It is interesting to note that in the ^{19}F NMR experiments separate signals for the oxidized and $S = {}^3/_2$ forms of FeMo-cofactor were observed in the same solutions (Figure 19a).[86] This requires that the electron exchange between them is slow on the NMR time scale at the concentrations employed (~1 mM). The observation that the resonances did not move toward each other when both oxidation states were present placed an upper limit on the rate constant for electron transfer between oxidized and reduced FeMo-cofactor–thiolate complex molecules of 5×10^3 M^{-1} s^{-1}.

Some protein-bound [FeS] clusters exhibit pH-dependent reduction potentials apparently because they exist in both protonated and deprotonated forms.[107] Because FeMo-cofactor solutions are in NMF, no detailed pH titrations have been carried out on this species. It is probable, however, that the oxidation experiments discussed above were carried out at alkaline pH values because only alkaline NMF will extract the FeMo-cofactor from the protein matrix. This was demonstrated for the ^{19}F NMR experiments where the free *p*-$CF_3C_6H_4S^-$ was shown to be deprotonated throughout the course of data collection.[86] Recently, Newton et al. have reported that the addition of acid to alkaline FeMo-cofactor solutions results in the formation of two new forms of FeMo-cofactor which exhibit different $S = {}^3/_2$ EPR signals and reduction potentials.[108] Figure 20 compares the EPR signals of electrochemically reduced, acidified FeMo-cofactor to that observed at alkaline pH. The alkaline form has apparent g values of 4.6, 3.4, and 2.0 while the two acid forms which appear together have g values of 4.5, 3.6, and 2.0, and 4.9, 3.1, and 1.9. All three forms were reported to have corresponding one-electron-oxidized states and to have different differential pulse voltammetric reduction peak values of −0.37 V vs SHE (al-

kaline), −0.32 V (acid, g = 4.5, 3.6, 2.0) and −0.43 V (acid, g = 4.9, 3.1, 1.9).[108] One possible explanation for these results is that isolated FeMo-cofactor can be protonated at two sites which have different pK_a values and that the acid-treated FeMo-cofactor was a mixture of species that were protonated at the higher pK_a site and at both sites. Note that protonation/deprotonation must be slow if two species are observed. It is not known if these sites would be available for protonation when the FeMo-cofactor is bound to the MoFe protein or if the one-electron oxidation of the protonated species would be accompanied by deprotonation.

B. In the Reducing Direction

For scientists interested in the possibility of using isolated FeMo-cofactor (or a synthetic analogue) as a catalyst for N_2 reduction, it is encouraging that isolated FeMo-cofactor can undergo the same reversible one-electron-oxidation reaction as the M-center in the MoFe protein. However, this transition is unlikely to occur in vivo under substrate-reducing conditions. Rather, during substrate reduction, the $S = {}^3/_2$ M-center is further reduced to an EPR-silent, (S = integer) state.[99-101] For the MoFe protein, this state can only be produced upon the addition of the physiological electron donor, the reduced Fe protein, and MgATP.[99-101] Thus, the EPR-silent, substrate-reducing state of the M-center has never been produced electrochemically or with any artificial electron donor.

In contrast to this result, an early report suggested that a presumably analogous, fully reduced state of isolated FeMo-cofactor could be produced in the presence of CO, using 5-deazaflavin as the photoactivated reductant and EDTA as the electron donor.[58] The disappearance of the $S = {}^3/_2$ EPR signal was reported to require these strongly reducing conditions together with CO, a potent inhibitor of N_2 reduction by nitrogenase, and was said to be reversed upon removal of the CO atmosphere.[58] However, a subsequent report demonstrated that the loss of the EPR signal depended only upon the addition of EDTA and occurred in the dark and in the absence of 5-deazaflavin and CO.[85] Thus, the reaction did not represent the simple reduction of isolated FeMo-cofactor to a fully reduced state. Attempts to reduce isolated FeMo-cofactor with $Fe_4S_4(SC_2H_5)_4^{3-}$, which has a reduction potential of −1.3 V vs SHE, also failed to remove the $S = {}^3/_2$ FeMo-cofactor EPR signal.[109] Thus, there is currently no evidence that a fully reduced state of isolated FeMo-cofactor, analogous to the substrate reducing state of the M-center, has been achieved by addition of chemical reductants.

One recent report indicated that such a state may have been produced electrochemically. In addition to the $S = {}^3/_2$ to S = 0 transition discussed above, cyclic voltammetry (Figure 21) of FeMo-cofactor at a glassy carbon electrode showed a second quasi-reversible reduction wave with a formal potential of −1.00 V vs SHE.[10] The production of this reduced species also appeared to require a single electron.[10] A determination of whether or not this species represents a state of FeMo-cofactor analogous to the fully reduced S = integer state of the MoFe protein must await its production in bulk for parallel spectroscopic studies. If it does represent fully reduced FeMo-cofactor; however,

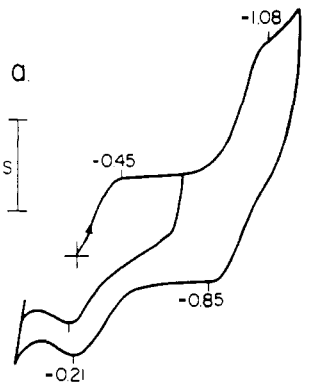


Figure 21. Cyclic voltammogram of FeMo-cofactor in NMF at a glassy carbon electrode. Peak positions are marked in volts vs SHE.[10]

it is interesting to note that unlike nitrogenase it is not catalytic with respect to H_2 production from H^+.

C. Interactions with Substrates and Inhibitors

In addition to its physiological substrates N_2 and H^+, nitrogenase catalyzes the reductions of a large number of nonphysiological substrates (e.g. HN_3, N_3^-, N_2O, NO_2^-, C_2H_2, HCN, CH_3NC).[110] It is generally accepted that all of these substrates bind to a metal atom or atoms in the MoFe protein of nitrogenase.[19,47,110,111] As shown in Figure 1, that protein contains P-clusters as well as FeMo-cofactor centers. This section discusses the currently available circumstantial evidence that within the MoFe protein, FeMo-cofactor is the site of substrate binding and reduction.

1. Biophysical Evidence

At present, direct evidence for the binding of substrates to the $S = {}^3/_2$ center of the MoFe protein is extremely limited. It includes the observation that the $S = {}^3/_2$ EPR signal is pH-dependent and that the pK_a of this signal is displaced in the presence of C_2H_2.[65,112] This phenomenon can be at least partially inhibited by CO, which is an inhibitor of substrate reduction by nitrogenase but not an inhibitor of H_2 evolution. These observation have been interpreted as evidence for the binding of C_2H_2 and CO to the $S = {}^3/_2$ center of the MoFe protein.[112] A transient EPR signal, observed during turnover in the presence of C_2H_2, has also been interpreted as evidence for C_2H_2 binding to the FeMo-cofactor center of the MoFe protein.[113] However, Mo K absorption edge and EXAFS spectra for the dithionite-reduced MoFe protein are identical before and after the addition of C_2H_2, CO, N_2, NaN_3, NaCN, or CH_3NC.[114] Thus, if these species do bind to the $S = {}^3/_2$ center of the MoFe protein, they do not appear to bind to Mo.[114]

One possible reason for the lack of direct spectroscopic evidence for substrate binding is that some substrates may only bind to the MoFe protein under turnover conditions. In fact, a large body of steady-state and pre-steady-state kinetic data have been used to argue that N_2, and possibly other substrates, can only bind after the MoFe protein has been reduced by more than one electron.[110,115] During nitrogenase turnover, after the addition of the reduced Fe protein and MgATP, the $S = {}^3/_2$ center of the MoFe protein is reduced to an EPR-silent, but paramagnetic, species.[99-101] Unfortunately, even in the absence of added substrates this

reduced MoFe protein catalyzes the reduction of $2H^+$ to H_2 gas. Consequently, a reduced form of the MoFe protein cannot be isolated and has never been used for substrate-binding experiments.

Another possible reason for the lack of direct evidence for substrate binding may be that the FeMo-cofactor center of the MoFe protein is somehow buried within the protein and physically inaccessible to substrate binding except under turnover conditions. If this is the case, however, substrates should bind to the $S = {}^3/_2$ state of isolated FeMo-cofactor in NMF. In fact, direct evidence for CN^- and CH_3NC binding has recently been obtained via two independent routes.[26,114] First, in 1985 Smith et al. showed that the $S = {}^3/_2$ EPR signal exhibited by isolated FeMo-cofactor in NMF changed upon addition of CN^- and that the change was completed at a FeMo-cofactor:CN^- ratio of less than 2.5.[26] Second, using ^{19}F NMR as a probe and p-$CF_3C_6H_4S^-$ as the reported ligand, Conradson et al. showed that both CN^- and CH_3NC bind to the isolated FeMo-cofactor–thiolate complex without displacing the thiolate ligand.[114] Thus, CN^- and CH_3NC bind to isolated FeMo-cofactor but at a site distinct from the thiolate binding site. The NMR study showed that their binding increased the electronic relaxation time of the FeMo-cofactor–thiolate complex and increased the lifetime of the FeMo-cofactor–p-$CF_3C_6H_4S^-$ bond from ca. 0.1 to 1.0 s.

Although the EPR data were originally interpreted in terms of two CN^- binding sites,[26] the NMR data were taken to mean that FeMo-cofactor had a single CN^- or (CH_3NC) binding site with a finite formation constant.[114] Parallel Mo K XANES and EXAFS experiments showed that CN^- did not bind to the Mo atom in isolated FeMo-cofactor.[114] On the basis of prior kinetic studies, cyanide and CH_3NC had both been proposed to bind to the FeMo-cofactor site of the MoFe protein in two different ways.[116] In their productive binding modes, cyanide was reduced by six electrons to methane plus ammonia, while CH_3NC was reduced by six electrons to methane plus methylamine. In their nonproductive binding modes CN^- and CH_3NC prevented electron transfer to substrate, causing inhibition of electron flow but not of ATP hydrolysis. The direct evidence for CN^- and CH_3NC binding just discussed did not address whether these species were bound to isolated FeMo cofactor in their productive or nonproductive modes.

CO can both inhibit cyanide (or CH_3NC) reduction and relieve CN^- (or CH_3NC) inhibition of electron flow through nitrogenase.[115,116] However, unlike the situation for CN^- and CH_3NC, no change in the ^{19}F NMR spectrum of the FeMo-cofactor–p-$CF_3C_6H_4S^-$ complex was observed upon addition of 3 atm of CO. This observation strongly suggests that CO does not bind to the isolated FeMo-cofactor–p-$CF_3C_6H_4S^-$ complex in NMF. Thus, the CO binding site must be distinct from the CN^- (or CH_3NC) binding site. These observations have also led to the suggestion that the CO binding site exists only when the FeMo-cofactor is bound to the MoFe protein.[114]

2. Biochemical/Genetic Evidence

In 1984, Hawkes, McLean, and Smith performed the experiment that is most often quoted as providing the best evidence that FeMo-cofactor is the site of N_2 binding and reduction.[47] First they purified the MoFe protein from a Nif V^- strain of *K. pneumoniae*. As discussed in section III.B., this protein could reduce C_2H_2 but could not fix N_2. It also had altered reactivity toward CO such that CO inhibited H_2 evolution by the Nif V^- protein but not by the wild type MoFe protein. Hawkes et al. isolated the FeMo-cofactor from purified Nif V^- MoFe protein and used it to activate the inactive FeMo-cofactor-deficient MoFe protein as synthesized by a Nif B^- strain. The resulting holoprotein was indistinguishable from the Nif V^- protein (i.e. it reduced C_2H_2 but not N_2, and CO inhibited H_2 evolution). Thus, the inability of the Nif V^- protein to fix N_2 was due to a defect in the FeMo-cofactor, not in the protein. That defect has subsequently been shown to be the substitution of citrate for homocitrate (section III.B.).

The Nif V^- experiment demonstrated that a subtle alteration in the environment of FeMo-cofactor caused a change in its reactivity toward N_2 and CO. It is not known if that change was a direct result of ligation by citrate instead of homocitrate or if the effect was indirect. For example, the citrate form of FeMo-cofactor might not "fit" into the protein properly such that other protein interactions or the reduction potential of the cluster might have been affected. Possible support for the latter idea has come with the recent construction of a site-directed mutant of the MoFe protein α-subunit, where a glutamine at position 191 was substituted for a lysine.[117] The MoFe protein from this site-directed mutant had the same phenotype as the Nif V^- protein in that it reduced C_2H_2 but not N_2, and CO inhibited its H_2 evolution. However, the FeMo-cofactor from that protein was the same as the wild type FeMo-cofactor. Taken together the above data demonstrate that subtle alterations in the environment of the FeMo-cofactor cluster by changing either the endogenous ligand homocitrate or the protein residues near the cluster can cause changes in the substrate reactivity of the enzyme.

3. Substrate Reduction by Isolated FeMo-Cofactor

As discussed in section VI.B., one electron electrochemically reduced FeMo-cofactor does not catalyze the reduction of H^+ to H_2, and no stable chemically reduced forms of isolated FeMo-cofactor have been produced. Nonetheless, the reduction of C_2H_2 to C_2H_4 by isolated FeMo-cofactor has been reported and interpreted in terms of providing evidence that FeMo-cofactor is the substrate reducing site of the enzyme.[118] In those experiments FeMo-cofactor was reduced by $NaBH_4$ in a borate–NaOH buffer at pH 9.6 and used as a catalyst for C_2H_2 reduction. The rate of C_2H_2 reduction in that system was ca. 8% of the enzymatic rate. This experiment does not prove that intact FeMo-cofactor was responsible for the observed C_2H_2 reduction activity, because numerous synthetic Fe and Mo compounds can also catalyze C_2H_2 reduction to C_2H_4 with $NaBH_4$ as the reductant.[118,119] O_2-degraded FeMo-cofactor also reduced C_2H_2 to C_2H_4 at similar rates, suggesting that an unknown FeMo-cofactor decomposition product(s) was (were) the actual catalyst in the $NaBH_4$ system.[118] In addition to C_2H_2 reduction, the $NaBH_4$/FeMo-cofactor system reduces the nitrogenase substrate cyclopropene.[120] However, unlike the enzyme, which reduced cyclopropene to a 2:1 ratio of propylene to cyclo-

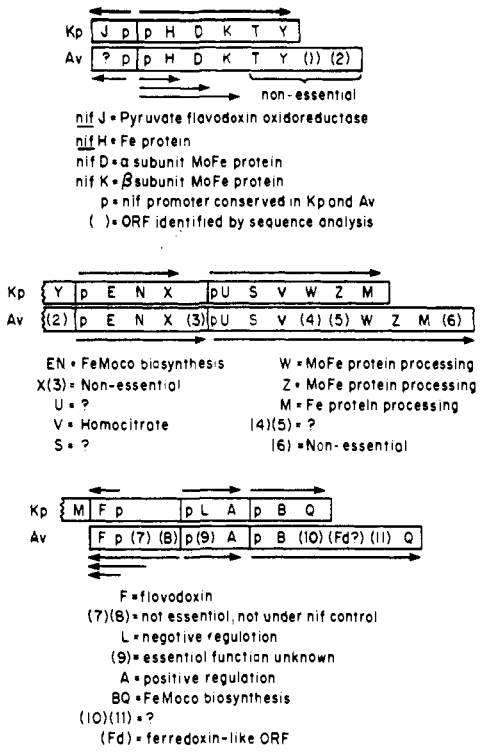


Figure 22. Nitrogen fixation (*nif*) gene cluster from *K. pneumoniae* and *A. vinelandii* showing function of each gene. Arrows represent the direction of mRNA transcript. (Note that in *K. pneumoniae* the genes are in one cluster on the chromosome while in *A. vinelandii* they are split into two clusters with a large piece of DNA between ORF(6) and *nif*F.)

propane,[121] the $NaBH_4$/FeMo-cofactor system only produced cyclopropane as a product.[120] Thus, these experiments do not provide compelling evidence that FeMo-cofactor is the site of substrate binding and reduction.

VII. FeMo-Cofactor Biosynthesis

A. In Vivo

FeMo-cofactor is a metal cluster of composition 1Mo:6–8Fe:8–9S:1homocitrate which is degraded in the presence of O_2 or H_2O. The challenge to the bacterial cell is to assemble the FeMo-cofactor from its component parts, while protecting it from the aqueous (and possibly aerobic in some cases) environment of the cell. One potential approach would be to use the MoFe protein polypeptides as scaffolds for the construction of FeMo-cofactor in place. However, it has been definitively established by three independent groups that FeMo-cofactor is not synthesized in this way.[122-124] Rather, FeMo-cofactor is accumulated in vivo in the absence of the MoFe protein polypeptides, which leads to the conclusion that it must be assembled elsewhere in the cell and later incorporated into the FeMo-cofactor-deficient MoFe protein.[122-124] This section briefly discusses what is known about the participation of nitrogen fixation (*nif*) specific gene products (Figure 22) in the synthesis and accumulation of FeMo-cofactor in vivo. This subject has recently been reviewed elsewhere.[125] *nif*-Specific gene products are only synthesized when cells are fixing N_2: the genes are turned off when the cells are supplied with a fixed form of nitrogen like NH_4^+.

1. Starting Material

In order to synthesize FeMo-cofactor, bacterial cells need iron, sulfur, molybdenum, and homocitrate. Iron is generally supplied in the medium as Fe^{2+} or Fe^{3+} salts and sulfur is generally added as SO_4^{2-}. Iron and sulfur are needed for numerous proteins in addition to nitrogenase, and each organism has a general system for the uptake, processing, and accumulation of these elements. There is no information to suggest that special nitrogen fixation specific gene products are required. In addition to FeMo-cofactor, nitrogenase, and many other bacterial proteins, contain [FeS] clusters. It is not known how these clusters are assembled in vivo and the process may well use currently unidentified enzymes. This putative biosynthetic pathway for [FeS] clusters could also be recruited, if necessary, for the synthesis of a portion of FeMo-cofactor, but there is currently no information to indicate whether or not that is the case.

The mechanisms involved with Mo uptake and processing are more specific than those for iron and sulfur because Mo is found in only a few bacterial enzymes (e.g. nitrogenase, formate dehydrogenase, sulfite oxidase, xanthine oxidase, nitrate reductase). It is generally supplied in the medium as MoO_4^{2-} and organisms which contain molybdenum enzymes have systems for MoO_4^{2-} uptake and accumulation.[126] In certain nitrogen-fixing organisms some genes which encode proteins involved in general MoO_4^{2-} processing appear to be under *nif* control. For example, *E. coli* is a non-N_2-fixing organism which needs MoO_4^{2-} to synthesize nitrate reductase and formate dehydrogenase. It contains a gene called *chl*D which is needed for MoO_4^{2-} uptake or processing. If there is a mutation in *chl*D, the organism can still synthesize its Mo enzymes provided a much higher than normal concentration of MoO_4^{2-} is present in the medium.[25] At least one N_2-fixing organism appears to have recruited this general Mo-processing gene into its *nif* region. Thus, the *nif* gene region of *Anabaena* 7120 has recently been shown to contain a piece of DNA encoding a protein with extensive sequence similarity to the *E. coli chl*D gene.[127] Similarly, the *nif* gene region of the *C. pasteurianum* chromosome contains an open reading frame with sequence similarity to the *E. coli chl*J gene, which is also involved in MoO_4^{2-} processing.[128]

In addition to iron, sulfur, and molybdenum, FeMo-cofactor biosynthesis requires a source of homocitrate. As discussed in section III.B. homocitrate synthesis requires the participation of the *nif*V gene product, which is believed to encode the enzyme homocitrate synthetase.

2. FeMo-Cofactor Assembly

At some point during FeMo-cofactor biosynthesis the Mo atom in MoO_4^{2-} must be reduced and its ligation sphere changed from all O to mixed S and O(N). Because this Mo environment is unique to nitrogenase and is not found in other Mo enzymes,[126] this process may involve one or more *nif*-specific enzymes. One *nif*-specific protein that has been proposed to be involved in molybdenum processing is the *nif*Q gene product. The *nif*Q encoded protein has never been identified, but the *nif*Q sequence has been determined.[129-131] *K. pneumoniae* strains with mutations in the *nif*Q gene

were able to fix N_2 only when extremely high (>10 μM versus 10 nM for wild type cells) MoO_4^{2-} concentrations were available.[132] The Nif Q$^-$ cells were not deficient in their ability to transport MoO_4^{2-}, but they accumulated lower levels of Mo than wild type cells.[132] When Nif Q$^-$ mutants were supplied with a reduced form of sulfur, by substituting SO_4^{2-} with cysteine in the medium, they fixed N_2 with lower levels of MoO_4^{2-} in the medium and they accumulated higher levels of molybdenum.[133] This apparent relationship between reduced sulfur and molybdenum in Nif Q$^-$ mutants has led to the proposal that the *nif*Q gene product may be involved in the synthesis of a Mo–S compound for eventual incorporation into FeMo-cofactor.[125]

Another gene which is unambiguously involved in FeMo-cofactor biosynthesis is the *nif*B gene. Organisms which have defects in the *nif*B gene accumulate an inactive FeMo-cofactor-deficient form of the MoFe protein but do not accumulate FeMo-cofactor. Their inactive MoFe protein can be activated in vitro by the addition of isolated FeMo-cofactor in NMF.[1] The *nif*B-encoded protein has never been purified, but the *nif*B sequence has been determined.[130,131,134,135] The ca. 50 000 M_r *nif*B polypeptide has also been observed on SDS–polyacrylamide gels of cell free extracts.[136] The distribution of cysteine residues[130,131,134,135] and the known O_2 sensitivity of the *nif*B product[137] suggest that it contains an [FeS] cluster binding site. Nitrogenase systems which do not use Mo still require the *nif*B gene product (see section VIII). Thus, although its function in FeMo-cofactor biosynthesis is unknown, the *nif*B gene product is probably involved in the synthesis of the [FeS] portion of FeMo-cofactor and not the Mo portion.

The *nif*Q and *nif*B gene products discussed above may be enzymes which carry out essential steps in FeMo-cofactor biosynthesis. Two other *nif* genes, *nif*E and *nif*N, are thought to participate in FeMo-cofactor biosynthesis in a different way by providing a site for FeMo-cofactor assembly.[138] This proposal was based on a comparison of the sequences of the *nif*EN gene products to those of the *nif*DK gene products which encode the α- and β-subunits of the MoFe protein of nitrogenase.[134,138–141] The conclusion from those comparisons was that the *nif*E gene shared sequence identity with the *nif*D gene while the *nif*N gene shared sequence identity with the *nif*K gene. This led to the proposal that *nif*EN might form an $\alpha_2\beta_2$-protein analogous to the $\alpha_2\beta_2$ *nif*DK-encoded MoFe protein,[138] with the putative *nif*EN protein containing a FeMo-cofactor assembly site analogous to the FeMo-cofactor binding site of the MoFe protein. Some support for this idea has come with the purification from *A. vinelandii* of the *nif*EN encoded protein complex which was shown to be an $\alpha_2\beta_2$-tetramer.[142] Although that protein appeared to contain Fe, it has not as yet been characterized with respect to the presence or absence of intermediates of FeMo-cofactor biosynthesis, so that it is premature to conclude that it contains the site of FeMo-cofactor assembly. It should also be noted that the *nif*EN product was purified from a Nif B$^-$ mutant.[142] Thus, the possibility that the *nif*EN $\alpha_2\beta_2$-protein might normally be associated with the *nif*B gene product in vivo cannot be eliminated. Whatever the function of the *nif*EN product in FeMo-cofactor biosynthesis, it is clearly required. Strains with mutations in the *nif*E or *nif*N genes synthesize inactive FeMo-cofactor-deficient MoFe proteins but do not accumulate FeMo-cofactor. Those proteins can be activated in vitro by addition of isolated FeMo-cofactor.[1]

A final *nif* gene unambiguously required for FeMo-cofactor biosynthesis is, surprisingly, the *nif*H gene, which encodes the Fe protein of nitrogenase.[143–145] When the *nif*H gene was deleted from the *A. vinelandii* chromosome, the cells did not synthesize FeMo-cofactor but did accumulate an inactive FeMo-cofactor-deficient MoFe protein that could be activated in vitro by addition of isolated FeMo-cofactor.[144,145] During nitrogenase turnover the Fe protein forms a complex with the MoFe protein and serves as a specific electron donor to that protein. The sequence similarities between the $\alpha_2\beta_2$ *nif*DK-encoded MoFe protein and the $\alpha_2\beta_2$ *nif*EN-encoded protein make it tempting to speculate that during FeMo-cofactor biosynthesis the Fe protein binds to the *nif*EN protein. The specific function of the Fe protein in FeMo-cofactor biosynthesis is unknown and therefore it is not clear if the Fe protein serves as an electron donor for some essential step in FeMo-cofactor biosynthesis. It is known, however, that to be effective in FeMo-cofactor biosynthesis the Fe protein does not have to be catalytically active in nitrogenase turnover.[143–145]

In addition to the *nif*Q, -B, -N, -E, -V, and -H gene products, which have confirmed roles in FeMo-cofactor biosynthesis, a number of other less well characterized proteins may be involved. For example, when FeMo-cofactor was synthesized by mutant strains which did not synthesize the MoFe protein polypeptides, FeMo-cofactor accumulated on an unknown ca. 50 000 M_r protein, distinct from the *nif*NE protein.[146] This protein may therefore be involved in FeMo-cofactor assembly. Another example is the *nif*X gene found in the same operon as the *nif*EN genes in a number of organisms (Figure 22). This gene shares sequence identity with the *nif*B gene, leading to the suggestion that it also may be involved in FeMo-cofactor biosynthesis.[140] Finally, sequence analysis has also shown that in some, but not all, nitrogen-fixing organisms there is a gene in the same operon as the *nif*B gene, which encodes a ferredoxin-like protein.[129,130,134,147,148] In at least one case this gene is required for nitrogen fixation.[140] That observation, and the close proximity of this ferredoxin-like gene to the *nif*B gene, suggests that it also may be involved in FeMo-cofactor biosynthesis.

3. Sequence of Events

In vivo, the assembly of an active MoFe protein requires the synthesis of FeMo-cofactor, the synthesis of the FeMo-cofactor-deficient MoFe protein and the insertion of the former into the latter. The sequence of events in FeMo-cofactor biosynthesis is currently unknown. The most plausible scenario at present is that the *nif*Q product processes Mo while the *nif*B product assembles [FeS] to form a FeMo-cofactor precursor which is bound to the $\alpha_2\beta_2$ *nif*EN protein. It is likely that this protein also interacts in some way with the Fe protein. The synthesis of the FeMo-cofactor-deficient MoFe protein similarly takes place in a series of steps which are beyond the scope of this review. At present there is no evidence to establish when the indirect *nif*V

gene product, homocitrate, enters the scheme. Although it is likely to be during FeMo-cofactor biosynthesis or insertion, it could also be during the synthesis of the FeMo-cofactor-deficient MoFe protein. The biochemical data only require that homocitrate leaves with FeMo-cofactor when it is artifically extracted from denatured MoFe protein.

B. In Vitro

To date, no FeMo-cofactor intermediates have been isolated from mutants that are blocked in specific steps in FeMo-cofactor biosynthesis and only one altered form of FeMo-cofactor (the citrate-containing Nif V⁻ FeMo-cofactor) has been obtained by using the in vivo approach. A more promising approach appears to be the development of an in vitro system for FeMo-cofactor biosynthesis and such a system has recently been reported.[149] The system requires at a minimum the *nif*B, *nif*N, and *nif*E gene products, Fe protein, dithionite, MoO_4^{2-}, homocitrate, and MgATP.[125,142,149] The requirement for MgATP is interesting since the Fe protein contains two MgATP binding sites and normally hydrolyzes MgATP during its transfer of one electron to the MoFe protein.[110,115] Thus, the requirements for the Fe protein and for MgATP may be related. FeMo-cofactor has not been isolated directly from the in vitro system, but is identified after it has been incorporated into a FeMo-cofactor-deficient form of the MoFe protein.[125,142,149]

At present this in vitro system relies on the addition of cell-free extracts as a source of the *nif*B gene product and results in low yields of FeMo-cofactor when compared to in vivo synthesis. Hopefully, this system will soon be completely defined, with only purified components, making it possible to isolate and characterize FeMo-cofactor biosynthetic intermediates.[142] Despite its current limitations, this system has been successfully used by Hoover et al. to synthesize altered forms of FeMo cofactor with homocitrate derivatives, in attempts to determine which portions of the homocitrate molecule were necessary for FeMo-cofactor synthesis.[125] In the future, isotopically labeled FeMo-cofactor may be prepared in the same way for use in NMR or ENDOR experiments.

VIII. Nitrogenases without Molybdenum

Until recently it was generally accepted that biological N_2 fixation could only be catalyzed by the Mo-containing nitrogenase. In 1980, Bishop et al. published a controversial paper in which they provided evidence for the presence of an additional, Mo-independent system for N_2 fixation.[150] In that study they reported that a number of Nif⁻ mutants of *A. vinelandii* could be made to fix N_2 if they were grown on media that did not contain Mo. The strains tested included those with point mutations in the *nif*HDK gene cluster as well as regulatory mutants which did not synthesize the MoFe protein polypeptides. These data were interpreted as providing evidence that *A. vinelandii* had an alternative nitrogenase system which was not synthesized under normal growth conditions but became derepressed when the cells were starved for Mo.[150] The validity of this interpretation was definitively demonstrated in 1986.[151–153] In those studies, the DNA encoding the Mo

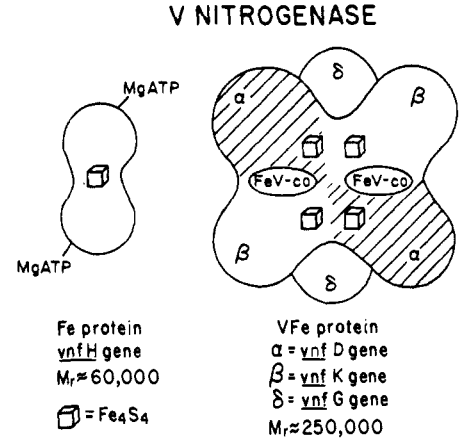


Figure 23. Schematic representation of V nitrogenase showing both component proteins and their metal centers.

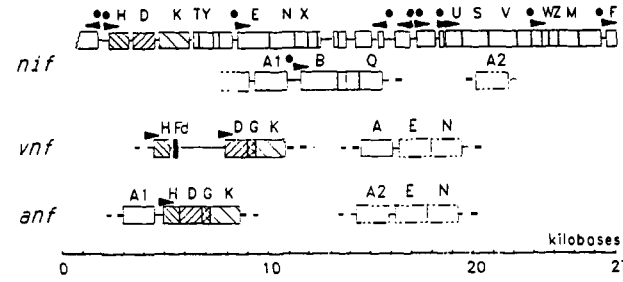


Figure 24. Physical map of nitrogenase genes in *A. vinelandii*. Nitrogenase structural genes are hatched. Genes associated with different nitrogenases are distinguished by different genotypic names: *nif* (Mo nitrogenase gene cluster), *vnf* (V nitrogenase gene cluster), *anf* (the third "alternative" nitrogenase gene cluster).[154c]

nitrogenase (*nif*HDK) structural polypeptides was specifically deleted from the *A. vinelandii* and *A. chroococcum* chromosomes by using recombinant DNA techniques. The resulting strains could still fix N_2 if Mo was not present in the medium.[151–153] Since that time it has become generally accepted that *Azotobacter* contains nitrogenase systems which do not use Mo. Here, the properties of these systems will be briefly reviewed with emphasis on what is known about their FeMo-cofactor-like clusters. This subject has recently been reviewed in more detail.[154]

A. Vanadium Nitrogenase

Following the construction of *Azotobacter* strains which were lacking the DNA encoding Mo nitrogenase, it was demonstrated that the ability of these strains to fix N_2 in the absence of Mo could be greatly enhanced by addition of vanadium (V) to the media.[154b,c] This observation quickly led to the purification of a vanadium–iron protein, (VFe) protein from both *A. chroococcum*[155,156] and *A. vinelandii*.[157,158] The V nitrogenase system (Figure 23) is similar to the Mo system in that it contains an Fe protein and a VFe protein.[154] The Fe protein is encoded by the *vnf*H gene, which is closely related to the *nif*H gene. The VFe protein has six subunits, two encoded by the *nif*D-like *vnf*D gene, two by the *nif*K-like *vnf*K gene, and two encoded by the *vnf*G gene, which does not have a counterpart in the *nif* system.[159] The organization of these genes relative to the Mo system is shown in Figure 24.

Like the MoFe protein, the VFe protein appears to contain two general types of metal clusters, unusual

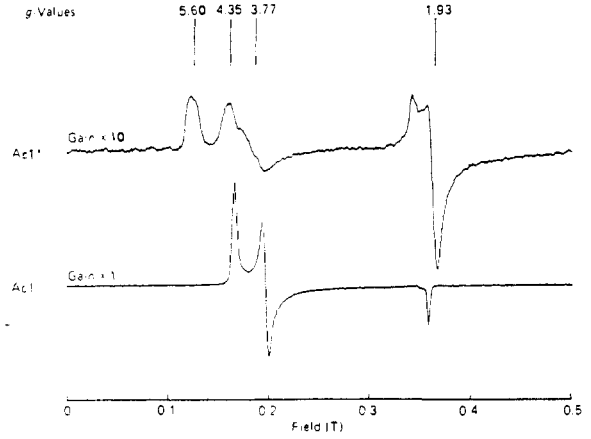


Figure 25. EPR spectra at 10 K of the *A. chroococcum* MoFe protein (bottom) and VFe protein (top).[156]

TABLE 6. Average EXAFS Results on the V Site in the VFe Protein[162,163]

	O shell		S shell		Fe shell	
	V–O dist, Å	no. of atoms	V–S dist, Å	no. of atoms	V–Fe dist, Å	no. of atoms
semireduced VFe protein	2.15	3 ± 1	2.31	3 ± 1	2.75	3 ± 1

[FeS] clusters and FeV-cofactor centers, analogous to FeMo-cofactor.[154] Much of the currently available information about the FeV-cofactor comes from spectroscopic characterization of the VFe protein. The dithionite-reduced VFe protein has been characterized with respect to the temperature dependence of its MCD spectra.[160] The data were consistent with the presence of a paramagnetic cluster with an $S = {}^3/_2$ ground state. However, the electronic and magnetic properties of the vanadium $S = {}^3/_2$ center were quite distinct from those of the molybdenum $S = {}^3/_2$ center.[160] An $S = {}^3/_2$ ground state for the dithionite-reduced FeV-cofactor center of the VFe protein was also assigned on the basis of EPR experiments.[154,157,161] The EPR signal of the VFe protein for *A. chroococrum* has *g* values of 5.6, 4.35, 3.7, and 1.93 (Figure 25) and is significantly different in shape from the MoFe protein signal. The VFe protein EPR signal is also weaker than the corresponding $S = {}^3/_2$ MoFe protein signal,[156,161] but both signals integrate to one spin per V (or Mo).[161] The environment surrounding the V atom in the VFe protein has also been investigated by V K edge XAS studies and the quantitative EXAFS data are shown in Table 6.[162,163] Like the Mo atom in FeMo-cofactor, the V atom in the VFe protein probably has pseudo-octahedral geometry and is surrounded by three O(N) atoms at 2.15 Å and three S atoms at 2.31 Å in the first coordination shell with ca. three Fe atoms at 2.75 Å. As shown in Figure 26 the best available model for this V site is the synthetic V complex $[Me_4N][VFe_3S_4Cl_3(DMF)_3]$.[164]

The spectroscopic data for the VFe protein strongly suggest that the VFe protein contains a FeV-cofactor analogous to the FeMo-cofactor. In fact, this FeV-cofactor has been isolated in NMF from the VFe protein using the original FeMo-cofactor isolation procedure.[165] Like FeMo-cofactor, the isolated FeV-cofactor could activate inactive FeMo-cofactor-deficient forms of the MoFe protein.[162] The FeV-cofactor was reported to have a stoichiometry of 1V:5–6Fe:4–5S. Judging from the history of analytical measurements on FeMo-cofactor (section III), it is premature to decide whether those analytical ratios are significantly different from FeMo-cofactor. No complete spectroscopic characterization of isolated FeV-cofactor has yet appeared. In addition to this biochemical evidence there is mounting genetic evidence which supports a close relationship between the Mo- and V-containing cofactors. Thus, the FeMo-cofactor biosynthetic *nif*B gene is required for both systems[130] while genes analogous to the nif*EN* genes are also found in the *vnf* system (Figure 24).

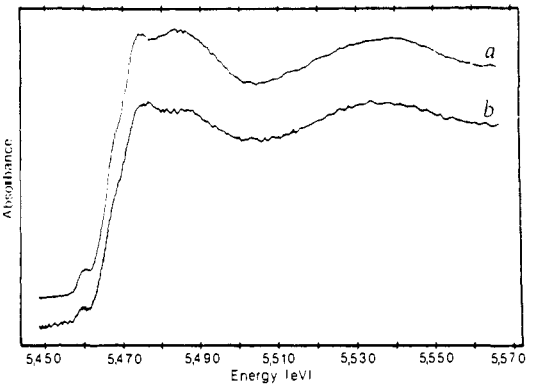


Figure 26. V K edge and XANES of $[Me_4N][VFe_3S_4Cl_3(DMF_3)]$[164] (a) and the VFe protein from *A. chroococum* (b).[164]

The substrate reduction pattern for the V nitrogenase has not yet been studied in detail. One significant difference from the Mo system is that C_2H_2 reduction by Mo nitrogenase is more successful in competing with H_2 evolution for electrons than is C_2H_2 reduction by V nitrogenase.[156] The products of C_2H_2 reduction by the two enzymes are also different, such that the Mo system takes C_2H_2 exclusively to C_2H_4, while the V system produces significant (ca. 2%) C_2H_6 as an additional product.[166] The appearance of C_2H_6 has been proposed as a test for the presence of V nitrogenase in vivo.[166] This proposal should be viewed with caution, however, on the basis of the recent demonstration of C_2H_6 production by a site-directed mutant of Mo nitrogenase which contains a normal FeMo-cofactor.[96] These data suggest that it is not necessarily the substitution of V for Mo that causes the production of C_2H_6 but rather a change in the protein environment of the cluster. It is interesting to note that when the FeV-cofactor is used to activate the inactive FeMo-cofactor-deficient MoFe protein, the resulting hybrid protein still produces C_2H_6 from C_2H_2. Preliminary experiments indicate that the redox properties of the FeV-cofactor in the VFe protein are generally similar to those of the FeMo-cofactor.[163]

B. Nitrogenase without either Mo or V

In 1936 Bortels first reported that V could substitute for Mo in biological N_2 fixation by *A. vinelandii*.[167] It has taken the scientific community 50 years to accept that all nitrogenases do not contain Mo. There is now definitive evidence that in addition to the V nitrogenase system there is a third form of nitrogenase that does not require either V or Mo. This possibility was first indicated by Bishop et al., who initially observed that Nif$^-$ mutants of *A. vinelandii* could grow under N_2-fixing conditions in the absence of Mo or V.[150] Pau et al. provided definitive evidence for a third nitrogenase by removing the DNA encoding both the Mo nitrogenase and the V nitrogenase systems from the *A. vine-*

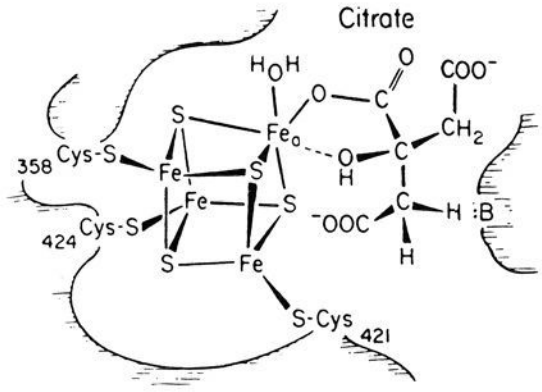


Figure 27. Scheme summarizing available information on the active site of aconitase and the binding of substrate to the [FeS] cluster. The figure shows citrate bound. When isocitrate is bound, the molecule is flipped by 180 ° so that the CH_2COO^- group points downward and the carboxyl at the bottom in the figure now becomes bound to Fe_a.[171]

landii chromosome and showing that the resulting strain could still fix N_2.[168] The same type of experiment demonstrated that *A. chroococcum* only has the Mo and V systems. The *A. vinelandii* genes encoding the nitrogenase structural proteins for this third system have now been sequenced and their organization is shown in Figure 24.[169]

It is not yet known what type of cofactor serves at the active site of this third system. It must have some properties in common with FeMo-cofactor because its biosynthesis requires the *nif*B gene product[130] and separate versions of the *nif*NE genes.[154] The protein analogous to the MoFe and VFe proteins has been partially purified from this third system and shown to reduce H^+, N_2, and C_2H_2 (to C_2H_4 and C_2H_6).[170] Although the partially purified protein contained only very low levels of metals other than Fe, it also had very low substrate-reducing activity, so it is premature to conclude that it is an Fe-only nitrogenase.

IX. Outlook

Most of what we know about the structure of FeMo-cofactor has come from spectroscopic experiments and this information is summarized in section IV.F. In the future these techniques will continue to increase our understanding of FeMo-cofactor. XAS at the Mo, Fe, S, and V edges using highly active samples may provide the best information about the positions of those metal atoms relative to each other. EPR-related techniques and NMR experiments on isotopically labeled FeMo-cofactor should continue to provide detailed information not only about the metal atoms but also about homocitrate. It is also possible that the availability of the V nitrogenase (and the third nitrogenase system) may make the cofactor problem accessible to other spectroscopic techniques.

In spite of the wealth of information that has come, and will continue to come, from spectroscopic experiments, it is unlikely that they will provide a complete three-dimensional structure of FeMo-cofactor in the near future. One way that structure may be obtained is through the chemical synthesis of a synthetic FeMo-cofactor. It is likely that the recent discovery of homocitrate, as an endogenous organic component of FeMo-cofactor, will stimulate chemists to produce additional model complexes. In this regard, it is not known if homocitrate is a terminal ligand to Mo or to one of the Fe atoms in FeMo-cofactor or if it bridges two portions of the cluster. In considering the use of

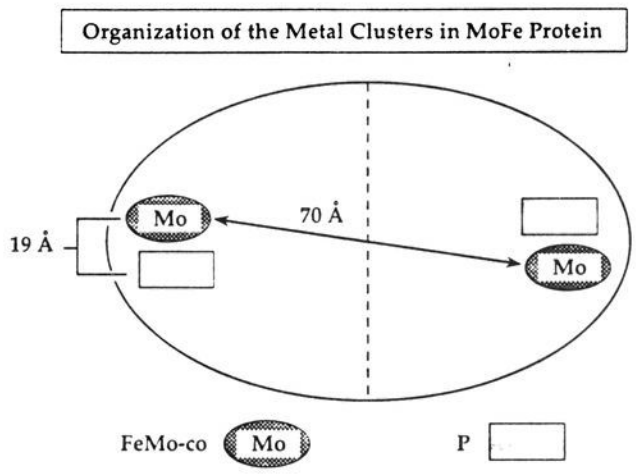


Figure 28. A schematic representation of the spatial arrangement of the metal–sulfur cluster bound to the *C. pasteurianum* MoFe protein as determined by X-ray anomalous scattering.[172] The representation of the large, "8-Fe" cluster with a P symbol indicates that it contains the Fe atoms normally assigned to P-clusters.

homocitrate for chemical synthesis, however, chemists may take a clue from biology. Thus, another enzyme, aconitase, has recruited an $[Fe_4S_4]$ cluster to participate in the conversion of citrate to isocitrate. Figure 27 shows how citrate is believed to bind to a single Fe atom at the active $[Fe_4S_4]$ cluster site of aconitase.[171]

The complete three-dimensional structure of FeMo-cofactor may also be obtained from the structure of the MoFe protein. As shown in Figure 28 determination of the structure of the MoFe protein from *C. pasteurianum* is in progress and a 5-Å resolution map has been derived.[172] These data confirm the independence of the two FeMo-cofactor clusters within the $\alpha_2\beta_2$ MoFe protein tetramer, show that they are 70 Å apart, and suggest that they may be close to the surface of the protein. They also indicate that the FeMo-cofactor is within electron-transfer distance, 19 Å away from the P-clusters contained within the MoFe protein. While the refinement of the protein structure may soon be available, the scientific community probably should not rely solely on the protein data to resolve the positions of individual metal atoms within the FeMo-cofactor. Rather, they should continue vigorously in attempts to crystallize isolated FeMo-cofactor in order to obtain a high-resolution structure. It is likely that the recent publication of simpler isolation procedures, especially those which use solvents other than NMF, will stimulate many groups to attack this problem.

Once the structure of FeMo-cofactor is known and synthetic FeMo-cofactor is available, the challenge will be to reduce FeMo-cofactor and use it as a catalyst for substrate reduction. It is encouraging that isolated FeMo-cofactor does have some of the redox properties of the FeMo-cofactor center of the MoFe protein and that it interacts directly with an electrode. The production of catalytically active FeMo-cofactor, however, may not be as simple as electron transfer and may require knowledge of how the task is accomplished within the enzyme nitrogenase. This information will continue to come from a combined biochemical/genetic/spectroscopic approach.

X. Acknowledgments

We are grateful to Drs. Jeff Bolin, Barry Smith, and William E. Newton for providing research results prior to publication and to NIH Grant No. GM43144 for

funding our recent research on FeMo-cofactor.

XI. Abbreviations

ATP, adenosine 5′-triphosphate; Bu, butyl; DEAE, (diethylamino)ethyl; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetracetic acid; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance, EXAFS, extended X-ray absorption fine structure; Fe protein, the iron protein component of nitrogenase; FAB, fast atom bombardment; M-center, the species which gives rise to the $S = {}^3/_2$ EPR signal of the MoFe protein; MCD, magnetic circular dichroism; MoFe protein, the molybdenum–iron protein component of nitrogenase; M_r, molecular weight; NMF, *N*-methylformamide; *o*-phen, 1,10-phenanthroline; ORF, open reading frame (a piece of DNA encoding an unknown protein); P-cluster, the non-FeMo-cofactor, [FeS] clusters of the MoFe protein; SHE, standard hydrogen electrode; THF, tetrahydrofuran; tris, tris(hydroxymethyl)aminomethane; UV/vis, ultraviolet/visible; VFe protein, the vanadium iron protein of nitrogenase; XAS, X-ray absorption spectroscopy; XANES, X-ray adsorption edge and near edge.

Registry No. Nitrogenase FeMo-cofactor, 72994-52-6.

References

(1) (a) Shah, V. K.; Brill, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 3249. (b) Shah, V. K. *Methods Enzymol.* **1980**, *69*, 792.
(2) Pienkos, P. T.; Shah, V. K.; Brill, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5468.
(3) Lough, S. M.; Jacobs, D. B.; Parker, S. F.; McDonald, J. W.; Watt, G. D. *Inorg. Chem. Acta* **1988**, *151*, 227.
(4) Burgess, B. K.; Jacobs, D. B.; Stiefel, E. I. *Biochim. Biophys. Acta* **1980**, *614*, 196.
(5) Smith, B. E. In *Molybdenum Chemistry of Biological Significance*; Newton, W. E., Otsuka, S., Eds.; Plenum: New York, 1980; p 179.
(6) Yang, S.-S.; Pan, W.-H.; Friesen, G. D.; Burgess, B. K.; Corbin, J. L.; Stiefel, E. I.; Newton, W. E. *J. Biol. Chem.* **1982**, *257*, 8042.
(7) Walters, M. A.; Chapman, S. K.; Orme-Johnson, W. H. *Polyhedron* **1986**, *5*, 561.
(8) La Planche, L. A.; Rogers, M. T. *J. Am. Chem. Soc.* **1964**, *86*, 337.
(9) Newman, R. C., Jr.; Jonas, V.; Anderson, K.; Barry, R. *Biochem. Biophys. Res. Commun.* **1971**, *44*, 1156.
(10) Schultz, F. A.; Gheller, S. F.; Burgess, B. K.; Lough, S.; Newton, W. E. *J. Am. Chem. Soc.* **1985**, *107*, 5364.
(11) Conradson, S. D.; Burgess, B. K.; Newton, W. E.; Mortenson, L. E.; Hodgson, K. O. *J. Am. Chem. Soc.* **1987**, *109*, 7507.
(12) McLean, P. A.; Wink, D. A.; Chapman, S. K.; Hickman, A. B.; McKillop, D. M.; Orme-Johnson, W. H. *Biochemistry* **1989**, 9402.
(13) Lough, S. M.; Jacobs, D. B.; Lyons, D. M.; Watt, G. D.; McDonald, J. W. *Biochem. Biophys. Res. Commun.* **1986**, *139*, 740.
(14) Wink, D. A.; McLean, P. A.; Hickman, A. B.; Orme-Johnson, W. H. *Biochemistry* **1989**, *28*, 9407.
(15) McLean, P. A.; Papaefthymiou, V.; Münck, E.; Orme-Johnson, W. H. In *Nitrogen Fixation: Hundred Years After*; Bothe, H., deBruijn, F. J., Newton, W. E., Eds.; Fischer: Stuttgart, 1988; p 101.
(16) Frank, P.; Ghellar, S. F.; Newton, W. E.; Hodgson, K. O. *Biochem. Biophys. Res. Commun.* **1990**, *163*, 746.
(17) Orme-Johnson, W. H.; Wink, D. A.; McLean, P. A.; Harris, G. S.; True, A. E.; Hoffman, B.; Münck, E. *Rec. Trav. Chim. Pays-Bas* **1987**, *106*, 299.
(18) Holm, R. H.; Simhon, E. D. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; John Wiley & Sons: New York, 1985; p 1.
(19) Shah, V. K.; Davis, L. C.; Gordon, J. K.; Orme-Johnson, W. H.; Brill, W. J. *Biochim. Biophys. Acta* **1973**, *292*, 246.
(20) Shah, V. K.; Imperial, J.; Ugalde, R. A.; Ludden, P. W.; Brill, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1636.
(21) Joerger, R. D.; Bishop, P. E. *J. Bacteriol.* **1988**, *176*, 1475.
(22) Hawkes, T. R.; Smith, B. E. *Biochem. J.* **1983**, *209*, 43.
(23) Robinson, A. C.; Dean, D. R.; Burgess, B. K. *J. Biol. Chem.* **1987**, *262*, 14327.
(24) Nagatani, H. H.; Shah, V. K.; Brill, W. J. *J. Bacteriol.* **1974**, *120*, 697.
(25) Hawkes, T. R.; Smith, B. E. *Biochem. J.* **1984**, *223*, 783.
(26) Smith, B. E.; Bishop, P. E.; Dixon, R. A.; Eady, R. R.; Filler, W. A.; Lowe, D. J.; Richards, A. J. M.; Thomson, A. J.; Thorneley, R. N. F.; Postgate, J. R. In *Nitrogen Fixation Research Progress*; Evans, H. J., Bottomley, P. J., Newton, W. E., Eds.; Martinus Nijhoff: Dordrecht, The Netherlands, 1985; p 597.
(27) Burgess, B. K.; Newton, W. E. In *Nitrogen Fixation: The Chemical-Biochemical-Genetic Interface*; Müller, A., Newton, W., Eds.; Plenum Press: New York, 1983; p 83.
(28) Shah, V. K. In *Nitrogen Fixation*; Newton, W. E., Orme-Johnson, W. H., Eds.; University Park Press: Baltimore, 1980; Vol. I, p 237.
(29) Shah, V. K.; Brill, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 3438.
(30) Shah, V. K.; Ugalde, R. A.; Imperial, J.; Brill, W. J. *J. Biol. Chem.* **1985**, *260*, 3891.
(31) Nelson, M. J.; Levy, M. A.; Orme-Johnson, W. H. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 147.
(32) Clark, L. J.; Axley, J. H. *Anal. Chem.* **1955**, *27*, 2000.
(33) (a) Cramer, S. P.; Hodgson, K. O.; Gillum, W. O.; Mortenson, L. E. *J. Am. Chem. Soc.* **1978**, *100*, 3398. (b) Cramer, S. P.; Gillum, W. O.; Hodgson, K. O.; Mortenson, L. E.; Stiefel, E. I.; Chisnell, J. R.; Brill, W. J.; Shah, V. K. *J. Am. Chem. Soc.* **1978**, *100*, 3814.
(34) Diehl, H.; Smith, G. F.; McBride, L.; Cryberg, R. In *The Iron Reagents*; The G. Frederick Smith Chemical Co.: Columbus, OH, 1985; p 1.
(35) Van De Bogart, M.; Beinert, H. *Anal. Biochem.* **1967**, *20*, 325.
(36) Brumby, P. E.; Miller, R. W.; Massey, V. *J. Biol. Chem.* **1965**, *240*, 2222.
(37) Tongager, S. R.; Averill, B. A. *Anal. Biochem.* **1980**, *102*, 13.
(38) Lovenberg, W.; Buchanan, B. B.; Rabinowitz, J. C. *J. Biol. Chem.* **1963**, *238*, 3899.
(39) Eady, R. R.; Imam, S.; Lowe, D. J.; Miller, R. W.; Smith, B. E.; Thorneley, R. N. F. In *Nitrogen Fixation*; Stewart, W. D. P., Gallon, J. R., Eds.; Academic Press: New York, 1980; p 19.
(40) Burgess, B. K.; Yang, S.-S.; You, C.-B.; Li, J.-G.; Friesen, G. D.; Pan, W.-H.; Stiefel, E. I.; Newton, W. E.; Conradson, S. D.; Hodgson, K. O. In *Current Perspective in Nitrogen Fixation*; Gibson, A. H.; Newton, W. E., Eds.; Australian Academy of Sciences: Canberra, Australia, 1981.
(41) Levchenko, L. A.; Poschupkina, O. S.; Sadkov, A. P.; Marakushev, S. A.; Mikhailov, G. M.; Borod'ko, Y. G. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 1384.
(42) McLean, P. A.; Dixon, R. A. *Nature* **1981**, *242*, 655.
(43) MacNeil, T.; MacNeil, D.; Roberts, G. P.; Supiano, M. A.; Brill, W. J. *J. Bacteriol.* **1978**, *136*, 253.
(44) Merrick, M.; Mechthild, F.; Dixon, R.; Elmerich, C.; Sibold, L.; Houmard, J. *J. Gen. Microbiol.* **1980**, *117*, 509.
(45) McLean, P. A.; Smith, B. E.; Dixon, R. A. *Biochem. J.* **1983**, *211*, 589.
(46) Smith, B. E.; Dixon, R. A.; Hawkes, T. R.; Liang, Y.-C.; McLean, P. A.; Postgate, J. R. In *Advances in Nitrogen Fixation Research*; Veeger, C.; Newton, W. E., Eds.; Martinus Nijoff/Junk, The Hague and Pudoc: Wageningen, The Netherlands, 1984; p 139.
(47) Hawkes, T. R.; McLean, P. A.; Smith, B. E. *Biochem. J.* **1984**, *217*, 317.
(48) Eidness, M. K.; Flank, A. M.; Smith, B. E.; Flood, A. C.; Garner, C. D.; Cramer, S. P. *J. Am. Chem. Soc.* **1986**, *108*, 2746.
(49) (a) McLean, P. A.; True, A. E.; Nelson, M. J.; Chapman, S.; Godfrey, M. R.; Teo, B. K.; Orme-Johnson, W. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1987**, *109*, 943. (b) True, A. E.; McLean, P.; Nelson, M. J.; Orme-Johnson, W. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1990**, *112*, 651.
(50) Hoover, T. R.; Shah, V. K.; Roberts, G. P.; Ludden, P. W. *J. Bacteriol.* **1986**, *167*, 999.
(51) Hoover, T. R.; Robertson, A. D.; Cerny, R. L.; Hayes, R. N.; Imperial, J.; Shah, V. K.; Ludden, P. W. *Nature* **1987**, *329*, 1.
(52) Hoover, T. R.; Imperial, J.; Ludden, P. W.; Shah, V. K. *J. Bacteriol.* **1988**, *170*, 1978.
(53) Hoover, T. R.; Imperial, J.; Ludden, P. W.; Shah, V. K. *Biochemistry* **1989**, *28*, 2768.
(54) Hoover, T. R.; Imperial, J.; Liang, J.; Ludden, P. W.; Shah, V. K. *Biochemistry* **1988**, *27*, 3647.
(55) Imperial, J.; Hoover, T. R.; Madden, M. S.; Ludden, P. W.; Shah, V. K. *Biochemistry* **1989**, *28*, 7796.
(56) Hedman, B.; Frank, P.; Gheller, S. F.; Roe, L. A.; Newton, W. E.; Hodgson, K. O. *J. Am. Chem. Soc.* **1988**, *110*, 3798.
(57) Stephens, F. J.; McKenna, C. E.; McKenna, M. C.; Nguyen, H. T.; Devlin, F. *Biochemistry* **1981**, *20*, 2857.

(58) Rawlings, J.; Shah, V. K.; Chisnell, J. R.; Brill, W. J.; Zimmerman, R.; Münck, E.; Orme-Johnson, W. H. *J. Biol. Chem.* **1978**, *253*, 1001.
(59) Shah, V. K.; Davis, L. C.; Gordon, J. K.; Orme-Johnson, W. H.; Brill, W. J. *Biochim. Biophys. Acta* **1973**, *292*, 246.
(60) Stiefel, E. I.; Cramer, S. P. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; John Wiley & Sons, New York, 1985; p 411.
(61) Collison, D.; Mabbs, F. E. *J. Chem. Soc. Dalton Trans.* **1982**, 1565.
(62) (a) Smith, B. E.; Lang, G. *Biochem. J.* **1974**, *137*, 169. (b) Münck, E.; Rhodes, H.; Orme-Johnson, W. H.; Davis, L. C.; Brill, W. J.; Shah, V. K. *Biochim. Biophys. Acta* **1975**, *400*, 32. (c) McLean, P. A.; Papaefthymiou, V.; Orme-Johnson, W. H.; Münck, E. *J. Biol. Chem.* **1975**, *262*, 12900.
(63) Newton, W. E.; Ghellar, S. F.; Sands, R. H.; Dunham, W. R. *Biochem. Biophys. Res. Commun.* **1989**, *162*, 882.
(64) Robinson, A. E.; Richard, A. J. M.; Thomson, A. J.; Hawkes, T. R.; Smith, B. E. *Biochem. J.* **1984**, *219*, 495.
(65) (a) Smith, B. E.; Lowe, D. J.; Bray, R. C. *Biochem. J.* **1973**, *135*, 331. (b) Euler, W. B.; Martinsen, J.; McDonald, J. W.; Watt, G. D.; Wang, Z.-C. *Biochemistry* **1984**, *23*, 3021.
(66) (a) Orme-Johnson, W. H.; Orme-Johnson, N. R.; Touton, C.; Emptage, M.; Henzl, M.; Rawlings, J.; Jacobson, K.; Smith, J. P.; Mims, W. B.; Huynh, B. H.; Münck, E.; Jacobs, G. S. In *Molybdenum Chemistry of Biological Significance*; Newton, W. E., Otsuka, S., Eds.; Plenum: New York, 1980; p 85. (b) Orme-Johnson, W. H. In *Current Perspectives in Nitrogen Fixation*; Gibson, A. H., Newton, W. E., Eds.; Australian Academy of Sciences: Canberra, 1981; p 79.
(67) Hoffman, B. M.; Roberts, J. E.; Orme-Johnson, W. H. *J. Am. Chem. Soc.* **1982**, *104*, 860.
(68) Venters, R. A.; Nelson, M. J.; McLean, P. A.; True, A. E.; Levy, M. A.; Hoffman, B. M.; Orme-Johnson, W. H. *J. Am. Chem. Soc.* **1986**, *108*, 3487.
(69) Hoffman, B. M.; Venters, R. A.; Roberts, J. E. *J. Am. Chem. Soc.* **1982**, *104*, 4711.
(70) True, A. E.; Nelson, M. J.; Venters, R. A.; Orme-Johnson, W. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1988**, *110*, 1935.
(71) (a) Kincaid, B. M.; Eisenberger, P. M.; Hodgson, K. O.; Doniach, S. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 2340. (b) Schulman, R. G.; Eisenberger, P.; Blumberg, W. E.; Stombaugh, N. A. *Ibid.* **1975**, *72*, 4003. (c) Eisenberger, P.; Shulman, R. G.; Brown, G. S.; Ogawa, S. *Ibid.* **1976**, *73*, 491. (d) Hu, V. W.; Chan, S. I.; Brown, G. S. *Ibid.* **1977**, *74*, 3821.
(72) Cramer, S. P.; Hodgson, K. O.; Stiefel, E. I.; Newton, W. E. *J. Am. Chem. Soc.* **1978**, *100*, 2748.
(73) Flank, A. M.; Weininger, M.; Mortenson, L. E.; Cramer, S. P. *J. Am. Chem. Soc.* **1986**, *108*, 1050.
(74) Stiefel, E. I. *Prog. Inorg. Chem.* **1977**, *22*, 1.
(75) Conradson, S. D.; Burgess, B. K.; Newton, W. E.; Hodgson, K. O.; McDonald, J. W.; Rubinson, J. F.; Gheller, S. F.; Mortenson, L. E.; Adams, M. W. W.; Mascharak, P. K.; Armstrong, W. A.; Holm, R. H. *J. Am. Chem. Soc.* **1985**, *107*, 7935.
(76) Wolff, T. E.; Berg, J. M.; Holm, R. H. *Inorg. Chem.* **1981**, *20*, 174.
(77) (a) Christou, G.; Garner, C. D. *J. Chem. Soc. Dalton Trans.* **1980**, 2354. (b) Huang, L.; Lin, S. *Jiegou Huaxue* **1984**, *3*, 25.
(78) (a) Weininger, M. S.; Mortenson, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 378. (b) Yamane, T.; Weininger, M. S.; Mortenson, L. E.; Rossman, M. G. *J. Biol. Chem.* **1982**, *257*, 1221.
(79) Antonio, M. R.; Teo, B. K.; Orme-Johnson, W. H.; Nelson, M. J.; Groh, S. E.; Lindahl, P. A.; Kawzlarich, S. M.; Averill, B. A. *J. Am. Chem. Soc.* **1982**, *104*, 4703.
(80) Arber, J. M.; Flood, A. C.; Garner, C. D.; Gormal, C. A.; Hasnain, S. S.; Smith, B. E. *Biochem. J.* **1988**, *252*, 421.
(81) Zumft, W. G. *Eur. J. Biochem.* **1978**, *91*, 345.
(82) Newton, W. E.; Gheller, S. F.; Hedman, B.; Hodgson, K. O.; Lough, S. M.; McDonald, J. W. *Eur. J. Biochem.* **1986**, *159*, 111.
(83) Kurtz, D. M., Jr.; McMillan, R. S.; Burgess, B. K.; Mortenson, L. E.; Holm, R. H. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4986.
(84) Teo, B. K.; Averill, B. A. *Biochem. Biophys. Res. Commun.* **1979**, *88*, 1454.
(85) Burgess, B. K.; Stiefel, E. I.; Newton, W. E. *J. Biol. Chem.* **1980**, *255*, 353.
(86) (a) Mascharak, P. K.; Smith, M. C.; Armstrong, W. H.; Burgess, B. K.; Holm, R. H. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 7056. (b) Conradson, S. D.; Burgess, B. K.; Holm, R. H. *J. Biol. Chem.* **1988**, *263*, 13743.
(87) Newton, W. E.; Gheller, S.; Shultz, F. A.; Burgess, B. K.; Conradson, S. D.; McDonald, J. W.; Hedman, B.; Hodgson, K. O. In *Nitrogen Fixation Research Progress*; Evans, H. J., Bottomley, P. J., Newton, W. E., Eds.; Martinus Nijoff: Dordrecht, The Netherlands, 1985; p 604.
(88) Conradson, S. D.; Burgess, B. K.; Ghellar, S. F.; Newton, W. E.; Holm, R. H.; Hodgson, K. O., unpublished results.
(89) Brigle, K. E.; Newton, W. E.; Dean, D. R. *Gene* **1985**, *37*, 37.
(90) Dean, D. R.; Brigle, K. E.; May, H. D.; Newton, W. E. In *Nitrogen Fixation: Hundred Years After*; Bothe, H., de-Bruijn, R. J., Newton, W. E., Eds.; Ficsher: Stuttgart, 1988; p 107.
(91) Brigle, K. E.; Setterquist, R. A.; Dean, D. R.; Cantwell, J. S.; Weiss, M. C.; Newton, W. E. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7066.
(92) Kent, H. M.; Ioannidis, I.; Gormal, C.; Smith, B. E.; Buck, M. *Biochem. J.* **1989**, *264*, 257.
(93) Tesler, J.; Hoffman, B. M.; Lo Brutto, R.; Ohnishi, T.; Tsai, A.-L.; Simpkin, D.; Palmer, G. *FEBS Lett.* **1987**, *214*, 117.
(94) Thomann, H.; Morgan, R. V.; Jin, H.; Burgmayer, S. J.; Bare, R. E.; Stiefel, E. I. *J. Am. Chem. Soc.* **1987**, *109*, 7913.
(95) Jin, H-Y.; Ph.D. Thesis, City University of New York, 1989.
(96) Scott, D. J.; May, H. D.; Newton, W. E.; Brigle, K. E.; Dean, D. R. *Nature* **1990**, *343*, 188.
(97) Palmer, G.; Multani, J. S.; Cretney, W. C.; Zumft, W. G.; Mortenson, L. E. *Arch. Biochem. Biophys.* **1972**, *153*, 325.
(98) Stephens, P. J. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; John WIley & Sons: New York, 1985; p 117 and references therein.
(99) Orme-Johnson, W. H.; Hamilton, W. D.; Ljones, T.; Tso, M.-Y. W.; Burris, R. H.; Shah, V. K.; Brill, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 3142.
(100) Mortenson, L. E.; Zumft, W. G.; Palmer, G. *Biochim. Biophys. Acta* **1973**, *292*, 422.
(101) Smith, B. E.; Lowe, D. J.; Bray, R. C. *Biochem. J.* **1973**, *135*, 331.
(102) (a) Watt, G. D.; Burns, A.; Lough, S. *Biochemistry* **1980**, *19*, 4926. (b) Watt, G. D.; Burns, A.; Tennent, D. L. *Biochemistry* **1981**, *20*, 7272. (c) Lough, S.; Burns, A.; Watt, G. D. *Biochemistry* **1983**, *22*, 4062.
(103) Johnson, M. K.; Thomson, A. J.; Robinson, A. E.; Smith, B. E. *Biochim. Biophys. Acta* **1981**, *671*, 61.
(104) O'Donnell, M. J.; Smith, B. E. *Biochem. J.* **1978**, *173*, 831.
(105) Newton, W. E.; Schultz, F. A.; Gheller, S. F.; Lough, S.; McDonald, J. W.; Conradson, S. D.; Hedman, B.; Hodgson, K. O. *Polyhedron* **1986**, *5*, 567.
(106) Schultz, F. A.; Gheller, S. F.; Newton, W. E. *Biochem. Biophys. Res. Commun.* **1988**, *152*, 629.
(107) Armstrong, F. A.; George, S. J.; Thomson, A. J.; Yates, M. G. *FEBS Lett.* **1988**, *234*, 109.
(108) Newton, W. E.; Gheller, S. F.; Feldman, B. J.; Dunham, W. R.; Schultz, F. A. *J. Biol. Chem.* **1989**, *264*, 1924.
(109) Newton, W. E.; Burgess, B. K.; Cummings, S. C.; Lough, S.; McDonald, J. W.; Rubinson, J. F.; Conradson, S. D.; Hodgson, K. O. In *Advances in Nitrogen Fixation Research*; Veeger, C., Newton, W. E., Eds.; Nijhoff/Junk, The Hague and Pudoc: Wageningen, The Netherlands, 1984; p 160.
(110) Burgess, B. K. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; John Wiley & Sons: New York 1985; p 161.
(111) Hageman, R. V.; Burris, R. H. *J. Biol. Chem.* **1979**, *254*, 1189.
(112) Smith, B. E. In *Nitrogen Fixation: The Chemical–Biochemical–Genetic Interface*; Müller, A., Newton, W. E., Eds.; Plenum Press: New York, 1983; p 23.
(113) Hawkes, T. R.; Lowe, D. J.; Smith, B. E. *Biochem. J.* **1983**, *211*, 495.
(114) Conradson, S. D.; Burgess, B. K.; Vaughn, S. A.; Roe, A. L.; Hedman, B.; Hodgson, K. O.; Holm, R. H. *J. Biol. Chem.* **1989**, *264*, 15967.
(115) Thorneley, R. N. F.; Lowe, D. J. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; John Wiley & Sons: New York, 1985; p 161.
(116) (a) Li, J.-G.; Burgess, B. K.; Corbin, J. L. *Biochemistry* **1982**, *21*, 4393. (b) Rubinson, J. F.; Corbin, J. L.; Burgess, B. K. *Biochemistry* **1983**, *22*, 6260.
(117) Scott, D. J.; Newton, W. E.; Brigle, K. E.; Dean, D. R. In *Nitrogen Fixation: Achievements and Objectives*; Gresshoff, R., Stacey, J., Newton, W. E., Eds.; Chapman & Hall: New York, in press.
(118) Shah, V. K.; Chisnell, J. R.; Brill, W. J. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 232.
(119) (a) Weathers, B. J.; Grate, J. H.; Strampach, N. A.; Schrauzer, G. N. *J. Am. Chem. Soc.* **1979**, *101*, 925. (b) Corbin, J. L.; Pariyadath, K.; Stiefel, E. I. *J. Am. Chem. Soc.* **1976**, *98*, 7862. (c) Newton, W. E.; Corbin, J. L.; Schneider, P. W.; Bulen, W. A. *J. Am. Chem. Soc.* **1971**, *93*, 268.
(120) McKenna, C. E.; McKenna, M.-C.; Huang, C. W. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4773.
(121) McKenna, C. E.; McKenna, M.-C.; Higa, M. I. *J. Am. Chem. Soc.* **1976**, *98*, 4657.
(122) Ugalde, R. A.; Imperial, J.; Shah, V. K.; Brill, W. J. *J. Bacteriol.* **1984**, *159*, 888.
(123) Filler, W. A.; Kemp, R. M.; Ng, J. C.; Hawkes, T. R.; Dixon, R. A.; Smith, B. E. *Eur. J. Biochem.* **1986**, *160*, 371.
(124) Robinson, A. C.; Burgess, B. K.; Dean, D. R. *J. Bacteriol.* **1986**, *166*, 180.
(125) Hoover, T. R.; Imperial, J.; Ludden, P. W.; Shah, V. K. *Biofactors* **1988**, *1*, 199.
(126) Cramer, S. P.; Stiefel, E. I. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; John Wiley & Sons: New York, 1985; p 161.

(127) Haselkorn, R., as presented at the 8th International Symposium on N_2-fixation, Knoxville, TN, May, 1990.
(128) Chen, J-S. In *Nitrogen Fixation: Achievements and Objectives*; Gresshoff, R., Stacy, J., Newton, W. E., Eds.; Chapman & Hall: New York, in press.
(129) Buikema, W. J.; Klingensmith, J. A.; Gibbons, S. L.; Ausubel, F. M. *J. Bacteriol.* **1987**, *169*, 1120.
(130) Joerger, R. D.; Bishop, P. E. *J. Bacteriol.* **1988**, *170*, 1475.
(131) Arnold, W.; Rump, A.; Klipp, W.; Priefer, U. B.; Puhler, A. *J. Mol. Biol.* **1988**, *203*, 715.
(132) Imperial, J.; Ugalde, R. A.; Shah, V. K.; Brill, W. J. *J. Bacteriol.* **1984**, *158*, 187.
(133) Ugalde, R. A.; Imperial, J.; Shah, V. K.; Brill, W. J. *J. Bacteriol.* **1985**, *164*, 1081.
(134) Klipp, W.; Reilander, H.; Schlüter, A.; Krey, R.; Puhler, A. *Mol. Gen. Genet.* **1989**, *216*, 293.
(135) Evans, D.; Jones, R.; Woodley, P.; Robson, R. *J. Gen. Microbiol.* **1988**, *134*, 931.
(136) Puhler, A.; Aguilar, M. O.; Hynes, M.; Muller, P.; Klipp, W.; Priefer, U.; Simon, R.; Weber, G. In *Advances in Nitrogen Fixation Research*; Veeger, C., Newton, W. E., Eds.; Nijhoff/Junk, The Hague and Pudoc: Wageningen, The Netherlands, 1984; p 609.
(137) Shah, V. K.; Hoover, T. R.; Imperial, J.; Paustian, T. D.; Roberts, G. P.; Ludden, P. W. In *Proceedings of the VIIth International Congress on Nitrogen Fixation*; Newton, W. E., Bothe, H., Eds.; Fischer Verlag: Stuttgart, 1988; p 115.
(138) Dean, D. R.; Brigle, K. E. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 5720.
(139) Brigle, K. E.; Weiss, M. C.; Newton, W. E.; Dean, D. R. *J. Bacteriol.* **1987**, *169*, 1547.
(140) Moreno-Vivian, C.; Schmehl, M.; Masepohl, B.; Arnold, W.; Klipp, W. *Mol. Gen. Genet.* **1989**, *216*, 353.
(141) Setterquist, R.; Brigle, K. E.; Beynon, J.; Cannon, M.; Ally, A.; Cannon, F.; Dean, D. R. *Nucleic Acids Res.* **1988**, *16*, 5215.
(142) Paustian, T. D.; Shah, V. K.; Roberts, G. P. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6082.
(143) Filler, W. A.; Kemp, R. M.; Ng, J. C.; Hawkes, T. R.; Dixon, R. A.; Smith, B. E. *Eur. J. Biochem.* **1986**, *160*, 371.
(144) Robinson, A. C.; Burgess, B. K.; Dean, D. R. *J. Bacteriol.* **1986**, *166*, 180.
(145) Robinson, A. C.; Dean, D. R.; Burgess, B. K. *J. Biol. Chem.* **1987**, *262*, 14327.
(146) Ugalde, R. A.; Imperial, J.; Shah, V. K.; Brill, W. J. *J. Bacteriol.* **1984**, *159*, 888.
(147) Ebeling, S.; Noti, J. D.; Hennecke, H. *J. Bacteriol.* **1988**, *170*, 1999.
(148) Mulligan, M. E.; Buikema, W. J.; Haselkorn, R. *J. Bacteriol.* **1988**, *170*, 4406.
(149) Shah, V. K.; Imperial, J.; Ugalde, R. A.; Ludden, P. W.; Brill, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6636.
(150) Bishop, P. E.; Jarlenski, D. M. L.; Hetherington, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 7342.
(151) Bishop, P. E.; Hawkins, M. E.; Eady, R. R. *Biochem. J.* **1986**, *238*, 437.
(152) Bishop, P. E.; Premakumar, R.; Dean, D. R.; Jacobson, M. R.; Chisnell, J. R.; Rizzo, T. M.; Kopczynski, J. *Science* **1986**, *232*, 92.
(153) Robson, R. L. *Arch. Microbiol.* **1986**, *146*, 74.
(154) (a) Bishop, P. E. *Trends Biol. Sci.* **1986**, *11*, 225. (b) Eady, R. R.; Robson, R. L.; Smith, B. E. In *The Nitrogen and Sulfur Cycles*; Cole, A. J., Ferguson, S., Eds.; Society for General Microbiology Symposium 42; Cambridge University Press: Cambridge, 1987; p 363. (c) Pau, R. N. *Trends Biol. Sci.* **1989**, *14*, 183.
(155) Robson, R. L.; Eady, R. R.; Richardson, T. H.; Miller, R. W.; Hawkins, M.; Postage, J. R. *Nature* **1986**, *322*, 388.
(156) Eady, R. R.; Robson, R. L.; Richardson, T. H.; Miller, R. W.; Hawks, M. *Biochem. J.* **1987**, *244*, 197.
(157) Hales, B. J.; Case, E. E.; Morningstar, J. E.; Dzeda, M. F.; Manteres, L. A. *Biochemistry* **1986**, *25*, 7251.
(158) Hales, B. J.; Langosch, D. J.; Case, E. E. *J. Biol. Chem.* **1987**, *261*, 15301.
(159) Robson, R. L.; Woodley, P. R.; Pau, R. N.; Eady, R. R. *EMBO J.* **1989**, *8*, 217.
(160) Morningstar, J. E.; Johnson, M. K.; Case, E. E.; Hales, B. J. *Biochemistry* **1987**, *26*, 1795.
(161) Morningstar, J. E.; Hales, B. J. *J. Am. Chem. Soc.* **1987**, *109*, 6854.
(162) (a) Arber, J. M.; Dobson, B. R.; Eady, R. R.; Stevens, P.; Hasnain, S. S.; Garner, C. D.; Smith, B. E. *Nature* **1987**, *325*, 327. (b) Arber, J. M.; Dobson, B. R.; Eady, R. R.; Hasnain, S. S.; Garner, C. D.; Matsushita, T.; Nomura, M.; Smith, B. E. *Biochem. J.* **1989**, *258*, 733.
(163) (a) Garner, C. D.; Arber, J. M.; Harvey, I.; Hasnain, S. S.; Eady, R. R.; Smith, B. E.; deBoer, E.; Wever, R. *Polyhedron* **1989**, *8*, 1649. (b) Garner, C. D.; Arber, J. M.; Hasnain, S. S.; Dobson, B. R.; Eady, R. R.; Smith, B. E. *Physica B.* **1989**, *158*, 74.
(164) Kovacs, J. A.; Holm, R. H. *J. Am. Chem. Soc.* **1986**, *108*, 340.
(165) Smith, B. E.; Eady, R. R.; Lowe, D. J.; Gormal, C. *Biochem. J.* **1988**, *250*, 299.
(166) Dilworth, M. J.; Eady, R. R.; Robson, R. L.; Miller, R. W. *Nature* **1987**, *327*, 167.
(167) Bortels, H. *Zentralbl. Bakteriol. Parasitenkd. Abt.* **1936**, *1195*, 193.
(168) Pau, R. N.; Mitchenall, L. A.; Robson, R. L. *J. Am. Chem. Soc.* **1989**, *171*, 124.
(169) Joerger, R. D.; Jacobson, M. R.; Premakumar, R.; Wolfinger, E. D.; Bishop, P. E. *J. Bacteriol.* **1989**, *191*, 1075.
(170) Chisnell, J. R.; Premakumar, R.; Bishop, P. E. *J. Bacteriol.* **1988**, *170*, 27.
(171) Beinert, H.; Kennedy, M. C. *Eur. J. Biochem.* **1989**, *186*, 5.
(172) Bolin, J. T.; Ronco, A. E.; Mortenson, L. E.; Morgan, T. V.; Williamson, M.; Xuong, N.-h. In *Nitrogen Fixation: Achievements and Objectives*; Gresshoff, R., Stacey, J., Newton, W. E., Eds., Chapman & Hall: New York, in press.