Preparation of Isotopically Labeled MoFe Protein of Nitrogenase

by

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Submitted to the Department of Chemistry on September 22, 1995 in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Chemistry

Abstract

The study of nitrogen fixation is driven by the goal of understanding how the nitrogenase system is able to break the very stable triply bonded dinitrogen. Even after studying the biological system during the last fifty years, the molecular details of how the enzyme functions are incomplete. To examine the mechanism of nitrogenase, isotopically labeled MoFe proteins of nitrogenase were constructed, either with a deuterated homocitric acid at the active site (FeMoco) or by the incorporation of iron isotopes into the P cluster and FeMoco.

The wild-type growth phenotype of *Klebsiella pneumoniae* (UN) can be restored to the *nifV*- mutant (deficient in homocitrate synthase) of *K. pneumoniae* (UN 1990) by the addition of the open form of homocitric acid to the growth media. This ability was utilized to incorporate a ^{2}H-homocitrate label into the MoFe protein for Electron Spin Echo Envelope Modulation (ESEEM) studies. These studies revealed a ESEEM spectra consistent with a ^{14}N-containing residue within close proximity to FeMoco. The deuteron on the homocitrate was not detected. This result is harmonious with the spin moment of the cluster being distant from the Mo end of the cofactor, an observation that tallies with difficulty earlier observed in detecting ^{95}Mo hyperfine coupling on the Electron Paramagnetic Resonance (EPR) of the cofactor center in nitrogenase.

The iron hybrid proteins were produced by incorporating extracted cofactor from wild-type *Azotobacter vinelandii* with a cellular extract from a cofactorless *nif*^{B}- mutant of *A. vinelandii* (UW45). The use of a double phase fermentation for the growth of UW 45 was investigated for optimal apoMoFe protein production. UW45 apoMoFe protein appears to be not as stable as the protein produced from a *nifB*- mutant of *K. pneumoniae* (UN106), but since UW 45 was able to produce up to 16 times as much apoMoFe protein per cell weight than UN 106, UW 45 is the better choice as the source of large quantities of apoMoFe protein. It was also discovered that the addition of iron to the derepression medium is not required. Elimination of iron citrate in the derepression medium does not affect the nitrogenase activity and greatly reduces the cost of incorporating ^{57}Fe into the hybrid MoFe proteins.

To produce large quantities of extracted FeMoco, a systematic examination of the cofactor extraction process was examined. The optimal amount of DE-52 required to bind 100 mg of homogeneous MoFe protein is

4.0 grams. The binding of the protein to the DE-52 is an equilibrium process that takes about 60 minutes. It can be demonstrated through the use of the spectroscopically active compound $Ru(bpy)_3Cl_2$ that the salt does indeed extract the cofactor from the unfolded protein. The interaction of the salt and FeMoco is also an equilibrium process, with the time required for the process to reach equilibrium varying from 30 - 60 minutes depending on the size of the column poured. The best way to extract large quantities of FeMoco is to use a batch-load, column extract method.

The production of isotopically labeled hybrid MoFe proteins enabled the P cluster and FeMoco to have different isotopes of iron incorporated. These hybrids were then used to examine the site of CO inhibition which was known to be at an iron containing cluster in the MoFe protein. The production of the CO-inhibited state of MoFe protein using these hybrids and globally enriched ^{57}Fe MoFe protein was investigated using EPR and ^{57}Fe Electron-Nuclear Double Resonance (ENDOR) spectroscopy. These studies of the CO-inhibited state of MoFe protein strongly suggests that the site of CO inhibition is FeMoco.

Thesis Supervisor: Dr. William H. Orme-Johnson
Title: Professor of Chemistry

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This thesis would not have been possible without the help and support of several people.

I came to MIT with the intention of studying nitrogenase in the lab of Professor W.H. Orme-Johnson. I felt very privileged to have the opportunity to do research in his laboratory. He gave me the opportunity to explore and determine a variety of scientific approaches to solve interesting chemical and technical problems. He allowed me to work independently but was available for consultation when it was required.

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experiments were a collaboration between three graduate students, David Wright, Trish Humiston and myself. It was the collective of the three minds which enabled the improved method to be developed. The 20 L homocitrate incorporation experiments were a collaboration between Thomas Collet, David Wright and myself. The EPR experiments would not have been possible but for the patience and expertise of Jim Simms and Scott Gardner, who never seemed to tire of answering my endless questions about liquid He EPR. I would like to thank Dr. Brian Hoffman, Dr. Vickie DeRose and Dr. Hong In Lee of Northwestern University for the expertise in ESEEM and ENDOR.

My family have been very encouraging and supportive through the years of education it took for me to come to this time and place. My parents, Dave and Dianne Christie, never let me lose faith in my abilities and convictions even when it seemed difficult.

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Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 4

List of Tables

Chapter 1: Nitrogenase Review

I. Introduction

The bioavailability of nitrogen is of vital importance since nitrogen is a component of proteins and nucleic acids in every life form on earth,. Nitrogen fixation is the process by which free atmospheric dinitrogen is converted into ammonia which is subsequently incorporated into biomolecules. Even though the conversion of dinitrogen to ammonia is overall a thermodynamically favorable one, formation of the intermediates in the reaction is thermodynamically unfavorable. There are several means by which nitrogen can be fixed. Atmospheric dinitrogen fixation occurs during lightning storms accounting for about 15% of the total amount of nitrogen fixed (Burns and Hardy, 1975). During an electric storm, dinitrogen combines with oxygen to form oxides of nitrogen which are hydrolyzed, further react with atmospheric and soil components and are then absorbed into the soil (Toon and Ellis, 1983). Industrially, nitrogen is chemically reduced to ammonia by the following reaction:

$$N_2\,(g) + 3H_2(g) \rightleftharpoons 2NH_3(g)$$

This is known as the Haber process and is carried out in the presence of an iron oxide and aluminum catalyst at pressures of 10^2 to 10^3 atm and temperatures of 400 to 500 ^oC (Cotton and Wilkinson, 1988). In 1994, there were 37.93 billion pounds of ammonia produced, making it the 6th most produced chemical in the world, ranking first on a molar basis (C & EN, 1995). Ammonia is subsequently incorporated into various chemical products including fertilizers, household supplies and cosmetics. Nevertheless, industrial production accounts for only 25% of the total amount of fixed nitrogen produced on the earth by all processes (Burns and Hardy, 1975).

In the biosphere nitrogen is reduced by a variety of bacteria at soil temperatures and pressures. This accounts for 60% of the total amount of dinitrogen fixed (Burns and Hardy, 1975). Prokaryotic microorganisms are the

only known living organisms capable of reducing dinitrogen (Peters, 1987). Principal nitrogen fixers include certain free-living soil bacteria, free-living cyanobacteria on soil surfaces or in water, cyanobacteria in symbiotic associations with fungi in lichens or with fern, mosses and liverworts and bacteria or other microbes associated symbotically with roots, especially those of legumes (Salisbury and Ross, 1985; Peters, 1987).

The study of nitrogen fixation is driven by the goal of understanding how these microorganisms are able to break the very stable triply bonded dinitrogen under such mild conditions. Even after studying the biological system during the last fifty years, the molecular details of how the enzyme nitrogenase functions are incomplete. The commonly studied bacterial systems are *Azotobacter vinelandii, Klebsiella pneumoniae* and *Clostridium pasteuranium.* The stoichiometry of the reaction is thought to be:

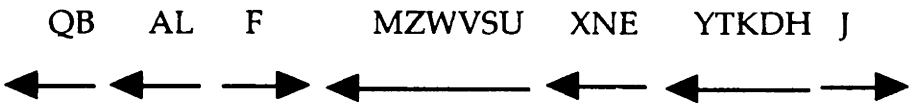
$$N_2 + 8e^- + 8H^+ + 16\ MgATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16\ MgADP + 16Pi$$

It is known that the biological system consists of at least 20 different gene products. The enzyme itself is composed of two separable components, called the MoFe protein and the Fe protein. There are three different types of nitrogenases which differ by the type of metal in the active site of the enzyme. The molybdenum-containing enzyme, which has a component known as the Molybdenum Iron (MoFe) protein, is the system which has been studied the most thoroughly. Its active site for N_2 reduction contains $MoFe_7S_8$ and an organic moiety, homocitric acid. There is also a vanadium iron (VFe) protein containing vanadium in the active site cluster instead of molybdenum. The genes responsible for the VFe protein are induced under low molybdenum concentrations. The third nitrogenase, with its own subset of genes, appears to contain an all-iron active site (Smith and Eady, 1992). The DNA coding for all three is present in the genomes of several organisms.

II. The Biosynthesis of Nitrogenase

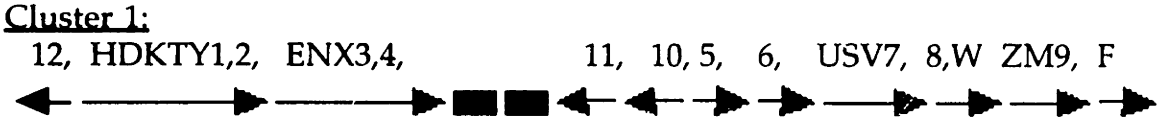
To understand how the nitrogenase system functions, mutants of both *Azotobacter* and *Klebsiella* have been produced and systematically examined. The nitrogenase system of *Klebsiella* consists of 20 different genes clustered in a 23 kB region and are arranged in 7 operons (Arnold, et al., 1988).

Figure 1.1: The *Klebsiella nif* gene cluster



The positioning of the *nif* genes in the *Azotobacter* species is more complicated. There is one large cluster with 15 of the *nif* genes with 12 open interspersed reading frames (indicated by the numbers) (Jacobson et al., 1989). There is a second *nif* cluster which contains *nifJ, L, A, B,* and *Q,* shown below (adapted from Das, 1993).

Figure 1.2: The two *nif* clusters from *Azotobacter*.



(ORF = Open Reading Frame, Fd = flavodoxin)

The *nif* gene products and their possible role in nitrogen fixation are included in table 1.1 (adapted from Allen et al., 1994). These roles have been deduced from the constitution and function of specifically mutated strains.

Table 1.1: The *nif* Gene products and their role in Nitrogen Fixation

Gene	Identity / Role
nifJ	Pyruvate: flavodoxin (ferredoxin) oxidoreductase, electron donation
nifF	Flavodoxin: physiological electron donor to Fe protein
nifH	Fe protein, catalyzes ATP-dependent electron transfer to MoFe protein; involved in FeMoco synthesis and apoMoFe maturation
nifM	Processing of Fe protein
nifD	MoFe protein α subunit; FeMoco binding unit
nifK	MoFe protein β subunit
nifQ	Involved in FeMoco synthesis, probably an early step in MoO_4^{2-} processing
nifY	In *K. pneumoniae,* attaches to MoFe protein to allow FeMoco insertion
nifE	Subunit of nifNE complex; involved in FeMoco synthesis
nifN	Subunit of nifNE complex; involved in FeMoco synthesis
nifB	Involved in FeMoco, FeVco and FeFeco synthesis
nifV	Putative homocitrate synthase; involved in FeMoco synthesis
nifU	Mobilizing iron for metallocluster synthesis
nifS	Cysteine desulfurase; involved in synthesis of Fe-S centers
nifA	Positive regulatory transcription factor
nifL	Negative regulatory element
nifX	Negative regulatory element
nifT	Unknown function
nifW	Involved in homocitrate transport to the site of biosynthesis
nifZ	Involved in FeMoco biosynthesis or stabilization of MoFe polypeptides

A. The Regulation of Biological Nitrogen Fixation

There are two levels of control for nitrogenase synthesis, a general level and a *nif* specific level. The general control of nitrogenase synthesis is exerted by *ntrA*, *ntrB* and *ntrC* gene products. Additionally, expression of the *nif* genes is also controlled by *nif* specific regulatory proteins (Merrick, 1983). This regulation occurs at the levels of transcription, mRNA stability and protein stability.

Transcription of the *nifLA* operon is controlled by the *ntr* gene products. The *ntr* gene products function to control the overall balance of many nitrogen-assimilation enzymes. *ntrB* and *ntrC* genes are linked to the structural gene for glutamine synthetase *glnA*, while the *ntrA* is unlinked. Merrick (1983) has shown that *ntrA* gene product is required for expression of the activator function of both the *ntrC* and *nifA* gene products, while Espin and coworkers (1982) experimentally demonstrated that *ntrC* is required for activation of transcription of the *nifLA* operon

The *nifA* gene product is a positive regulator and the *nifL* gene product is a negative regulator of *nif* expression. Using *K. pneumoniae* mutants which constitutively express *nifA*, Buchannan-Wollaston and co-workers (1981) were able to show that even in the presence of fixed nitrogen and oxygen, the *nif* genes were expressed. In *K. pneumoniae*, **NIFL** will bind to **NIFA** and inactivate it in the presence of greater than ~5 μM ammonia. At concentrations greater than ~200 μM ammonia, the expression of the *nifLA* operon does not occur (Merrick et al., 1982). Through their work on mutations in the *nifL* region as well as *nif-lac* fusions, Merrick and coworkers (1982) demonstrated that the *nifL* product acts as a *nif*-specific repressor in response to ammonia, amino acids, oxygen and possibly also to temperature. Eydmann and coworkers (1995) have shown *in vitro* for *Azotobacter vinelandii* that **NIFL** is able to modulate the activity of **NIFA** through an adenosine nucleotide switch. When ADP is bound to **NIFA**, the resulting change in conformation leads to a productive interaction with **NIFL**.

Conversely, when ATP is bound to **NIFA**, interaction is not favoured (Eydmann et al., 1995). When Gosnik and coworkers (1990) made an insertional mutation in the *nifX* gene of *K. pneumonia*, and introduced conditions to repress the nitrogenase system, the nitrogenase system continued to produce the proteins longer than the wild type organism. The overexpression of *nifX* caused the *nif* proteins not to be synthesized. They conclude that NIFX has a negative regulatory effect on the *nif* system (Gosnik et al., 1990).

There are many environmental stimuli which affect the activity of nitrogenase. The nitrogenase system of proteins are very sensitive to oxygen. If nitrogenase-producing cultures of the facultative anaerobe, *Klebsiella pneumonia*, are aerated, nitrogenase synthesis is repressed (St. John et al., 1974). Regulation of the synthesis at the level of transcription of *nif* genes is by a mechanism that involves the *nifL* gene product as a negative controlling element. At high temperatures (i.e. 41^{0}C), the transcription of the operons *nifKDH*, *nifMVSU* and *nifJ* are inhibited due to the reversible inactivation of the *nifA* gene product (Brooks et al., 1984).

B. The Biosynthesis of the Iron Protein of Nitrogenase

The Fe protein of nitrogenase is an α_2 dimer which is the product of *nifH*. In *Azotobacter vinelandii* (*Av*), *nifU*, *nifS* and *nifM* are all required for the full activation or the catalytic stability of the Fe protein (Jacobson et al., 1989). To discover what the minimum number of genes required to make active Fe protein, plasmids carrying various genes were placed into *Escherichia coli*. When only *nifH* was on the plasmid, inactive polypeptides were formed. The presence of both *nifH* and *nifM* produced active Fe protein suggesting that the maturation of the Fe protein involves **NIFM** (Howard et al., 1986; Roberts et al., 1978). There are several hypotheses of how **NIFM** helps in this process. It has been suggested that **NIFM** inserts the Fe-S cluster into the Fe protein. Another hypothesis is that **NIFM** isomerizes the Fe-S

cluster once it has been inserted between the two subunits (Howard et al., 1989). A third conjecture is that **NIFM** helps the two subunits of the Fe protein become properly orientated so that the Fe_4S_4 cluster is able to be inserted properly (Georgiadis et al., 1992). The cluster is formed through the actions of **NIFS** and **NIFU**. These two genes will be discussed in detail below.

C. The Biosynthesis of the Molybdenum Iron Protein of Nitrogenase

At least 9 of the 20 genes *nif* genes are involved in the biosynthesis of the MoFe protein. This is a coordinated event of protein synthesis and is very tightly regulated. The process can be split into two parts, the biosynthesis of the structural genes (*nifD* and *K*) and the biosynthesis of the FeMoco with its subsequent insertion into the polypeptide.

The protein backbone of the MoFe protein are encoded by *nifD* and *nifK*. The α subunit is a product of *nifK* and the transcription of *nifD* produces the β subunit. These proteins must be combined in a specific tertiary structure and have the FeMoco and P clusters inserted into them.

There is a well characterized *in vitro* FeMoco synthesis system that has illuminated many aspects of FeMoco biosynthesis (Shah et al., 1986). A pictorial view of the gene products involved in FeMoco biosynthesis are provided in figure 1.2 (adapted from Dean et al., 1993).

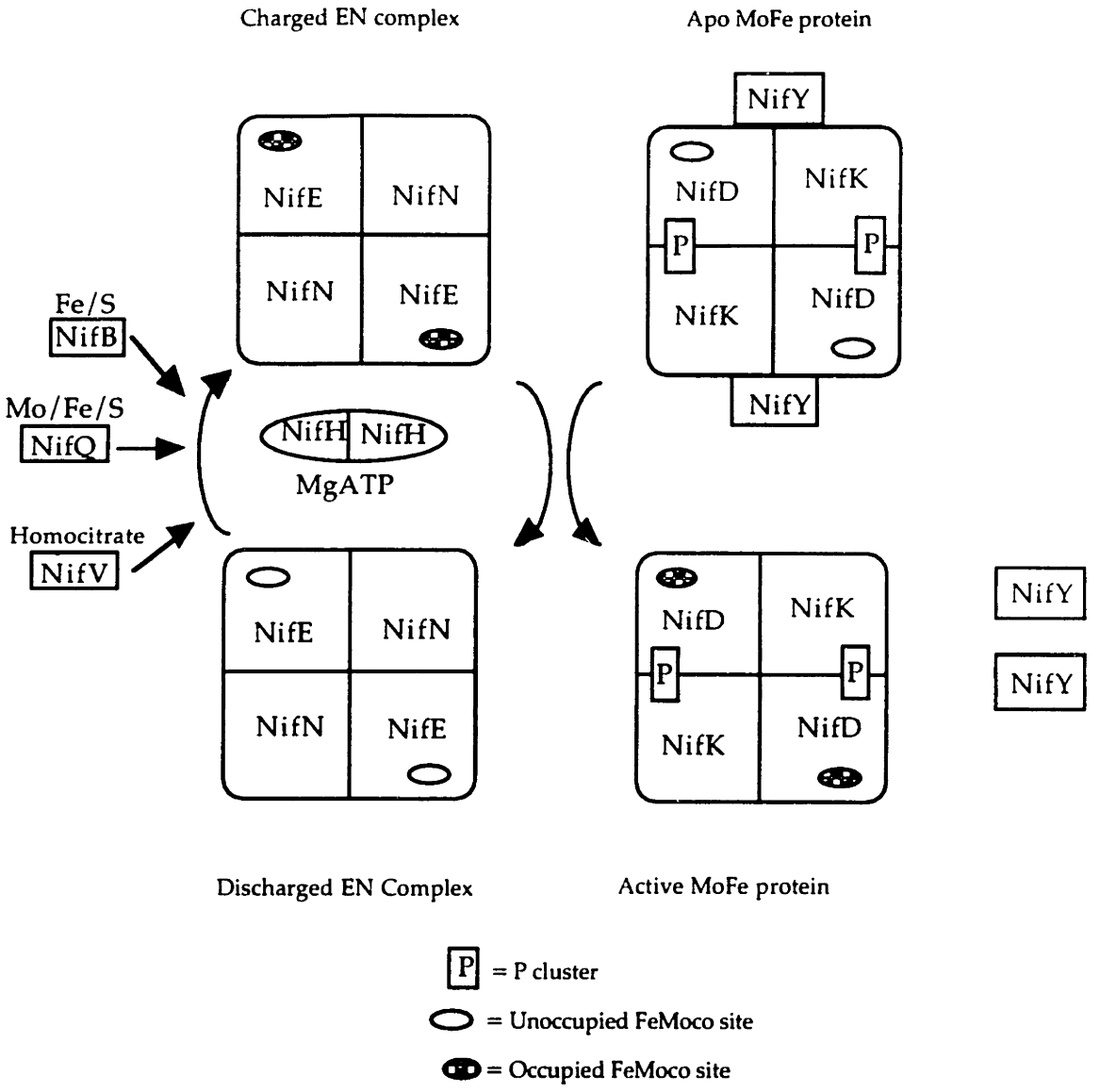


Figure 1.2: Gene products involved in the MoFe protein biosynthesis.
NIFB, NIFQ and **NIFV** are involved in the biosynthesis of FeMoco. With the aid of **NIFH** and MgATP, FeMoco is inserted onto the **NIFEN** complex. The apoMoFe protein consists of an $\alpha_2\beta_2\gamma_2$ complex with the P clusters inserted. **NIFY** functions as a molecular doorstop, holding open the site for FeMoco attachment and when FeMoco is transferred to the apoMoFe protein, **NIFY** dissociates from the complex.

The processing of molybdenum for the incorporation into FeMoco is the task of the *nifQ* gene product (Imperial et al., 1984). There are two Mo-processing genes present in the bacteria, *nifQ* and *mol*. The *mol* gene product, in addition to being required for synthesis of FeMoco, is involved in the synthesis of Mo-co, the cofactor of other molybdoenzymes, for instance nitrate reductase. *nifQ* mutants are not defective in molybdenum cofactor biosynthesis for other molybdenum containing enzymes (Imperial et al., 1985). It has been suggested that the **NIFQ** is involved in a the biosynthetic step in FeMoco biosynthesis in which the Mo-S moiety is produced (Dean and Jacobson, 1992). An interesting feature of *nifQ*- mutants is that at high molybdenum concentrations, no effect on the nitrogenase system is observed. This may well be because the reaction

$$S^{2-} + MoO_xS_y \Leftrightarrow MoO_{x-1}S_{y+1}$$

occurs in aqueous solutions without the need of protein catalysis. It is only at low molybdenum concentrations that there is an apparent problem in the nitrogenase system (Imperial et al., 1984). It has been difficult to observe the phenotype of a *nif Q*- mutant due to the low levels of Mo starvation that the mutants have to be exposed to before the phenotype is expressed. The *nif Q*- mutant is seen more easily in *Klebsiella* than in *Azotobacte*r because *Azotobacter* has the ability to store high quantities of molybdenum in a storage protein. In *K. pneumoniae*, Mo accumulation is tightly regulated and is under the control of *nif* gene expression (Pienkos and Brill., 1981).

How **NIFB** functions in the biosynthesis of FeMoco is not completely understood. NifB- mutants are regularly used to assay for the activity of extracted FeMoco, so the ability of *NifB*- mutants to incorporate premade FeMoco into the apoMoFe is not affected. It is known that **NIFB** from *K. pneumonia* is membrane bound and has an extractable cofactor, called NifB-co, in which the only detectable metal is iron (Shah et al., 1994). From observations of how NifB-co behaves in the *in vitro* FeMoco synthesis system, Shah et al., (1994) has suggested that the role of *NifB* in FeMoco

biosynthesis is to provide all the iron to FeMoco. They have also suggested that the iron is donated in a pre-formed FeS cluster to the NIFEN complex (Allen et al., 1994).

The precise role of **NIFNE** is still under debate. Based on sequence homology between *nifN* and *nifK* with *nifE* and *nifD* , Brigle et al. (1987) suggested that **NIFNE** is used as a scaffold upon which FeMoco is either partially or wholly biosynthesized and then transferred to **NIFDK**. But when Paustian et al., (1989) used the **NIFNE** purified from UW45 (a *nif B*-mutant), they discovered that some moiety on **NIFNE** was utilized during the *in vitro* biosynthesis of FeMoco, thus indicating that it was more than just a scaffold upon which FeMoco is synthesized. **NIFNE** contains 4.6 moles of Fe, 1.2 moles of Zn and 0.63 moles of Cu per mole of protein and contains an absorbance characteristic of an Fe-S center. It has yet to be determined if Zn and Cu play a role in **NIFEN**. It has been suggested that **NIFNE** might donate Fe, S or specify the metal (i.e. Mo, V or Fe) incorporated into FeMoco, (Paustian et al., 1989), but since no Mo was detected in purified **NIFEN**, it seems that a probable role for **NIFEN** would be to donate an Fe-S moiety to FeMoco.

NIFH also has a role in the biosynthesis of FeMoco. *nifH* encodes the polypeptide of the Fe protein, and the fully mature Fe protein is not required for the biosynthesis of FeMoco (Roberts et al., 1978). Tal and coworkers (1991) suggest that Fe protein/magnesium adenosine 5'-triphosphate (MgATP) complex binds to the apoMoFe and modifies it in such a way that the cofactor site becomes accessible for FeMoco insertion. The support for this theory comes from the observation that a mutant with *nifH* deleted (a Δ*nifH* mutant) is able to produce wild type levels of MoFe activity upon the mixing of FeMoco, Fe protein and MgATP (Tal et al., 1991). Robinson and coworkers (1987) suggest that the Fe protein transfers an electron to the EN-cofactorless complex in order for the reduction potential to be obtained for the insertion of FeMoco (Robinson et al., 1987). It has been shown in *Azotobacter*

vinelandii that **NIFH**'s role is to promote the association of additional subunits (γ) with apoMoFe. Once the additional subunits have associated to the apoMoFe, it is ready for FeMoco insertion (Allen et al., 1993). In *K. pneumoniae,* that additional subunit is the product of the *nifY* gene (White et al., 1992). White and coworkers (1992) were able to show electrophoretically that upon insertion of one FeMoco, one subunit dissociates from the complex. When an additional FeMoco was added an additional subunit dissociates, thus forming holoMoFe.

NIFV is a putative homocitrate synthase. The *NifV* gene product has attracted a lot of research interest due to the ability of *NifV*- mutants to be able to reduce acetylene but not dinitrogen (McLean and Dixon, 1981). One can reproduce the substrate specificity of FeMoco extracted from a NifV$^-$ mutant in the *in vitro* FeMoco biosynthetic system by replacing homocitrate with a variety of hydroxyltricarboxylic acids (Hoover et al., 1988b). This has resulted in a vast body of research on the altered substrate specificity (for a compilation see Allen et al., 1994) suggesting that homocitric acid plays a role in the modulation of redox state of FeMoco by either donating protons or transferring electrons (Kim and Rees, 1992). From comparisons between the *NifV* and acetyl-CoA binding synthases, it has been suggested that *NifV* encodes a homocitrate synthase and that its substrates are acetyl-CoA and α-ketoglutarate (Dean and Jacobson, 1992). It is a peculiar fact that demonstration of neither the synthetase activity nor the back reaction, CoA-homocitrate lyase, has been reported yet.

The roles of **NIFW** and **NIFZ** are believed to be related to the formation or accumulation of active MoFe protein. It has been shown in *K. pneumonia* and *A. vinelandii* that *nifW* and *nifZ* mutants were still able to reduce dinitrogen albeit at reduced activities (Paul and Merrick, 1989; Jacobson et al., 1989). Paul and Merrick (1989) have shown that these gene products are not involved in the accumulation or utilization of Mo or Fe for FeMoco biosynthesis. When a deletion mutant of *nifMZ* was produced, extracts had

negligible Fe protein activities (because *nifM* is involved in the maturation of Fe protein), and very low activities of MoFe protein. Extracts did, however have significant amounts of FeMoco-activatable apoMoFe (Paul and Merrick, 1989). This last result suggests a role for **NIFZ** in the FeMoco biosynthesis or a function in the stabilization of the polypeptides that form the MoFe protein (like **NIFM** does for the Fe protein). When a deletion double mutant of *nifV* and *nifW* was produced in *Rhodobacter capsulatus*, a higher than normal concentration of homocitric acid was required for FeMoco biosynthesis *in vitro*, this suggested that **NIFW** could be involved in homocitrate transport or incorporation into FeMoco (Masephol et al., 1993). Kim and Burgess (1994) produced a mutant in *A. vinelandii* in which *nifW* was deleted. This mutant produced wild type Fe protein and a MoFe protein which appeared to be wild type by the standard biophysical characterization (i.e. EPR, UV/Vis, SDS-PAGE gel electrophoresis). The MoFe protein did have altered activities and showed slightly altered substrate inhibition. Kim and Burgess (1994) concluded that the Δ*nifW* mutant phenotype was due to one of three possibilities: it has an organic acid other than homocitric acid in the FeMoco, the way that homocitric acid is coordinated to FeMoco has been altered, or there has been a modification of the polypeptide near FeMoco which is not detectable by electrophoresis.

NIFS is a pyridoxal phosphate-containing homodimer which catalyzes the desulfurization of L-cysteine to yield L-alanine and elemental sulphur (Zheng et al., 1993). This suggests a role of **NIFS** to provide the sulphur required for both the P cluster and FeMoco. Zheng and Dean (1994) demonstrated *in vitro* that the *NifS* was able to assemble a Fe_4S_4 cluster into an Fe protein which had had its cluster removed with α,α'-dipyridyl. The *in vitro* system contained the apoFe protein, **NIFS**, L-cysteine, ferrous ion, dithiothretitol and MgATP, and was able to reconstitute the Fe protein to 80-95% of its original activity. Allen et al. (1994) suggest that the reason for low MoFe activity in a *NifS*-mutant could be because there is no Fe protein in the

cell, to help in the maturation process of MoFe protein, and that **NIFS** could possibly be involved in both of the MoFe and Fe proteins' cluster assemblies. It is this researcher's opinion that the cell would not evolve two separate means to produce Fe_4S_4 core clusters for the clusters of the Fe protein and the P cluster and M center of the MoFe protein. Further, Allen and coworkers conclude that not being able to produce those clusters is what causes the *nifS* phenotype. When *nifS*'s protein sequence was compared to other proteins that use the cofactor pyridoxal-5-phosphate (PLP) and L-cysteine as a substrate, there was a high degree of homology around the PLP binding site and active cysteinyl residues (Zheng et al., 1993). These researchers further speculate that this represents a general means by which the cell may activate the sulphur in metallocluster formation.

NIFU's role in MoFe biosynthesis appears to be in the mobilization of iron for metallocluster synthesis (Dean et al., 1993). Based on the analysis of the purified gene product, it appears to have one Fe_2S_2 cluster and four additional cysteinyl residues which could be used to sequester Fe for the Fe-S cluster assembly. Dean and coworkers (1993) suggest three different roles for **NIFU** including the donation of its Fe_2S_2 cluster directly to immature forms of MoFe and Fe proteins. The Fe_2S_2 cluster of NIFU could also be used to mediate the redox states of the other iron bound to the protein, thus making the iron available to be incorporated into the other clusters. A third theory is that **NIFU** could have a redox role in the release of sulfide from **NIFS**. Determining the exact role of **NIFU** will require the development of an *in vitro* Fe-S cluster assembly assay (Dean et al., 1993).

III. Biophysical Studies of Nitrogenase.

A. The Fe Protein of Nitrogenase: Structural Studies.

The amino acid sequence of Fe proteins from 20 different nitrogenases have been examined and have shown to be highly conserved in both the conventional and alternative nitrogenases (Normand and Bousquet, 1989).

For instance, when the sequence homology of the Fe protein of *Thiobacillus ferrooxidans* was compared to *Parasoibua andersonii*, there was 74% sequence homology and a 54% sequence homology with *Clostridium pasteuranum* (Pretorius et al., 1987). The Fe protein is a dimer of two identical subunits with a total molecular weight of 60,000. There is also a Fe_4S_4 cluster as well as having two nucleotide-binding sites.

A crystal structure from a single species could be representative of all of Fe proteins. The crystal structure of the Fe protein from *Azotobacter vinelandii* has been determined to 2.9 Å (Georgiadis et al., 1992). It shows that the Fe_4S_4 cluster covalently links the two subunits of the Fe protein through two cysteines (97 and 132) from each subunit. The overall structure of the protein has been described as an iron butterfly or an iron lung with the cluster representing the head or heart, respectively. The protein has a sequence motif which is common to other nucleotide-binding proteins. This motif is referred to as Walker's Motif A and it has the sequence G-X-X-X-X-G-K-S/T (Walker et al., 1982). The Fe protein also contains the β-strand-loop-α -helix structure which is common to mononucleotide-binding proteins (Schulz, 1992).

Before the crystal structure determination, there had been exhaustive spectroscopic and chemical examinations of the Fe protein. Through 16 separate Fe analyse by various researchers, the ratio of iron to protein was determined to be 3.7± 0.4 Fe atoms/protein molecule (compiled in Lindahl, 1985). It was suspected that the iron was organized as a cubane cluster. In core extrusion studies, the iron was displaced as [4Fe-4S] clusters (Orme-Johnson et al., 1977; Gillum et al., 1977). Gillum and coworkers (1977) used benzenethiol to extract a 4-Fe site from the iron protein isolated from *C. pasteurianum*. They even suggested that this Fe_4S_4 cluster could act as a bridge between the two subunits. CD and MCD of the reduced Fe protein also suggested that there was a 4-Fe cluster present (Stephens et al., 1979).

Spectroscopic Studies

The Electron Paramagnetic Resonance (EPR) spectrum of Fe protein as isolated has a broadened spectrum with resonances at g_x=1.88, g_y=1.94 and g_z=2.05, generally referred to as g=1.94. The difficulty in the analysis of this signal arose when spin quantitization was attempted. Instead of achieving the expected one spin per 4Fe, the signal repeatably had a value of 0.3 spins/4Fe. Watt and McDonald (1985) were able to correlate the signal height with degree of oxidation or reduction of the Fe protein based on either the addition of oxidizing dyes to dithionite reduced Fe protein or by the addition of reducing equivalents from dithionite to dye oxidized Fe protein, but were unable to achieve more than 0.25 spins per mole of Fe protein. Lindahl et al. (1985) examined this issue and came to the conclusion that the native reduced Fe protein contained a mixture of two types of $[4Fe\text{-}4S]^{1+}$ clusters. One has a familiar S=1/2 ground state with the characteristic EPR signals at g=1.94 and the second form has an S=3/2 ground state with EPR signals around 5.8 and 5.1 (Lindahl, et al., 1985). When Morgan and coworkers (1986) integrated both the S=1/2 and S=3/2 signals together, they were able to assign a value of 1 spin per 4 Fe, the expected result. Hagen and coworkers (1985) analyzed the S=3/2 signal and were able to correlate the number of spins to the sum of the S=1/2 and S=3/2 spin states.

Extended X-ray absorption fine structure (EXAFS) and X-ray Absorption Near-Edge Structure (XANES) are techniques that can be used to derive structural information about metal centers. From an EXAFS experiment, an Fe-S distance of 2.31 Å with a coordination number of 4.0 and for Fe-Fe distance of 2.73 Å with a coordination number of 2.4 was derived (Lindahl, et al., 1987). This data supported the idea of a [4Fe-4S] cluster with four external thiol ligands. In Lindahl et al. (1987) EXAFS study of oxidized and reduced Fe protein they suggested that the cluster was held between the two subunits due to the longer than expected Fe-S bonds.

Mutagenetic Studies

Site-specific mutagenesis of the conserved cysteines in the Fe protein determined that four cysteines are required to produce active Fe protein (Howard et al., 1989). Although, there are five conserved cysteines in all the examined species (Pretorius et al., 1987), it was subsequently determined that cysteines 97 and 132 were the ligands to the cluster, due to the absence of nitrogenase activity when serine substitutions were made at those sites. When serine substitutions were made at the other three cysteines, moderate levels of activity were observed. Since the Fe protein is a dimer, this accounts for the anchoring thiols in the Fe_4S_4 cluster.

To examine the reactivity of the cysteines present in the Fe protein, the Fe protein was reacted with iodo[2-^{14}C]acetic acid (IAA) under various conditions. A cysteinyl residue when exposed to IAA will have its thiol carboxymethylated (Hausinger and Howard, 1983). It was determined that Cys-97 and Cys-132 were probable ligands to the Fe cluster because they were selectively labeled in parallel to the cluster degradation as well as being the only residues modified during the Mg-ATP dependent chelation of the cluster. Cys-85 was suggested to be part of the MgATP binding pocket due to MgADP protection of this thiol residue. Both the Fe protein and apoFe protein (without the Fe_4S_4 cluster) underwent conformational changes upon binding of MgATP (Hausinger and Howard, 1983).

Binding Studies

When MgATP binds to the Fe protein, the conformation of the protein changes. This can be seen in the lowering of the cluster's midpoint potential upon nucleotide binding (Zumft et al., 1973; Morgan et al., 1986) as well as the increase in accessibility of the cluster to chelators. (Walker and Mortenson, 1974; Ljones and Burris, 1978). The CD and MCD spectra also change (Stephens et al., 1979) and the ^{57}Fe quadrupole splittings of the cluster decreases when MgATP binds (Lindhal et al., 1985). The EPR spectrum also changes upon binding of MgATP (Lindahl et al., 1987) with the g=1.94 signal

changes from rhombic to axial symmetry and the other low field features change shape and intensity as well (Orme-Johnson et al., 1972). A g=4.3 signal is also observed. This signal arises from an S=5/2 state from a [4Fe-4S]$^{1+}$ cluster present when MgATP is bound. This state accounts for ~2% of the total protein. Zumft and coworkers (1974) suggested that two ATP molecules could bind per Fe protein based on binding experiments and observations of mid-point potential shifts of MgATP binding to the Fe protein.

The MgATP binding site on the Fe protein has been of interest to many researchers. Using site directed mutagenesis studies, Seefeldt and coworkers (1992) created a mutant in which the lysine at position 15 had been changed to a glutamine. The resulting protein had the normal iron content, and the characteristic EPR spectrum of the native species with the 4Fe-4S center. The differences only became apparent upon the addition of MgATP. When MgATP was added, the expected change in the EPR spectrum from a rhombic to an axial signal did not occur. There was also no increase in accessibility of the cluster to iron chelators when MgATP was added. This mutant protein was able to bind MgADP at wild type protein levels. A lysine to arginine mutant resulted in the characteristic 4Fe-4S center and EPR spectrum, but had no significant affinity for either MgATP and MgADP (Ryle et al., 1985). Ryle and coworkers suggest that, in the Fe protein, there is a salt bridge between Lys-15 and Asp-125 that gets disrupted upon binding of MgATP. Asp-125 could then interact with Mg^{2+} while Lys-15 interacts with the γ- and β-phosphates of MgATP. The relative position of Cys-132, which binds the Fe_4S_4 cluster to the polypeptide, could be affected, which in turn affects the cluster's chemistry (Ryle et al., 1995). This is plausible but requires further testing.

Fe protein$(MgATP)_2$ complex binds to MoFe protein. While in this complex, the Fe protein transfers an electron and the MgATP is hydrolyzed to MgADP and inorganic phosphate. One residue which might play a role in

this process is Ala-157. Gavini and Burgess (1992) produced a mutant in which Ala-157 had been changed to a serine. This mutant has a normal 4Fe-4S cluster and has a normal MgATP binding ability but when the MgATP binds, it does not induce a conformational change seen by the increase in accessibility of the cluster to chelators, produces Fe protein which is unable to productively electron transfer to the MoFe protein in an activity assay and MgATP is not hydrolyzed because inorganic phosphate is not released from the protein. Using small-angle x-ray scattering, which is used to study tertiary/quaternary structure of biomolecules in solution, Chen and coworkers (1994) showed that there was a decrease in the radius of gyration with wild type Fe protein but no decrease in the radius of gyration with the mutant Fe protein.

Based on Electron Spin Echo Envelope Modulation (ESEEM) Spectroscopy, it was suggested that the MgATP did not bind directly to the cluster (Morgan et al., 1990). In this study, Morgan and coworkers searched for possible non-covalent interaction via a ^{31}P of MgATP and the Fe-S cluster. They were not able to detect any interactions between ^{31}P and the Fe cluster. They did observe some weak interactions between ^{14}N of the peptide and the cluster, suggesting that the ^{31}P-ATP atoms are not as near the cluster as are the peptide nitrogen atoms.

B. The Molybdenum Iron Protein of Nitrogenase: Structural Studies

The MoFe protein of nitrogenase is a 240,000 kD protein arranged in a $\alpha_2\beta_2$ tetramer with a Fe_8S_8 cluster and a $MoFe_7S_8$(homocitrate) in each $\alpha\beta$dimer. When the sequence homologies of the α and β subunits are compared among the MoFe proteins of 10 different species, there is a high degree of sequence homology and predicted secondary structure (Dean and Jacobson, 1992).

There was considerable difficulty determining the protein structure to a level of detail at which the metal clusters' structure and composition can be

determined. It was unknown whether the P cluster's 8-Fe-8-S were part of one super-cluster or as two separate cubanes. Part of the problem in solving the MoFe protein crystal structure was a difficulty in obtaining heavy atom derivatives. Without heavy atom derivatives, it was difficult to measure difference signals useful for locating the anomalous scatters and solving the crystallographic phase problem.

Organization of the Metal Clusters in MoFe Protein

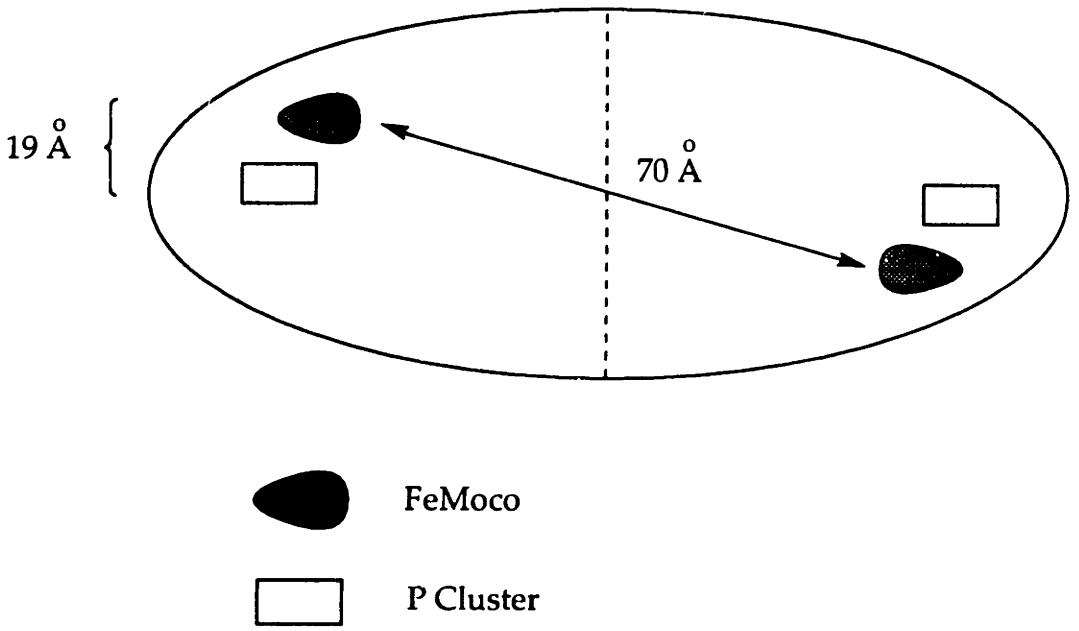


Figure 1.4: The spatial relationship between FeMoco and the P cluster. Bolin and coworkers (1990) showed that the P cluster consists of a super cluster of Fe_8S_8. The P cluster is separated from FeMoco by 19 Å. The FeMoco in each half of the αβdimer is separated by 70 Å.

Bolin and coworkers (1990) were finally able to solve the *C. pasteuranium* MoFe structure to 5 Å resolution using synchrotron source of X-rays at two wavelengths. At the 5 Å level, it was determined that the metals in the protein are arranged in four clusters (2 P clusters and 2 FeMoco) (Bolin et al., 1990, Bolin et al., 1993b). FeMoco is located in the α subunit and the P cluster is situated at the interface between the α and β subunits. The relationship

between the two metal centers are shown in the figure 1.4 (Bolin et al., 1990). To determine the molecular connectivity of FeMoco and the P cluster, a more refined structure was required.

The crystal structure of the MoFe protein has been solved in two different species, *Azotobacter vinelandii* (Kim and Rees, 1992) and *Clostridium pasteurianum* (Bolin et al., 1993a; Kim et al., 1993) each to 2.2 Å resolution. The overall structures of the two proteins are very similar, with conserved tertiary structure around the metal centers. The α and β subunits show similar polypeptide folds consisting of three domains of the α helical/β sheet type. In between the three domains in each subunit exists a wide, shallow cleft.

A model of both the FeMoco and the P clusters has been proposed. Bolin et al. (1993a) and Kim and Rees (1992) were independently able to determine the structures for the metal centers. After some initial differences, the two crystallographic groups have reached a similar conclusion for the structures. For clarity, the *A. vinelandii* numbering system will be used throughout this discussion. FeMoco consists two cubane fragments with the composition of 4Fe:3S in one and 1Mo:3Fe:3S in the other. Connecting the two cubane fragments are a waist of three sulfides. FeMoco is anchored to the protein by Cysα275, bound to the apical Fe atom at one end and Hisα442 through a Mo atom at the other. The homocitric acid is coordinated to the molybdenum through a hydroxyl and a carboxyl oxygen. This coordinately saturates the Mo atom. The most suprising feature of this structure is the presence of six trigonally coordinated iron atoms in the center of FeMoco. The thought that one or more of these under-coordinated Fe atoms may be the site of N_2 binding is inevitable. There are also several side chain residues around FeMoco. Hisα195 and Glnα191 are within 5 å of the FeMoco trigonal irons but do not appear to be ligated to the cluster. Other residues near the cluster are Argα359 and Argα96.

Structural Model for FeMoco as Proposed by Kim and Rees

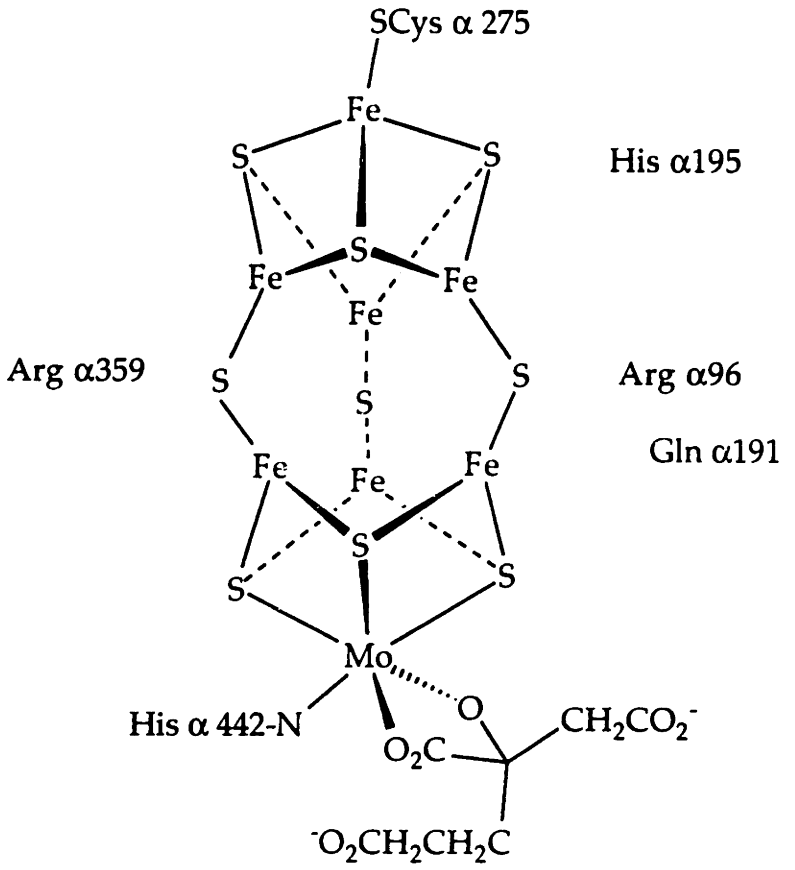


Figure 1.5: FeMoco model proposed by Kim and Rees (1992a).
The cluster is tethered to the polypeptide through a thiol-iron bond at one end and the Mo is connected through His-442. Homocitric acid is coordinated to Mo through hydroxyl and carboxyl oxygens. Sulfide ligands connect the trigonal irons.

The P cluster structure has also been modeled. They are two Fe_4S_4 cubanes held together by a disulphide bridge and two bridging cysteine sulphurs (Cysβ95 and Cysα88). The P clusters are joined to the protein through cysteine thiols bonded to iron atoms in the cubes.

The Consensus Model of the P cluster of Nitrogenase

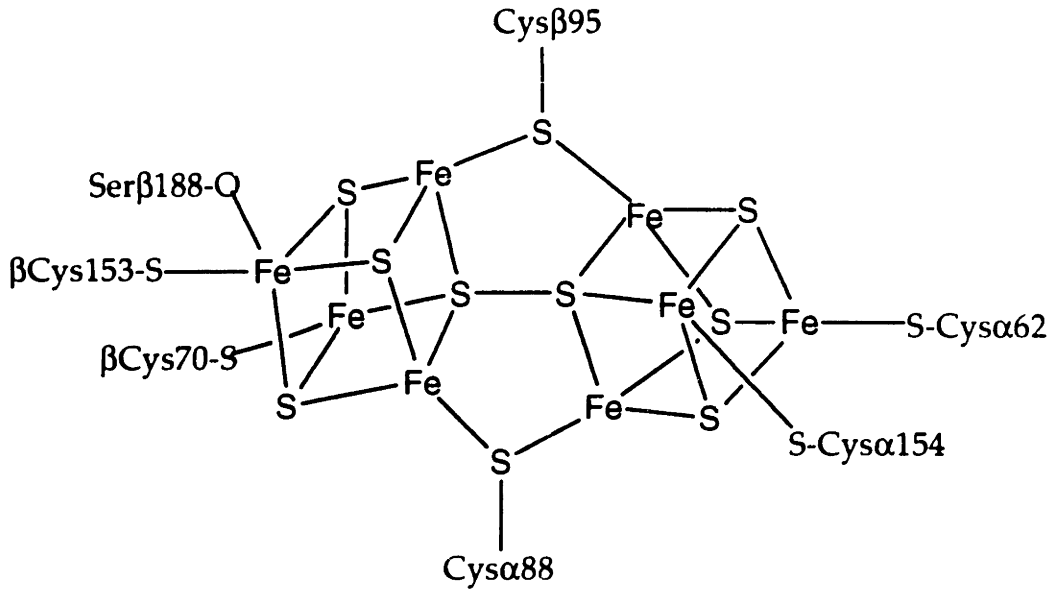


Figure 1.6: Model of the P cluster in the MoFe protein of nitrogenase. Two Fe_4S_4 cubanes are ligated together with a disulphide bond from a corner sulphur atom from each cube, and two bridging thiols. The cluster is connected to the protein through two thiol cysteines on one cube and two thiol cysteines and a hydroxyl serine on the other.

Even before the crystal structure of the MoFe protein had been determined, a vast amount of information was known about the MoFe protein. These studies can be separated into several categories: structural information about the protein, information about the interaction between the MoFe and Fe proteins, studies on the FeMoco cluster, and studies on the P cluster.

Mutagenic Studies

Structural information of the protein environment around FeMoco has been investigated. One approach has been to specifically substitute certain amino acid residues located around the cofactor either in the binding pocket or as direct ligands (Scott et al., 1990; Thomann et al., 1991). The researchers were able to demonstrate that the polypeptide environment of FeMoco plays

an important role in directing its substrate-reducing properties. Scott and coworkers (1992) showed that altering either Hisα195 or Glnα191 created a protein with altered substrate-reduction activity, inhibitor susceptibility and spectroscopic properties. Through extraction and reconstitution assays, it was demonstrated that the cofactor was not altered in either its composition or structural arrangement. Using the same kind of approach in *K. pneumoniae*, Kent et al. (1990) suggested that Cysα275 was a probable ligand to FeMoco. If a mutant has produced a protein which is able to bind FeMoco, when its altered MoFe protein is mixed with FeMoco, it will produce a protein which is able to reduce acetylene and dinitrogen. When the mutant in which Cysα275 was changed to Alaα275, the resulting protein was unable to bind with FeMoco to reduce the substrate.

The way in which the P cluster is tethered to the β-subunit of the MoFe protein has also been the subject of site-directed mutagenesis studies (Dean et al., 1990; May et al., 1991). May and coworkers (1991) looked at the conserved cysteine residues at positions 70, 95, and 153 in the β-subunit. The results are summarized in the table 1.2:

Table 1.2: Mutagenetic Studies on the conserved cysteines of the MoFe protein

		EPR	
Mutation made	Nif phenotype	S=3/2 signal	% of wild type
Cysβ70→Ala	Nif-	present	~2% wt
Cysβ70→Ser	Nif-	absent	
Cysβ95→Ser	Nif-	absent	
Cysβ153 →Ala	Nif-	present	~25% wt
Cysβ153→Ser	Nif+	present	~45% wt
Cysβ153→Δ	Nif+	present	~45% wt

From the results of their study, it is apparent that the cysteines at 70, 95 and 153 play an important role in the MoFe protein. Altering the cysteines at either 70 or 95 eliminates the ability of nitrogen fixation. May and coworkers (1991) suggest that when the cysteine at position 153 is changed to serine, that

the hydroxyl of the serine is still able to form a bond with the P cluster. When Cys153 is completely eliminated (Cysβ153→Δ), they suggest that there is a slight conformational change in the peptide in such a way that a new bond is formed (May et al., 1991). By examining the crystal structure, the iron at which Cys153 forms a bond is the only iron in the cluster which has two polypeptide bonds formed at it, so it is conceivable for the cluster to remain intact without the βCys-153 being present.

To examine the probable ligands to the P cluster from the α subunit of the MoFe protein, Dean and coworkers (1990) made numerous substitutions at the residues Cysα62, Cysα83, Cysα88 and Cysα154. The results indicated that Cysα62 and Cysα154 are probably essential for MoFe protein activity but that Cysα88 and Cysα83 are not. Upon elimination of the latter two residues, a new cluster-ligand arrangement is formed. If there were substitutions at either Cysα62 and Cysα154, the ability of the organism in the majority of the cases to produce MoFe protein was eliminated. If MoFe protein was detected, it was at ≤ 2% the level of wild type MoFe protein activities. When Cysα88 was substituted with an amino acid that did provide an alternative ligand, for instance an oxygen, the polypeptides appeared to be unable to compensate for it (seen in Nif^+ growth). If a substitution was made such that no alternative ligand was provided, for instance a glycine, the resulting phenotype was Nif^-. Upon examination of the crystal structure, it was shown that Cysα62 and Cysα154 did provide direct ligands to the P cluster. It is interesting to note that Cysα88 is a bridging ligand between the P clusters and in some of the mutants, the bridging thiol appeared to have been replaced by a hydroxyl oxygen.

The effect of Hisα195 on FeMoco has had an interesting history. It was originally proposed that Hisα195 was coordinated to the cofactor because a nitrogen-14 coupling to the cofactor g=3.771 signal was observed in ESEEM

(Electron Spin Echo Envelope Modulation) spectroscopy with wild-type protein appeared to have disappeared when substitutions were made (Thomann et al., 1991). When the crystal structure of the MoFe protein was determined, Hisα195 was not coordinated to the FeMoco, but indeed was in the vicinity of the cluster (Kim and Rees, 1992). The experiments were then repeated. Whenever a substitution was made at Hisα195 , the resulting mutant was Nif-, but if the mutant cultures were derepressed, the altered MoFe proteins could be induced. Substitution of Hisα195 to Glnα195 creates a protein which is able to bind but not reduce dinitrogen (Kim et al., 1995). ESEEM studies were undertaken on purified protein from Hisα195 (wild type) as well as Asnα195 and Glnα195 (DeRose et al., 1995). When the histidine was substituted with asparagine, there was a decrease in intensity in the nitrogen modulation, but when the histidine was substituted with glutamine, there was not an apparent effect. DeRose and coworkers (1995) suggest that the observed nitrogen modulation is not directly associated with the Hisα195, but is due to another nitrogen moiety of a different residue whose proximity to FeMoco is sensitive to certain substitutions at the Hisα195 position. Examination of differences between the amino acids glutamine, histidine and asparagine suggests that Hisα195 and Glnα195 can provide hydrogen bonding to FeMoco but not Asnα195, due to its decreased side chain length (De Rose et al., 1995). The suggested roles of Hisα195 are: to form a hydrogen bond with one of the sulphurs in the cofactor, to help maintain the "correct" orientation of FeMoco in the polypeptide matrix and to help fine tune the electronic properties of the substrate binding and reduction site during catalysis (DeRose et al., 1995; Kim et al., 1995).

Interactions between the Fe protein and MoFe protein

There has been interest in how the MoFe and Fe proteins interact. When Arg100 of the Fe protein was changed to either tyrosine or histidine, the resulting Fe proteins had some interesting features (Wolle et al., 1992). The histidine replacement (R100H) produced a protein that was inhibited by the salts present in the normal acetylene *in vitro* assay. This substitution also uncouples substrate reduction to MgATP hydrolysis. Wolle and coworkers (1992) suggest that the Arg100 role is to provide the stability of the complex between the MoFe and Fe proteins for maximum efficiency in substrate reduction. When the Fe and MoFe proteins were cross-linked with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, it was found that one of the γ subunits of the Fe protein was connected to one of the β subunits of the MoFe protein (Willing et al., 1989). In the same study, Willing and coworkers suggest that electrostatic forces are critical to the formation of the electron transfer complex.

Seefeldt (1994) also examined the docking of the MoFe and Fe proteins. Using site-directed mutagenesis Arg 140 and Lys 143 of the Fe protein were changed to the neutral amino acid glutamine. The resulting mutants (R140Q and K143Q) had a significant decrease in nitrogenase activity. When Fe protein is mixed with MoFe protein and MgATP, the MoFe protein protects the Fe protein's cluster from extrusion by α-α'-dipyridyl. When the mutant Fe proteins were examined under these conditions, the cluster was still able to be extruded from the protein. The mutant Fe proteins both demonstrated an increase in sensitivity to increasing ionic strength. Compared to the wild type complex hydrolyzing 2.5 MgATP per electron transfer, the R140Q and K143Q mutants hydrolyzed 6 and 31 MgATP per electron respectively. Seefeldt (1994) suggests that these residues function in aligning the Fe protein with the MoFe protein for the electron transfer.

Fe protein from *C. pasteuranium* (Cp2) when combined with MoFe protein from *A. vinelandii* (Av1) under assay conditions, no substrate is

reduced due to the formation of a catalytically inactive Cp2Av1 complex (Emerich and Burris, 1978; Emerich et al., 1978). Building upon this observation, Peters and coworkers (1994) created a *A. vinelandii* Fe protein hybrid which had residues 59 through 67 replaced with the corresponding residues from Cp2, denoted by AvCp2. The hybrid protein exhibited half the specific activity as Fe protein from *A. vinelandii* (Av2). The resulting hybrid protein did not form a tight non-dissociating complex with Av1. Instead it dissociated from Av1 at half the rate of Av2 from Av1. This indicated that there is more to the protein-protein interaction than just these residues.

Spectroscopic Studies

Spectroscopic studies have been utilized to deduce the properties and to characterize the MoFe protein. The MoFe protein as isolated has a characteristic EPR signal, with g values of g~4.3, g~3.7 and g~2.0. As isolated, the EPR signal is due to the FeMoco cluster, the P cluster is in an EPR silent state. The FeMoco is paramagnetic with an S=3/2 signal. There is hyperfine present on the signal when the sample is enriched for either ^{57}Fe (which produces broadening of the signal) or ^{95}Mo (which produces six hyperfine lines). These hyperfine interactions have been observed in MoFe protein isolated from *C. pasteurianum* (George et al., 1989), and *A. vinelandii* (McLean et al., 1987).

The MoFe protein can undergo redox reactions without Fe protein. The midpoint potentials of the oxidation of the g=3.7 signal are 0 mV for *C. pasteuranium*, -42 mV for *A vinelandii* and *A. chroococcum*, -95 mV for *Bacillus polymyxa*, and -180 mV for *K. pneumonia* (O'Donnel and Smith, 1978). O'Donnel and Smith (1978) rationalize the differences in the midpoint potentials are due to the environments in which these organisms grow. *A. chroococcum* and *A. vinelandii* are strict aerobes, *C. pasteurianum* is a strict anaerobe and *K. pneumonia* and *B. polymyxa* are facultative anaerobes which only synthesize nitrogenase in the absence of oxygen. There would have been evolutionary pressure to select for the organism which has a potential which

was lower than the surrounding environment so metabolic energy would not be wasted to have to keep the MoFe protein in the reduced state.

The MoFe protein can undergo both electrochemical and chemical oxidations. The oxidations occur in two phases. Initially the diamagnetic (S=O), EPR silent P-clusters are oxidized to a paramagnetic (S=3/2-9/2), EPR active state followed by the oxidation of the paramagnetic (S=3/2) M-centers (FeMoco) to a diamagnetic state with the loss of the characteristic S=3/2 signal. When thionin is used to oxidize MoFe protein from *A. vinelandii*, it takes 3-4 equivalents to oxidize the P-clusters and then an additional 2-3 equivalents to oxidize the M-centers (Zimmerman et al., 1978; Watt et al., 1980). Watt and Wang (1989) were able to show that MoFe protein can undergo up to a 12 electron oxidation, depending on the strength of the oxidant and the presence of intermediate three-, six, and nine-electron-oxidized MoFe protein states have some inherent stability. They argue that these oxidation states indicate the facile accessibility of the clusters in the protein to external electron transfer agents.

In order to study the P cluster using EPR, the MoFe protein must first be oxidized. Hagen and coworkers (1987) examined thionine-oxidized MoFe proteins from several different organisms. An S=7/2 signal was attributed to the multi-iron exchange-coupled P clusters. When this signal was quantified relative to the S=3/2 signal from dithionite-reduced MoFe protein, the stoichiometry was one P cluster per FeMoco, but they were unable to determine whether the P cluster was one cluster or two separate clusters interacting with one another.

In examination of *NifB-Kp1* (MoFe protein from a *K. pneumoniae* B-mutant), Robinson and coworkers (1984) were able to show that there was a structural and electronic similarity between the clusters in *NifB-Kp1* and wild-type Kp1. This showed that the P clusters are not significantly altered chemically or physically by the presence of the FeMoco. They also suggest that this lends support to the thought that the role of the P clusters to store electrons and mediate electron transfer to and from FeMoco.

Using progressive power saturation, Oliver and Hales (1992) followed the relaxation properties of the M-centers during thionin oxidation. They were able to see a perturbation of the magnetic properties of the M-centers from the diamagnetic P clusters. Based on modeling of the probable magnetic interactions of the P cluster and M center, Oliver and Hales (1992) suggested that during the oxidation of the P clusters, the P cluster acts as an interactive pair which was oxidized 1 equivalent at a time and in the singly and doubly oxidized state can be paramagnetic (Oliver and Hales, 1992).

In Mössbauer spectroscopy the ^{57}Fe nucleus is a probe whose interactions with surrounding electrons give structural and chemical information. This type of spectroscopy will see all iron atoms, regardless of their spin or valence state. In contrast, EPR only probes paramagnetic states (Münck et al., 1975). The first use of Mössbauer spectroscopy to analyze *K. pneumonia* MoFe protein was by Kelly and Lang in 1970 (Kelly and Lang, 1970). Since the techniques for purifying the MoFe and Fe proteins were not optimized at this time, this resulted in incorrect assignments of some of the signals due to the presence of both oxidized and reduced states of the MoFe protein (Smith and Lang, 1974). Using more active protein and better anaerobic techniques, Smith and Lang (1974) reinterpreted the earlier work, but maintained the signal assignments of their earlier work.

Upon examination of the *A. vinelandii* MoFe protein, Münck and coworkers (1975) were able to see strong similarities with the *K. pneumoniae* data of Smith and Lang (1974). This similarity of Mössbauer data would later be also correlated for *C. pasteuranium* (Huynh et al., 1980). Combining both EPR and Mössbauer data, Münck and coworkers (1975) were able to completely describe the Mössbauer spectrum using a simpler, more coherent nomenclature than that of Smith and Lang (1974) (see table 1.3).

Table 1.3: Spectral Components Observed in the MoFe Protein

Spectral component	Number of Fe atoms/molecule	Conditions of observation	Assignment
M	8-10	Native protein and reductant depleted fixing mixture	-associated with two EPR active centers of spin S=3/2 -magnetic spectra associated with ground doublet of S=3/2 spin system
D	9-11	Under all conditions investigated	-Almost temperature independent quadrupole splitting -isomeric shift suggests reduced iron -iron seems to be in a diamagnetic complex (S=0) -suggests that the iron is in spin coupled clusters
Fe^{2+}	3-4	Under all conditions investigated	-ΔE_o and S very similar to reduced rebredoxin, high-spin ferrous in character, with sulfur coordination. -behaves unlike rebredoxin in strong magnetic field. -seems to be incorporated into diamagnetic complex. -could be complexed with spectral component D
S	~1	Possibly present under all conditions	-ΔE_o~1.4 mm/s and S~0.6 mm/s indicates low-spin ferrous iron.

Using ^{57}Fe-labeled MoFe protein, they were able to determine that the MoFe protein contains 30 ± 2 iron atoms, with 16 iron atoms associated with the P clusters and the rest of the iron being associated with the M-centers (FeMoco). They also characterized the oxidation states of the MoFe protein as stated in figure 1.7 (after Zimmermann et al., 1978)

M center (FeMoco)

Oxidation state	M^{ox} $\overset{1\ e^-}{\rightleftharpoons}$	M^{N} $\overset{1\ e^-}{\rightleftharpoons}$	M^{R}
Electronic spin	S=0	S=3/2	S=interger
Mössbauer components	Doublet M^{ox}	Magnetic Mössbauer spectrum at 4.2 K	Doublet M^{R}

P-Cluster

Oxidation state	P^{ox} $\overset{1\ e^-}{\rightleftharpoons}$	P^{N}
Electronic spin	S>3/2, half-integer	S=0
Mössbauer component	M1 at 4.2 K: doublets I-IV at 170 K	D and Fe^{2+}

Figure 1.7: Oxidation states of FeMoco and P cluster.
These are the available oxidation states of the FeMoco and P cluster as described by Zimmerman and coworkers (1978).

There was debate over whether the S component of the Mössbauer spectra was a contaminant or a true component of either the P cluster or the FeMoco. McLean et al (1987b,1988) made isotopically labeled hybrids of MoFe protein. It is possible to grow mutants which are unable to biosynthesize FeMoco but are able to insert FeMoco into the apoMoFe protein. This would enable the P clusters of the apoMoFe protein to be labeled with one isotope of iron. The extracted FeMoco could then be labeled with another isotope. When isotopic hybrids of the MoFe protein with ^{57}Fe on the P cluster and ^{56}Fe on the M center were produced, the component S was present. The spectral components D, Fe^{2+} and S (see table 1.3) could then be all attributed to the P cluster because they all contributed to the ^{57}Fe Mössbauer spectrum and the source of the ^{57}Fe was the apoMoFe protein with the P clusters.

XANES Spectroscopy of the MoFe protein has been used as a tool to describe the structure around the FeMoco in the protein. Using Fe K-edge X-ray absorption spectroscopy, Chen and coworkers examined dithionite-reduced MoFe protein (1993) (see table 1.4). When the MoFe protein was oxidized with thionine, there was only a small contraction (2.32 and 2.73 Å to 2.29 and 2.7Å, respectively) observed in the Fe-S distance and possibly in the

Fe-Mo distance. What is not seen is a major cluster reorganization when the protein is oxidized (Chen et al., 1993). Since there is only one molybdenum in FeMoco, Mo-K edge EXAFS will give structural information around the molybdenum (see table 1.4). When Conradson and coworkers (1989) examined the MoFe protein in the presence of various inhibitors, including CO, N_2, C_2H_2, NaCN, CH_3NC or azide, they did not see any shifts in the molybdenum K-edge spectrum. One would expect to see a shift in the K-edge if a substrate bound to the molybdenum and the oxidation state of the atom changed.

Table 1.4: Estimated Distances in the MoFe protein derived from XANES experiments.

The number of atoms with the given distance in brackets (from Chen et al., 1993; Conradson et al., 1987; Liu et al., 1994):

Atoms	Crystallographic Distance (Å)	Distance Estimates	
		MoFe protein	FeMoco
Mo-O(N)		2.12(1.7)	2.10(3.1)
		2.22(3)	2.19(3)
Mo-S	2.42-2.50(3)	2.37(3)	2.37(3)
		2.37(4.5)	2.37(3.1)
Mo-Fe(short)	2.84-2.98(3)	2.37(3),	2.72(3)
		2.68 (3.5)	2.70(2.6)
Mo-Fe(long)	5.19-5.33(3)	5.06(3)	5.11(3)
Fe-O(N)		1.84(0.5)	
Fe-S		2.32(3.5)	2.20(3)
Fe-Fe	2.7-2.8	2.64(2.0)	2.64(2.2)
Fe-Mo		2.73(0.3)	2.70(0.8)
Fe-Fe	~3.8	3.78(1.4)	3.68(1.6)
Fe-S		4.28(2.2)	
Fe-Fe		4.68(0.4)	

Studies on extracted FeMoco

The active site of the MoFe protein is FeMoco. To comprehend how this enzyme is able to reduce dinitrogen, an understanding of the chemistry and structure of FeMoco is necessary. FeMoco can be extracted from the MoFe protein. Shah and Brill (1977) first developed a method that was later modified by Yang and coworkers (1982). The procedure involved citric acid and disodium hydrogen phosphate precipitation followed by centrifugation. The next step involved washing the pellet with dimethlyformamide (DMF), with an additional centrifugation step followed by washing the pellet with N-methylformamide (NMF) with a centrifugation step. The efficiency of the extraction varied from 45-90% (Burgess et al., 1980; Shah and Brill, 1977; Yang et al., 1982, Walters et al., 1986). The partial formulation of the resultant FeMoco is $MoFe_{6-8}S_{8-9}$ (Nelson et al., 1983). The extracted FeMoco can reconstitute acetylene reduction activity in mutants which lack FeMoco. This provides a functional assay for the activity and purity of the FeMoco preparations.

In the lab of Orme-Johnson (McLean et al., 1989; Wink et al., 1989), a new extraction procedure was developed. It involved binding the MoFe protein to DEAE-cellulose, denaturing the protein with an organic solvent, extruding the P clusters with an iron chelator and then extracting the FeMoco with an organic cation. This method can be done entirely in a glove box where the chance of oxygen and water damage was low. The extraction efficiency ranged from 50-90% with very high specific activities of the extracted cofactor. The excess salt can be removed by chromatographic separation on Sephadex LH-20. The extraction produced a cofactor with Mo:Fe ratios of 1:6± 1. This enabled FeMoco to be successfully extracted and available for study.

The characterization of isolated FeMoco has been of interest to researchers. Hoover and coworkers (1989) showed that homocitrate was a component of FeMoco. Using extracts of mutants unable to biosynthesize MoFe protein, they synthesized holoMoFe protein *in vitro* with the addition

of [^{3}H]homocitric acid. Upon purification of FeMoco from the MoFe protein, the ^{3}H was shown to be a component of FeMoco by NMR analysis. Huang and coworkers (1993) attempted to produce homogenous cofactor for characterization. Upon producing homogenous FeMoco, the minimal elemental composition was determined by several methods to be $MoFe_6S_6$. The molecular weight determined using calibrated columns was twice the value of the minimal elemental composition, suggesting that in solution, FeMoco exists as a dimer. The solution charge of FeMoco was determined to be -2 per Mo. They were unable to detect homocitric acid in their extensive purification of FeMoco, although it was initially present in the early stages of the extraction procedure. The crystal structure does indeed have homocitric acid attached to the Mo atom in the FeMoco.

Spectroscopic Studies

The first reported EPR spectra of isolated FeMoco was by Rawlings and coworkers (1978). It showed the characteristic S=3/2 signal that had been previously reported for MoFe protein. This demonstrated that EPR active M center of the MoFe protein had been isolated as a cluster. When the signal was integrated with respect to molybdenum, the result was one spin per Mo. The extracted FeMoco spectrum is grossly broadened when compared with the MoFe protein signal. This has been attributed to an unspecified change in ligation when the extraction has occurred in NMF due to the ligatation of this solvent to FeMoco. When thiophenol is added to isolated FeMoco, its EPR signal sharpens and more closely resembles that of the intact MoFe protein (Rawlings et al., 1978). When cyanide is added to the extracted FeMoco, there is a shift in the g values to produce an axial spectrum with sharper peaks. Richards and coworkers (1994) quantitated the amount of cyanide that binds to FeMoco to be 1.5 per Mo. If both cyanide and thiolate are added to FeMoco, both are able to bind, indicating that they bind to separate sites on the FeMoco (Richards et al., 1994).

Rawlings and coworkers (1978) also examined the isolated FeMoco using Mössbauer spectroscopy. Mössbauer experiments on the isolated FeMoco showed that it was iron containing cluster similar to the spectrum obtained for the S=3/2 M-center of the MoFe protein. This M center was shown to contain between five to seven iron atoms, but most likely six. All of the iron atoms were reported to have the same quadrupole splitting (ΔE_Q = 0.75 mm/s) and isomer shift (S= ± 0.37) (Rawlings et al., 1978). Newton and coworkers (1989) suggest that the observed changes in the Mössbauer spectrum when the FeMoco is isolated in NMF are due to the increased symmetry around FeMoco.

Another spectroscopic tool that has been used to examine the MoFe protein is Electron Nuclear Double Resonance (ENDOR). An ENDOR experiment provides an NMR spectrum of those nuclei that interact with the electron spin of the paramagnetic center that is being irradiated. The ENDOR frequencies directly give the electron-nuclear coupling constraints. The nuclear resonance transition is not being observed directly. Instead, observations of changes in the EPR signal intensity are examined. In this type of experiment, observations of the nuclei that have a hyperfine interaction with the electron-spin system are being observed, unlike the Mössbauer ^{57}Fe experiment in which all ^{57}Fe atoms are observed. This helps to better define the interaction of complex metal clusters (Hoffman, 1991).

ENDOR can be used to observe different nuclei of the FeMoco within the MoFe protein. Venters and coworkers (1986) examined the ^{1}H, ^{57}Fe, ^{95}Mo and ^{33}S ENDOR of the as-isolated MoFe proteins from *A. vinelandii*, *K. pneumoniae* and *C. pasteuranium*, expanding upon previous studies of ^{95}Mo and ^{1}H interactions in *A. vinelandii* by Hoffman and coworkers (Hoffman et al., 1982). The researchers were able to see five different types of iron sites associated with the FeMoco. They were also able to detect a single Mo in the MoFe protein spin system. The FeMoco appeared to be accessible to the solvent as observed by the presence of exchangeable protons. They were also

able to observe sulphur being coordinated to the MoFe spin system and were in a S^{2-} state. Upon development of new techniques for analyzing data, True and coworkers (1988) were able to describe all of the iron sites. The iron sites are A^1, A^2 and A^3, and B^1 and B^2. The isomer shift of the A sites are between the isomer shifts seen when Fe(II) or Fe(III) are tetrahedrally coordinated to sulphur. The B sites have isomeric shifts closer to isomeric shifts expected for Fe(III) coordinated to sulphur.

The Magnetic-Circular-Dichroism (MCD) spectroscopic experiments support the concept of the geometry of FeMoco being more ordered in the extracted state than when it is within the protein (Robinson et al., 1984). They do see an shoulder in the MoFe protein that is not present in the extracted FeMoco. Robinson and coworkers (1984) suggest that this may be due to a loss of a ligand or to a change in symmetry of the FeMoco cluster on extraction.

EXAFS has also been used to deduce the distances in the structure of isolated FeMoco. Upon extraction from the protein, the overall structure does not change dramatically. There appears to be change in the coordination environment around the FeMoco. Conradson and coworkers (1987) reported that there an increase of 1.7 to 3.1 O(or N) coordinating to the FeMoco after isolation but a decrease in Mo-S (4.5 down to 3.1) and in Mo-Fe (3.5 down to 2.6). When thiol is added to the extracted FeMoco, it did not coordinate directly to the molybdenum (Conradson et al., 1987). In a later study, Conradson and coworkers (1994) examined extracted FeMoco with selenophenol. Using Se-K edge EXAFS, they were able to show that the selenium and hence the thiol, binds to a iron in the FeMoco. What they could not determine was to which iron of FeMoco it was binding (Conradson et al., 1994). Similarly, when CO, N_2, C_2H_2, NaCN, CH_3NC or azide are added independently to solutions of FeMoco, the Mo EXAFS indicated that they did not bind directly to molybdenum (Conradson et al., 1989).

There have been numerous unsuccessful attempts to crystallize the isolated FeMoco. One explanation for the inability to produce crystals is the

inhomogeneity of the isolated FeMoco with respect to its oxidation state. There have been numerous redox states proposed for FeMoco (figure 1.8, after Schultz et al., 1985).

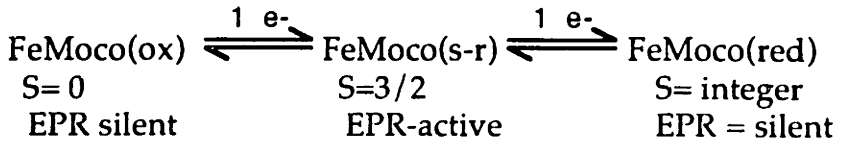


Figure 1.8: Proposed Redox states for FeMoco.

The FeMoco as isolated is in the semi-reduced (s-r) state. Schultz and coworkers (1985) showed that when FeMoco is isolated in NMF and stored under excess dithionite, that it "self-oxidized" completely to its EPR-silent state. This oxidation reaction was attributed to material, probably protic in nature in the solvent or the FeMoco preparation, which was capable of oxidizing FeMoco. Once FeMoco was oxidized, no $S_2O_4^{2-}$ was detected in the preparation. This reaction occurred quickly under anaerobic conditions at room temperature. Schultz and coworkers (1985) using cyclic voltametry, were able to observe that the FeMoco under went two reductions, one at $E_{1/2}$ = -0.32 and the other at $E_{1/2}$ = -1.00 V vs. the normal hydrogen electrode (Schutlz et al., 1988).

Examination of the P cluster

The other metallic cluster in the MoFe protein is the P cluster. Because the P clusters are EPR silent in the MoFe protein as isolated, to examine these clusters, the MoFe protein needed to be oxidized. Surerus and coworkers (1992) examined thionine oxidized MoFe protein from *A. vinelandii, K. pneumoniae, C. pasteuranium* and *Xanthobacter autotrophicus*. Using parallel mode EPR and Mössbauer spectrometry at various temperatures and applied fields, they were able to show that that oxidized P cluster (P^{OX}) consists of degenerate doublet (splitting Δ) of a system with an even number of

electrons. They then suggested that two electrons are removed from each P cluster in the transformation $P^N \rightarrow P^{OX}$. Because four electrons per MoFe protein are removed during thionine oxidation and there are 16 iron atoms in the P clusters, this then leads to the conclusion that the MoFe protein consists of two identical P cluster consisting of 8 Fe sites.

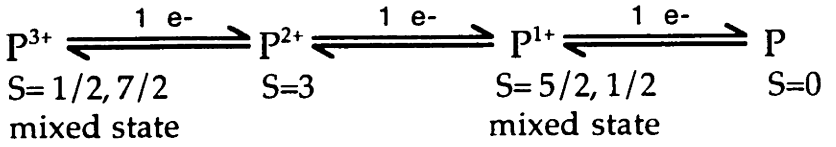
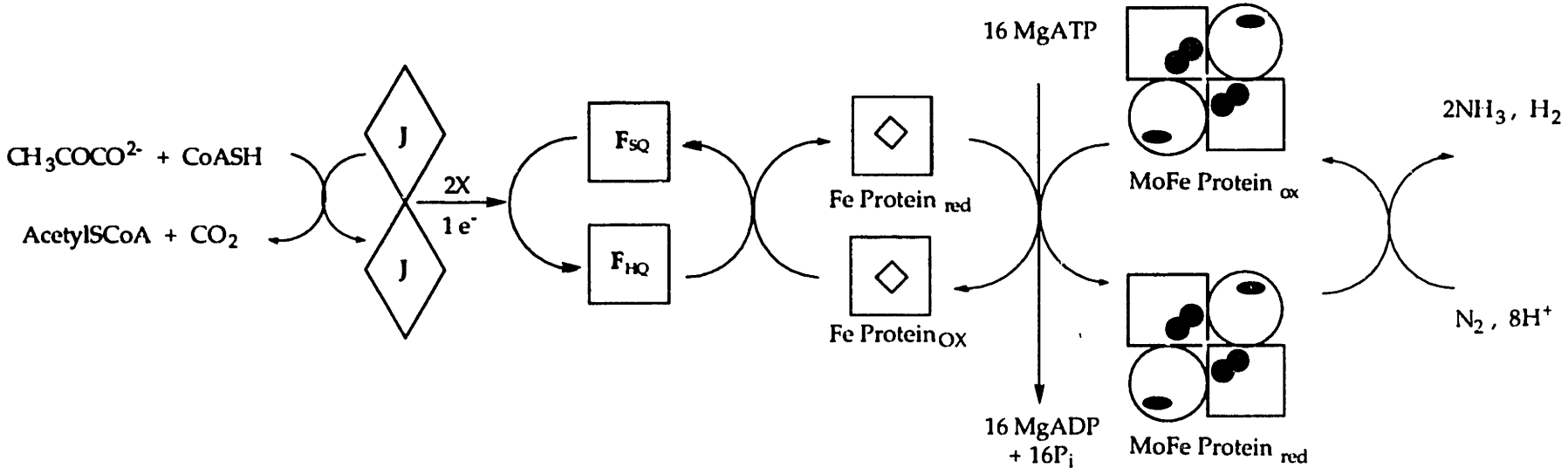


Figure 1.9: Proposed oxidation nomenclature for the P clusters of the MoFe protein.

Tittsworth and Hales (1993) set about to examine an intermediate in the thionine oxidation of MoFe protein. Using a controlled thionine oxidation, they were able to identify the state of the P cluster which was oxidized by 1 electron, as opposed to the two observed for P^{OX}. This revises the nomenclature for the oxidation states of the P cluster (figure 1.9). At the P^{1+} spin state, in addition to the S=3/2 signal (g=4.3,3.9, 2.0), there are features at g=2.06, 1.95 and 1.82 which are typical of ferredoxins and can be attributed to a S=1/2 spin system. There are also high field features (g=7.3, 6.67, 6.05 and 5.3) which can be attributed to a S=5/2 spin system. Pierick and coworkers (1993) oxidized the P clusters using redox titrations. By adjusting the potential of the MoFe protein to +236 mV vs NHE (normal hydrogen electrode), they were able to produce g= 10.4, 5.8, and 5.5 which they attributed to a S=7/2 spin state. The P clusters appear to have a varied electronic accessible redox states.

Figure 1.9: Electron Flow in *K. pneumoniae* nitrogenase

Electron Flow in *K. pneumoniae*



$$N_2 + 8H^+ + 8e^- + 16MgATP \longrightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

IV. The Mechanism of Nitrogenase

Biological nitrogen fixation is catalyzed by nitrogenase. The electron transport to the nitrogenase system (see figure 1.9) in *K. pneumoniae* starts with the oxidation of pyruvate and CoA to CO_2 and acetyl CoA which transfers electrons to **NIFJ** (pyruvate flavodoxin oxidoreductase). **NIFJ** then is able to reduce **NIFF** through the transfer of two electrons one at a time. The Fe protein is reduced by flavodoxin (**NIFF**) *in vivo* and by dithionite *in vitro*. The reduced Fe protein binds two MgATP and then upon binding to the MoFe protein, transfers one electron to the MoFe protein. The reduction of dinitrogen requires the transfer of eight electrons from a reduced Fe protein to the MoFe protein. The overall reaction catalyzed *in vivo* by nitrogenase can be represented by the following:

$$N_2 + 8e^- + 8H^+ + 16\ MgATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16\ MgADP + 16Pi$$

The Fe protein transfers one electron at a time to the MoFe protein. Since it takes 8 electrons for dinitrogen to be reduced and hydrogen evolved, eight Fe proteins must bind to a single MoFe protein during catalysis. The turnover rate of the enzyme, if one considers the physiological reactions, is formally 5 per second (Smith et al., 1973).

Besides being able to reduce dinitrogen, nitrogenase is able to catalyze the reduction of a variety of other substrates. There is a partial list in figure 1.10 (complied by Lowe et al., 1985). This list shows the various reduction reactions that nitrogenase is capable of. It is not meant to show the complete physiologically balanced reaction.

In the lab, the ability of nitrogenase to reduce acetylene to ethylene is exploited as a quick and easy way to test for the activity of the nitrogenase components (Postgate, 1972). The enzyme will reduce a number of triple-bonded systems. More unusual substrates for nitrogenase include NO_2^- (Vaughn and Burgess, 1989), N_2O (Mozen and Burris, 1954). Rivera-Ortiz and Burris (1975) describe four types of inhibition of nitrogenase: competitive, noncompetitive, unclassified and negative.

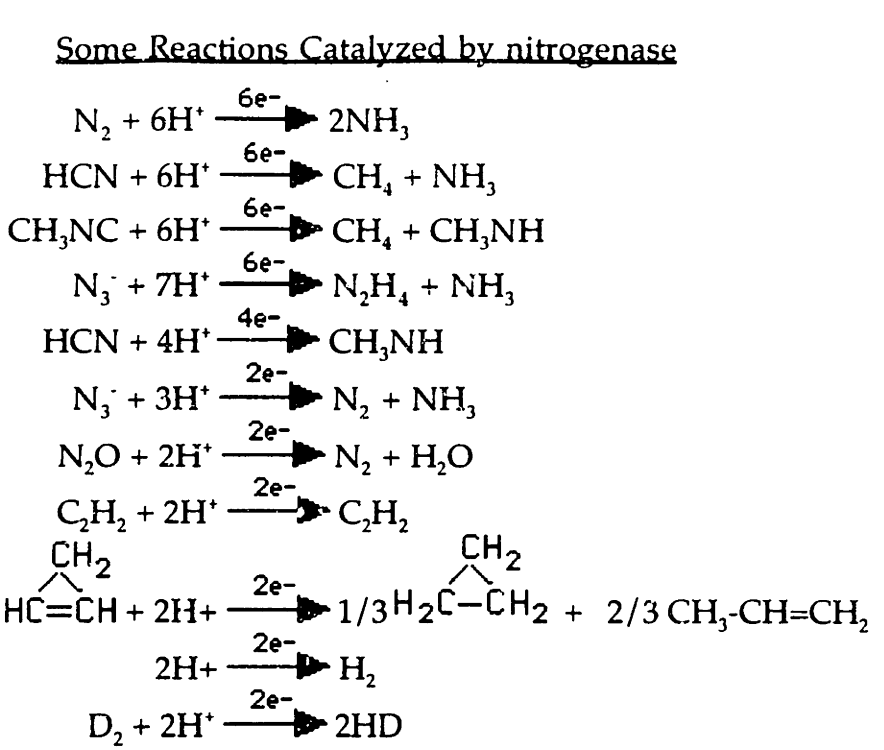


Figure 1.10: Substrates of nitrogenase.

Noncompetitive inhibitors may be binding to different oxidation states of the MoFe protein instead of at different sites on the enzyme (Yates, 1992). Inhibition of nitrogenase is accomplished by disrupting the electron flow (seen with CH_3NC, CN^-, metronidazole (2-methyl-5-nitroimidazole-1-ethanol) (Fham and Burgess, 1993; Peterson, 1988) or by binding at the state of the MoFe protein more oxidized than the one which binds N_2 (seen with CO, N_2O, NO) (Jensen and Burris, 1986; Liang and Burris, 1989).

Another use of ENDOR is to probe the active site in the presence of various inhibitors and substrates. Howes and coworkers (1994) examined the MoFe protein ^1H ENDOR in the presence of a wide variety of substrates and inhibitors (including N_2, C_2H_2, CO, NaCN, MgADP, MgATP). There were no perturbations in the ENDOR spectrum. There were two possible conclusions from these results. If the compounds did bind at this oxidation level of the MoFe protein, it was not close to the FeMoco environment. The second possible conclusion was that if the kinetic model of Thorneley and Lowe is

correct, substrate binding to FeMoco occurs at a more reduced state of the protein. They also looked at exchangeable protons near the FeMoco environment. When they soaked the MoFe protein in ^2H_2O, protons were exchanged for deuterium, something that Venters et al (1986) had previous observed. When they turned over the MoFe protein in ^2H_2O and no new exchangeable protons were present. This negative result strongly suggested that the hydrides that give rise to H_2 at the turnover oxidation level are present as ligands to FeMoco (Howes et al., 1994).

The current understanding of the kinetics of nitrogenase is the result of the excellent research done by on *K. pneumoniae* by the Unit of Nitrogen Fixation in England. Thorneley and Lowe developed a model of the mechanism for nitrogenase based on kinetic data and published their data as a series of papers in 1984 (Thorneley and Lowe, 1984a, 1984b; Lowe and Thorneley, 1984a, 1984b).

Fe protein's Kinetic and Mechanistic Role

The Fe protein's role in the reduction of dinitrogen is to provide electrons to the MoFe protein. The MgATP-induced electron transfer from the Fe protein to the MoFe protein has to occur eight times to reduce one molecule of dinitrogen. The Fe protein is able to bind independently to two separate sites on the MoFe protein. The Fe protein cycle is depicted in figure 1.11 (adapted from Thorneley et al, 1991). The MoFe in the figure 1.11 represents one of the independently functioning $\alpha\beta$ halves of the MoFe protein. The initial step in the cycle is the reduction of Fe protein by either flavodoxin *in vivo* or *in vitro* by dithionite, as pictured in figure 1.11. Then MgADP is replaced by MgATP (Ashby and Thorneley, 1987). Ashby and Thorneley (1987) also showed that when MgADP was mixed with dye-oxidized Fe protein and dithionite, that 2 MgATP bound cooperatively to the oxidized Fe protein and induced a conformational change such that the iron sulphur cluster was able to be reduced by $SO_3^{\cdot-}$ (the active species of dithionite.

The reduced Fe protein-MgATP complex then forms a complex with the oxidized MoFe protein. The MgATP is hydrolyzed to MgADP and P_i and the electrons are transferred from the Fe protein to the MoFe protein. The order in which these steps occur is still being actively researched. The dissociation of the oxidized Fe protein from the MoFe protein then occurs. This is the rate-limiting step in the substrate reduction. (Thorneley and Lowe, 1978). It is interesting that dissociation of the protein complex, $Fe^{OX}(MgADP + P_i)_2MoFe^{RED}$ is necessary for the reduction of the Fe protein, but not for the replacement of MgADP by MgATP (Thorneley, 1990).

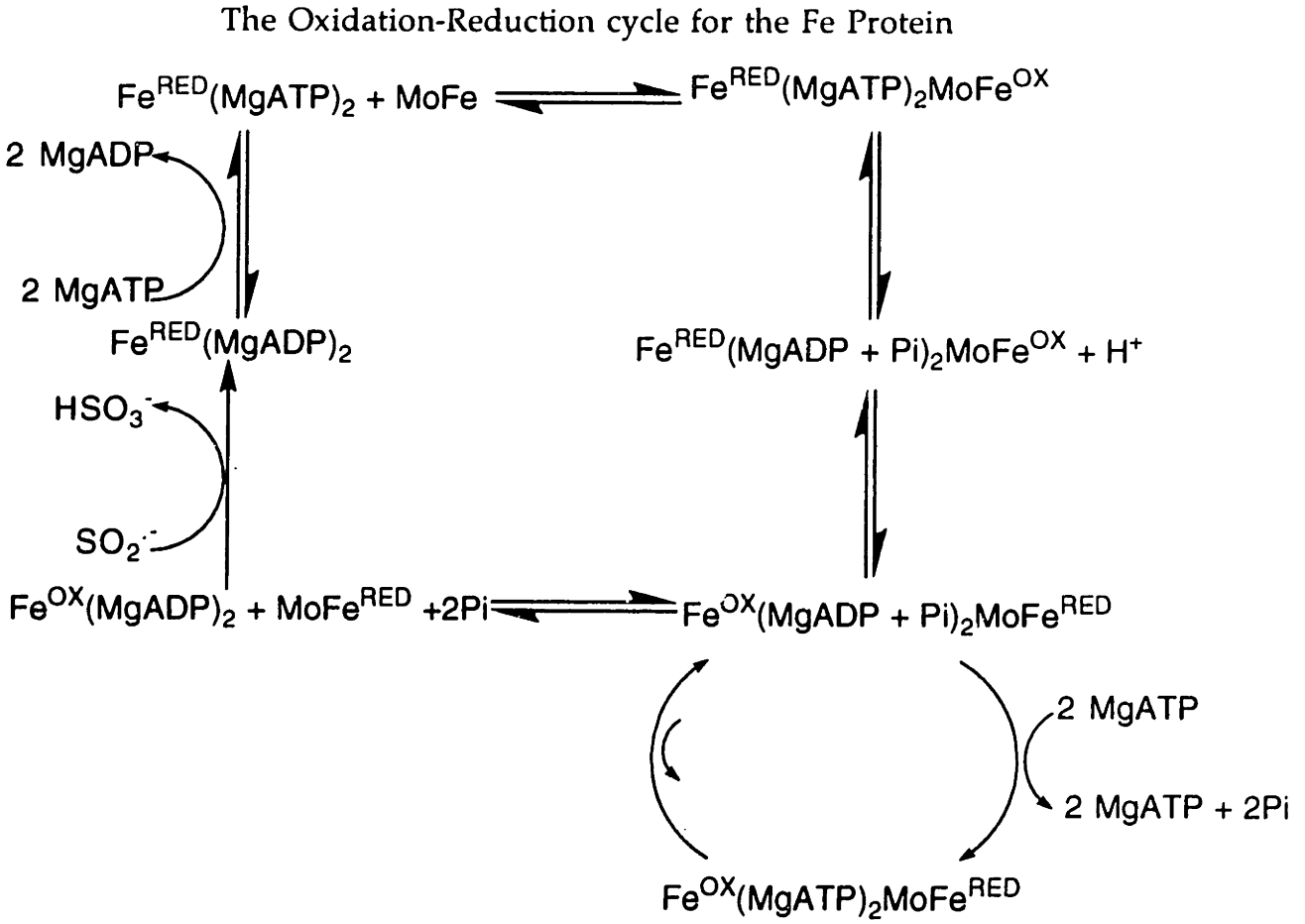


Figure 1.11: The oxidation-reduction cycle for the Fe protein.

MgATP and Electron Transfer

MgATP is required for substrate reduction. The MoFe and Fe proteins by themselves are unable to hydrolyze ATP (Imam and Eady, 1980). As early as 1966, Bulen and LeComte were able to show that the utilization of ATP is dependent upon the transfer of electrons to the substrate and requires both proteins present. EPR experiments on Fe protein and MoFe protein examined the question of electron transfer between the components. Orme-Johnson and coworkers (1972) were able to show that the reduced Fe protein would not reduce the MoFe protein but the reduced Fe protein-MgATP complex does reduce the MoFe protein. When substrates are present, the MoFe and Fe proteins in the presence of MgATP were oxidized when the reductant was exhausted. If dye-oxidized proteins were combined, MgATP was still hydrolyzed, suggesting that protein-protein electron transfer is not necessary (Cordewener et al., 1987)..

Eady (1973) was able to show using ultracentrifugation studies that a MgATP-hydrolyzing complex between MoFe and Fe protein was formed. There have been reports that MgATP was able to weakly bind to dithionite-reduced and thionine-oxidized MoFe protein (Miller and Eady, 1988; Miller and Eady, 1989; Miller et al., 1980; Kimber et al., 1982). Miller and coworkers (1993) experimentally showed that 3.9 mol of MgADP bound to MoFe protein that had been treated with the dye phenosafranine. Phenosafranine was able to oxidized the P cluster but did not oxidize FeMoco. This led Miller and coworkers (1993) to propose that the ADP binding sites were transiently filled during enzyme turnover by hydrolysis of ATP which was originally bound to the Fe protein. They further suggest the hydrolysis occurs on a bridging site on the MoFe-Fe protein complex. The MgADP was released once the P cluster was re-reduced by the reduced Fe protein-MgATP complex. This was suggested as a mechanism to prevent the reversal of electron transfer from the substrate binding site to the P cluster (Miller et al., 1993).

Eady and coworkers (1978) showed that electron transfer is coupled to the hydrolysis of MgATP. Using stopped-flow spectrometry, they followed

the formation of oxidized Fe protein and compared it with the rapid freeze quench data on the formation of inorganic phosphate, P_i. The two rates were found to be the same. But when stopped-flow calorimetry was used, Thorneley and coworkers (1989) were able to show that the Fe^{RED} (MgADP + $Pi)_2MoFe^{OX}$ is generated before electron transfer when the reaction is done at 6°C. Thus, at 6°C, hydrolysis of MgATP occurs before electron transfer. They suggest that the hydrolysis of MgATP plays a role in promoting electron transfer between the MoFe and Fe proteins and that it may induce the dissociation of the oxidized Fe protein from the reduced MoFe protein (Thorneley et al., 1989). In fact, it was shown that MgADP binds to oxidized Fe protein more tightly than to the Fe-MoFe protein complex (Thorneley et al., 1991).

Haaker's group in the Netherlands believe that electron transfer occurs before MgATP hydrolysis (Mensink and Haaker, 1992; Mensink et al., 1992; Duyvis et al., 1994). They based their conclusions on an examination of the MgATP-dependent pre-steady-state proton production by nitrogenase. They monitored the absorbance changes at 572 nm of the pH indicator *o*-cresolsulphon-phtalein in a weakly buffered solution. At 20°C, the MgATP-dependent H^+ production and electron transfer in the pre-steady-state phase was characterized by the observed rate constants of 9.4 s^{-1} and 104 s^{-1} respectively, indicating that the electron transfer is occurring at a faster rate than MgATP hydrolysis. Mensink and coworkers (1994) suggest a model where binding of MgATP allows electron transfer from Fe protein to MoFe protein, and ATP hydrolysis is obligatory for dissociation of the complex after electron transfer. They had suggested in an earlier paper (Mensink et al., 1992), that Thorneley and coworkers (1989) misinterpreted their data since Mensink and coworkers were unable to computer model the observations of the English group. Haaker's group concluded that to reconcile the differences between the two research groups, an investigation combining stopped-flow calorimetry and cresol red/stopped-flow spectroscopy would show which

comes first, hydrolysis of MgATP or electron transfer (Mensink et al., 1994). Experiments currently are underway!

MoFe protein Kinetic and Mechanistic Role

The MoFe protein cycle is more involved than the Fe protein cycle. Over the years, Thorneley, Lowe and coworkers have been able to estimate an internally consistent set of rate constants for the interconversion of different oxidation states of the MoFe protein using rapid-quench experiments, stopped-flow spectrometry and EPR time course experiments. The Fe protein cycle operates at a rate independent of the state of the MoFe protein. For instance when Fisher and coworkers (1991) examined the rate of electron transfer from Fe protein to an E_1 state of MoFe protein, there was no difference in rate from the transfer of electrons when the MoFe protein was in the E_0 state. To examine the early cycles in the reduction of the MoFe protein, the strategy has been to work at low electron flux through the MoFe protein by having a ratio of 1:100 of Fe protein to MoFe protein. Under these conditions, there is not sufficient electron flux to generate the E_3 and E_4 states so only hydrogen is evolved. It was under these conditions that Thorneley and Lowe (Lowe and Thorneley, 1984a) were able to calculate the rate constants for the early events in the proposed mechanism. To look at the later events in the mechanism, N_2 reduction must be favoured over H_2 evolution. This is seen under high turnover rates with a higher ratio of Fe protein to MoFe protein. The MoFe protein cycle is contained in the figure 1.12 (adapted from Smith and Eady, 1992). Each one of the E_N states of the MoFe protein represents the number of times the reduced Fe protein has transferred an electron. Hydrogen evolution occurs before the binding of dinitrogen and can occur at the E_2, E_3 and E_4 states. The evolution of H_2 causes the consumption of reducing equivalents and ATP that could be used to reduce N_2. The enzyme has tried to minimize the amount of hydrogen evolution. It has been suggested that this is the reason that the enzyme is so slow (Thorneley and Lowe 1982; Lowe and Thorneley, 1984a). The rate of hydrogen evolution

is proportional to the concentration of E_2. To keep the concentration of E_2 low, the rate of conversion to E_3 is maximized. This is accomplished by the reduced-Fe protein reacting at close to the diffusion controlled rate with the slow step in the reaction being the dissociation of oxidized Fe protein from the reduced MoFe protein. This type of mechanism predicts that high protein concentrations favor N_2 reduction over H_2 evolution and this could explain the very high *in vivo* concentrations of nitrogenase (Lowe et al., 1985).

Catalytic Cycle for the Reduction of N_2 by Mo nitrogenase of *K. pnuemoniae*

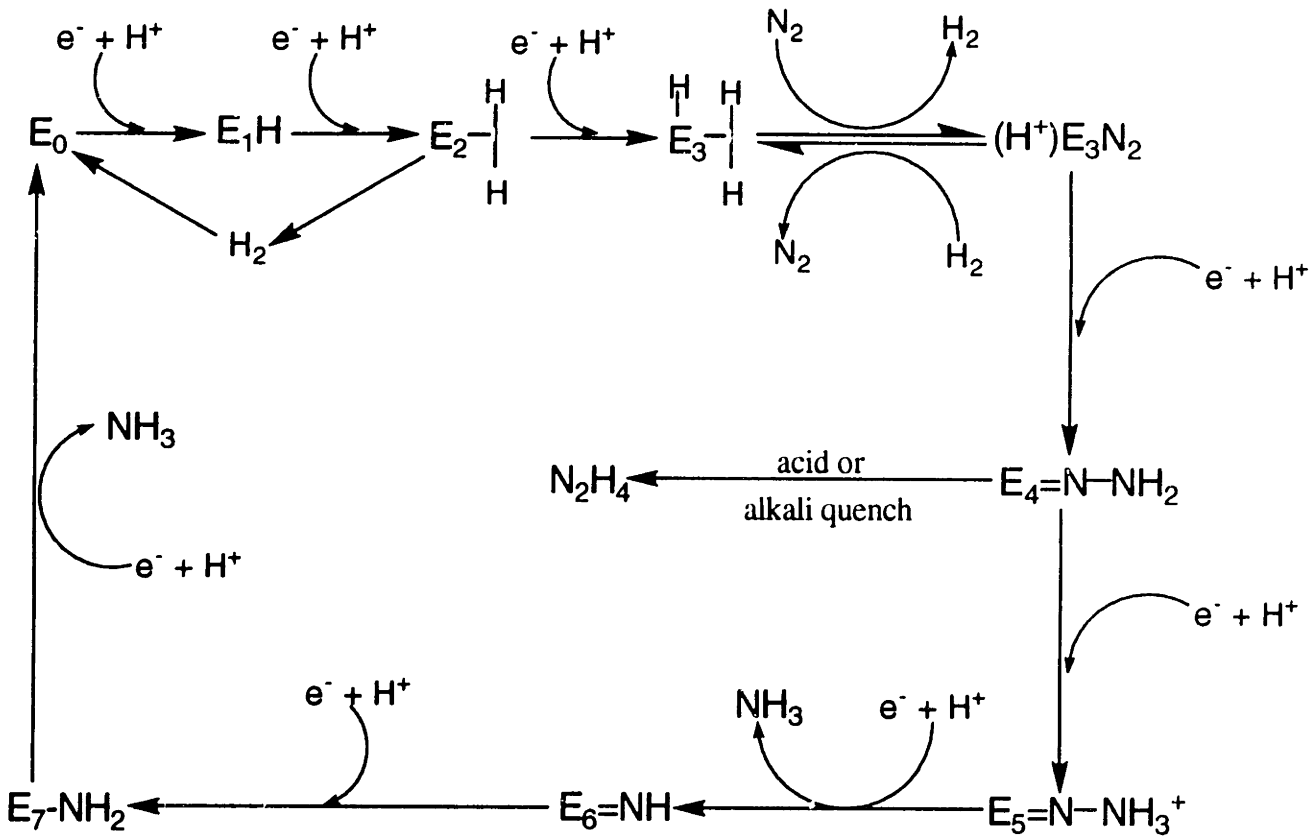


Figure 1.12: The MoFe protein cycle.

Dinitrogen, according to this analysis, does not bind until the MoFe protein has been reduced by at least 3 electrons. When it does bind, it displaces an inhibitory H_2. To examine the formation of this dihydrogen from this state of the MoFe protein, the enzyme is allowed to turnover in a H_2O

buffer system under an atmosphere of N_2 and $^2H_2$ (Burgess et al., 1981; Burgess et al. 1984). Under an atmosphere of $^3H_2$ and N_2, the amount of $^3H^+$ incorporated into the buffer system was less than 3% of the $^1H^3H$ formed. This indicates that the mechanism was not a reversible exchange process. When the enzyme was turned over under an atmosphere of $^1H^2H$ plus N_2, the amount of $^2H_2$ evolved was less than background levels. Lowe and Thorneley (1984b) tried to reconcile these experiments with their mechanism. They suggest that H_2 is evolved when one of the bound protons attacks a bound hydride and that the maximum number of exchangeable protons in the active site is two. This is supported by the ENDOR data of Venters et al (1981) and Howes et al (1994).

Table 1.5: Extinction Coefficient Changes Associated with MoFe protein during the Pre-steady-state Phase of Substrate Reduction

Reaction	$\Delta\varepsilon_{430}$ ($mM^{-1}cm^{-1}$)	Comment
E_0->E_1	<0.5	FeMoco reduced to EPR silent state
E_1->E_2	-2.2	Negative $\Delta\varepsilon_{430}$ assumed to be due to P center or FeMoco reduction
E_2->E_3	<0.5	
E_3->E_4	+6.7	P center oxidation shown by EPR under Ar and N_2, but not C_2H_2 when E_4 state is not achieved.

Lowe and coworkers (1993) combined stopped-flow spectrometry and parallel EPR studies to examine what is happening on the MoFe protein during E_0 to E_4. The results are summarized in table 1.5 (after Thorneley et al., 1993). The $E_1 \rightarrow E_2$ absorbance change is due to the electron density on a metal center instead of on the bound dihydrogen. At the E_4 state, the enzyme becomes committed to binding N_2 (Thorneley and Lowe, 1984a). When the E3→E4 reaction with various substrates was examined using parallel mode EPR, the results depended on the substrate present. Acetylene and carbon

monoxide bind to the FeMoco at either E_2 or E_4. Lowe and coworkers (1990) suggest that they are bound at the same site on the enzyme. The sharp signals observed at g =5.4 and g =5.7 are observed when the enzyme is able to produce the E_4 state (under Ar and N_2), but these signals are absent when CO and C_2H_2 are present. These signals have been attributed to the S=7/2 spin state signals observed when the P clusters are oxidized (Hagen et al., 1987). Lowe and coworkers (1993) conclude that when the E_4 state is reached, a transfer of electron density from the P cluster onto FeMoco occurs, generating increased reducing power. Thorneley and coworkers (1993) further state that it is this increase of electron density on FeMoco which is able to protonate one of the N atoms of dinitrogen and that this commits the MoFe protein to reduce dinitrogen.

The intermediates in the reduction of dinitrogen have not been isolated during turnover except when the enzyme is quenched with acid or alkali (i.e. $E_4N_2H_2 \rightarrow N_2H_4$ in the mechanism) (Thorneley et al., 1978). Hydrazine is formed upon the acid/base catalyzed reaction of dinitrogen hydride, an intermediate in N_2 reduction, with the protons or solvent. It was shown through time course experiments that the dinitrogen hydride forms after four slow steps (Lowe et al., 1985). This type of intermediate is supported by model chemistry (i.e. Chatt et al., 1978).

When the MoFe protein is quenched after five or six electrons have been transferred from the Fe protein, two moles of ammonia are released (Thorneley and Lowe, 1982). Since two of these added electrons have been used to produce H_2, this indicates that the MoFe protein produces a more oxidized state than as isolated (i.e. E_7, and E_6 states), something that the electrochemical experiments have previously shown (for instance see Watt et al., 1978).

The mechanism of nitrogenase is a complicated enzymatic process. Models have been proposed for the reduction of both the Fe and MoFe proteins. The reduction of dinitrogen and hydrogen evolution is modeled as

an eight electron process with the electrons being passed from the Fe protein to the MoFe protein. The role of the P cluster in catalysis is still not completely understood. From the examination of the crystal structure and available kinetic data, it can be hypothesized that the electrons are transferred from the Fe protein to FeMoco via the P cluster.

V. The Scope of This Thesis

The production of active protein from bacteria requires an understanding of the fermentation process used to grow the bacteria. Chapter two discusses strategies and experiments to optimize the growth of *A. vinelandii* and *K. pneumoniae*. The growth of mutants unable to synthesize holoMoFe protein is examined though experiments on restoration of the wild type growth phenotype of a *K. pneumoniae nifV-* mutant. The production of apoMoFe protein from *A. vinelandii* and *K. pneumoniae nifB-* mutants is studied to determine the optimal growth conditions for the production of apoMoFe protein.

Chapter three builds upon the work by others in this laboratory and reflects the collaborative efforts of Dr. A.B. Hickman, Dr. D.W. Wright and T.A. Humiston. This chapter demonstrates the understanding of how the column method of FeMoco extraction works, from the binding of the MoFe protein to the DE52 column to the extraction of FeMoco using different organic and inorganic salts.

The fourth chapter details the production of isotopically labeled MoFe proteins. Deuterated homocitric acid is incorporated into a *K. pneumoniae nifV-* mutant and studied using ESEEM spectroscopy. Building upon the work started in this laboratory by Dr. P.A. McLean, a hybrid in which the P cluster and FeMoco have different iron isotopes is produced through the use of extracted FeMoco from *A. vinelandii* and apoMoFe protein from an *A. vinelandii nifB-* mutant. This isotopically labeled hybrid is purified and its inhibition under CO is studied using EPR and ENDOR spectroscopies.

The appendices have laboratory guides outlining the skills and techniques required for successful research in nitrogenase. The Guide to Fermentations (Appendix A) includes from glycerol stock to frozen paste, hints and protocols for the growth of *A. vinelandii* and *K. pneumoniae*. Purification of the MoFe and Fe proteins from cell paste is the topic of Appendix B, Guide to Protein Purification. Appendix C, Guide to FeMoco extractions, describes in detail the batch-column method of extraction and subsequent purification of FeMoco that had been developed in the laboratory by Dr. D.W. Wright, T.A. Humiston and P.D. Christie.

Chapter Two: Fermentations of Wild-Type and apoMoFe proteins

I. Introduction

The goal of any successful enzyme fermentation is to produce the largest amount of the protein under study in an active or at least an activatable state. The regulation and biosynthesis of the nitrogenase enzyme is a very energy intensive biochemical process. There are major difficulties in the bioproduction of nitrogenase. The enzyme is a large molecule, catalytically very inefficient (turnover is about 1 N_2/sec), requires huge amounts of ATP to carry out an otherwise thermodynamically favorable reaction, and is inactivated by O_2. The cells synthesize nitrogenase to a level of several percent of the total protein in order to assimilate nitrogen into the cell. When cellular levels of nitrogen are sufficiently high, the enzyme synthesis is stopped. Furthermore, *Azotobacter vinelandii* is an aerobic organism producing this oxygen sensitive enzyme. It has developed very high respiratory rates which evidently serve to protect nitrogenase from oxygen damage (Yates and Jones, 1974). *Klebsiella pneumoniae* is a microaerophilic organism that can be grown anaerobically, which helps prevent enzyme degradation. This advantage is offset by the poor production of ATP, compared to organic substrate oxidized, typical of anaerobic life.

If there are sufficient quantities of fixed nitrogen available to the nitrogen fixing bacteria, it will not synthesize the nitrogenase system. The nitrogenase system is therefore repressed. The *nif* gene region is regulated by *nifA* and *nifL*. **NIFA** is a positive regulator and **NIFL** is a negative regulator. In *K. pneumoniae*, **NIFL** will bind to **NIFA** and inactivate it in the presence of greater than ~5 μM ammonia. At concentrations greater than ~200 μM ammonia, the expression of the *nifLA* operon does not occur (Merrick et al., 1982). Through their work on mutations in the *nifL* region as well as *nif-lac* fusions, Merrick and coworkers (1982) demonstrated that the *nifL* product acts as a *nif*-specific repressor in response to ammonia, amino acids, oxygen and possibly also to temperature. Eydmann and coworkers (1995) have shown *in*

vitro for *Azotobacter vinelandii* that **NIFL** is able to modulate the activity of **NIFA** through an adenosine nucleotide switch. When ADP is bound to **NIFA**, the resulting conformation facilitates interaction with **NIFL**.

When the fixed nitrogen levels fall below the levels required for cell growth, the biosynthesis of the nitrogenase system is initiated. The nitrogenase system is now being derepressed. The **NIFA** activates the *nif* promoters by binding to a specific DNA sequence which is usually located around 100 base pairs (bp) upstream of the transcriptional start site (deBruijn et al., 1990). **NIFA** expression in addition to being autoregulated is under control of the general cellular nitrogen regulation (*ntr*) system which consists of at least 6 different gene products. The **NTRA** (sigma-54) product, **NTRB** and **NTRC** are all required for the activation of the *nif* genes (Magasanik, 1988).

Growth of *Azotobacter* requires that the oxygen dissolved in the fermentation media be maintained within a certain range. Oxygen suppresses the synthesis of nitrogenase proteins (Postgate, 1982). At higher oxygen concentrations, the respiratory rate of the bacteria must increase to keep the levels of oxygen inside the cell low. The resulting reduction of the overall energy efficiency of the cell has been implicated in the protection against nitrogenase to oxygen damage (Dingler et al., 1988). When Post and coworkers (1983) subjected nitrogen-fixing cultures of *A. vinelandii* to high levels of oxygen, nitrogenase activity was eliminated. When the oxygen levels returned to normal levels, nitrogenase activity was restored. This "switch-off" was the result of the nitrogenase complex being associated with oxidized flavodoxin hydroquinone protein (**NIFJ**) which inactivated the enzyme by disrupting the nitrogenase specific electron transfer chain. The oxidized protein, when oxygen levels returned to normal, dissociated from the complex, and nitrogenase activity is restored (Robson and Postgate, 1980). Dingler and coworkers (1988) determined that at lower oxygen concentrations, the catalytic activity of nitrogenase was at its highest level. This is a very favorable growth condition for production of *A. vinelandii* biomass.

Characterization of fermentation protocols is important for the first step in enzyme production. The large scale production of *A. vinelandii* has been an area of interest between the Orme-Johnson group and Prof. Charles Cooney's research group in Chemical Engineering at MIT. It was found that there is an optimal cellular density (OD) which produced the most active nitrogenase. At higher OD's, enzyme degradation is quick and irreversible, so the decision of when to harvest is an important one. Through many 1000 L fermentations, the protocol was developed to produce the most active nitrogenase per biomass. Transferring the information from 1000 L fermentations to the 20 L scale may seem a step backwards, but 1000 L fermentations are often not feasible for the growth of mutants of *Azotobacter* and *Klebsiella*. With a 1000 L fermentation, the time required for harvest is too long and protein inactivation results. It can become a very time consuming process for little yield of active protein. On a 20 L scale, there is more facile control over the fermentation and its bioproducts.

The growth of *Klebsiella* requires that most of the oxygen be removed from the fermentation media. The oxygen levels are kept low by constant purging of the fermenter with dinitrogen gas. The organism will not grow if exposed to large amounts of oxygen.

Mutations in the *nif* genes usually produces organisms which are unable to reduce dinitrogen. These organisms require an additive of fixed nitrogen for growth. The induction of the mutant nitrogenase system will occur once the nitrogen source has either been removed or consumed. Monitoring the production of the mutant's nitrogenase system is not always easy. Mutations in the *nifV-* gene of *K. pneumoniae* produce a mutant which is able to reduce acetylene, but not dinitrogen. The levels of nitrogenase in this mutant can be monitored by the *in vivo* acetylene reduction assay. If the mutation creates a non-functioning MoFe protein, for instance a *nifB-* mutation, then it is not possible to perform an *in vivo* assay. It is, however, possible to assay the Fe protein produced, but this requires that the cells be spun down, frozen in liquid nitrogen, resuspended in reducing buffer,

disrupted anaerobically and then assayed *in vitro* using an ATP regeneration system and acetylene as the substrate. Many researchers do not follow the amount of nitrogenase produced. Once the fixed nitrogen source is consumed, the culture is allowed to derepress for 3 hours, either with a washing of the cells and the addition of fresh fixed-nitrogen depleted media (Robinson et al., 1986) or without (Shah et al., 1973).

Characterization of *nifV*- mutants and purification of **NIFEN** led to the discovery that homocitric acid is a component of FeMoco. The so-called V factor was purified from fermentation media of wild type *K. pneumoniae* (Hoover et al., 1987). Through polarimetry experiments, the configuration of the homocitrate was determined to be the R isomer. When derepressing cultures of *nifV*$^+$ strains of *K. pneumoniae* were examined for homocitrate content, it was determined that 80-90 % of the total homocitrate produced accumulated in the media at levels near 0.4 mM (Hoover et al., 1988). Additional experiments revealed that *nifV*- strains of *K. pneumoniae* could be cured (i.e. returned to a wild-type phenotype) if homocitrate was added to derepressing cultures.

In response to an iron deficiency, bacteria and fungi synthesize low molecular weight, virtually ferric-specific agents which are generically designated siderophores. A siderophore will spontaneously and rapidly associate with the ferric ion. Siderophores are multidentate, typically oxygen-containing ligands with affinity constants for ferric ion ranging from 10^{20} to 10^{30} and frequently have a molecular mass in excess of 600 da (Neilands, 1994). Once the siderophore is chelated to the iron, it is then transported across the cell membrane and the iron is transferred to a storage protein. One of the storage proteins is a type of ferritin. Ferritins are usually a multisubunit protein shell of outside diameter about 12.5 nm surrounding an inner non-haem iron-core in which a large amount of iron (up to 4500 Fe atoms/molecule is present in mammalian ferritins) can be stored in mineral form (Harrison et al., 1987).

Azotobacter vinelandii is an organism capable of surviving in areas where the availability of metal ions fluctuates. It has the ability to produce a variety of siderophores which will be excreted into the media and scavenge any required metal ions. Under iron-limiting conditions, *A. vinelandii* secretes at least three different siderophores (Corbin and Bulen, 1969). One of the excreted siderophore has been identified as a peptide with an attached yellow-green fluorescent chromophore. This peptide chain contains the uncommon amino acids homoserine and β-hydroxyaspartic acid (Bulen and LeComte, 1962). Once the siderophore has chelated the iron, there are outer membrane receptor proteins which recognize these complexes and transport then into the cell (Page and von Tigerstrom, 1982). The iron is stored intracellularly in a ferritin or bacterioferritin cytochrome $b_{557.5}$ (Watt et al., 1986; Stiefel and Watt, 1979). The storage proteins are composed of 24 subunits of 17 000 - 20,000 da, each with similar composition. both proteins are capable of accommodating up to 4000 Fe atoms in their interior cavities (Watt et al., 1986).

The experiments described in this chapter are designed to show strategies of fermentation methodologies. The goal of this chapter is to gain insight into the growth of various bacteria which will enable the isotopically labeled proteins of nitrogenase to be produced. *A. vinelandii* OP under nitrogen fixing conditions has a doubling time of 2-4 hours and the nitrogenase proteins constitute 7-10% of the cellular proteins produced. It is an organism optimally suited for nitrogenase production. The yields from *K. pneumoniae* are lower and development of a double phase fermentation (repression of the nitrogenase system followed by derepression of the nitrogenase system) was the strategy followed. This double phase fermentation process was also established as the best format for the production of apoMoFe protein, but first the amount of time required for derepression must be examined. Incorporation of iron isotopes was an area of interest. Development of the most efficient method of producing bacteria

with either ^{57}Fe or ^{56}Fe incorporated into the MoFe protein was also investigated. Finally, the combined techniques and experiences are included in Appendix A entitled Guide to Fermentations.

II. Materials and Methods:

All chemicals were either Mallinckrodt analytical reagent or Fluka Biochemical grade. Stock solutions of the non-volatile media components were made, autoclaved for sterilization and then utilized as required. Solutions of volatile compounds were steri-filtered through a 0.22μM filter prior to use. All bacterial work was performed in a flow hood equipped with a microbial filter. Standard sterile techniques were followed throughout the fermentations.

A. Equipment description

The 20 L Chemap Fermenter was equipped with an external automatic pH controller (New Brunswick) and an external dissolved oxygen controller (New Brunswick) with a galvanic probe (New Brunswick). The dissolved oxygen controller was not interfaced with the fermenter, so the oxygen content had to be manually adjusted through gas feed and agitation manipulations. The fermenter had been modified to enable a rapid chilling through a nitrogen gas feed which was chilled with liquid nitrogen before entering the fermenter. The water coolant was also chilled with a salt/ice bath. The 50 L Chemap Fermenter was monitored and controlled by both UNIX-based (Satt control, Alfa-Laval) and PC-based (Paragon control, Intec controls) control systems. Agitation was varied automatically to control dissolved oxygen at a partial pressure of 0.04-0.06 atm using an Ingold polarographic dissolved oxygen probe.

The fermentation products were harvested with either a Pellicon tangential flow membrane concentrator (Millipore) or a Sharples centrifuge. The Pellicon tangential flow membrane concentrator was equipped with a

Procon Pump and a 15 square foot Pellicon filter. The operational flow rate for concentrating the fermentation media was at least 18 liter per minute. Twenty liters of fermentation broth at OD_{660} = 3 would take approximately 5-8 minutes to concentrate down to 1-2 L. The Sharples Laboratory Presurtite Centrifuge operated with 25-30 psi of compressed air to the turbine drive. While the fermentation broth was pumped out of the fermenter with a high speed Amicon peristaltic pump, the centrifuge was purged with argon. The time required to achieve a spun down cell pellet was approximately 30 minutes. The cells were removed from the bowl in a glove bag under a blanket of nitrogen and immediately immersed into liquid nitrogen.

For the NUR experiments, a magnetic-sector mass spectrometer (model MGA 1600, Perkin-Elmer) was used. The instrument ionizes incoming gases using electron bombardment in a vacuum envelope of 10^{-6} torr. The ionized gases are directed into four Faraday cup collectors allowing quantification of ions over a mass-to-charge ratio (m/z) range from 2 to 120. In these experiments, the inlet and outlet gases were monitored for oxygen (m/z = 32), nitrogen (m/z = 28), argon (m/z = 40), carbon dioxide (m/z = 44) and water (m/z = 18). Carbon dioxide also ionizes to a CO fragment with a m/z equal to nitrogen that must be accounted for in the calibrate matrix of the instrument. The instrument was calibrated by feeding several mixtures of CO_2 and specialty gas (40% O_2, 45% N_2, 15% Ar).

B. Standard Growth Conditions:

Wild type *Azotobacter vinelandii* OP and wild type *Klebsiella pneumonia* (UN) were grown in Burk's nitrogen free media unless otherwise stated. Non-nitrogen fixing mutants, UN106 (a *K. pneumoniae nifB*- mutant), UN1990 (a *K. pnuemoniae nifV*- mutant) and UW45 (a *A. vinelandii nif B*- mutant) were grown initially with a fixed nitrogen source, ammonium acetate (1.21 g/L) for *K. pneumoniae* and urea (0.609 g/L) for *A. vinelandii*. For derepression media, L-arginine hydrochloride (0.5 g/L) was

added to cultures of UW45 and for *K. pneumoniae* cultures, L-serine (1.0 g/L) was added. Complete description of Burk's modified media is in Table 2.1. The media for the fermenter was the same except for the addition of antifoam (polyethylene glycol, PolySciences) at a concentration of 1 mL/L of media.

Table 2.1: Composition of Burk's Modified Media

Burk's Modified Media	Concentration per liter
Sucrose	20 g
K_2HPO_4	1.602 g
KH_2PO_4	0.408 g
NaCl	0.197 g
Fecitrate	3.0 mg
$MgSO_4.7H2O$	200 mg
$CaCl_2.2H2O$	88 mg
$NaMo_4.2H2O$	25 mg
H_3BO_3	0.88 mg
$ZnSO_4$	0.60 mg
$CoCl_2.6H2O$	0.60 mg
$MnSO_4.H2O$	0.60 mg
$CuSO_4.5H2O$	0.01 mg

Cultures were started from frozen glycerol stocks and plated on solid media. Typically multiple subcultures were taken to ensure that the organism had recovered from cell death due to freezing. Both solid and liquid cultures were grown at 30°C for *A. vinelandii* and 37°C for *K. pneumoniae* Single isolate colonies from the solid media were transferred to 5-10 mL liquid cultures then after 12-24 hours were transferred to 2 L baffled flasks. A summary of growth times are in the table 2.2:

Table 2.2: Typical Fermentation Growth times

Organism	1st subculture	time	2nd subculture	time
Av OP	5 mL	24 hrs	500 mL	24 hrs
UW45	5 mL	24 hrs	500 mL	24 hrs
Kp UN	10 mL	12-24 hrs	1 L	12 hrs
UN106	10 mL	12 hrs	1 L	12 hrs
UN1990	10 mL	12 hrs	1 L	12 hrs

The liquid cultures were shaken on an orbital shaker (New Brunswick or Queue) at 225-300 revolutions per minute. The pH of the cultures was monitored every 2-6 hours and adjusted to pH 7.4 as required.

The cultures were then used to inoculate a stirred bioreactor. During fermentations, OD_{660}'s were taken to monitor cell density. For growth of *A. vinelandii* OP, the fermentations were under nitrogen free conditions. All other fermentations (*K. pneumoniae* UN, UN106, UN1990 and UW45) were first grown in the presence of a fixed nitrogen source until the cell density was OD_{660}= 3.0-3.8, then the fixed nitrogen source was removed and the culture was derepressed. Where applicable, *in vivo* or *in vitro* acetylene reduction assays were used to monitor the derepression of nitrogenase. To harvest the cells from the fermentation, either a Pellicon tangential flow membrane concentrator (Millipore) with a 15 m^2 membrane or a Sharples centrifuge were utilized. The cells were then pelleted into liquid nitrogen. Detailed description of all techniques used for the fermentation experiments are included in the Appendix A , Guide to Fermentations.

C. Estimated Nitrogen Uptake Rates (NUR)

A. vinelandii wild-type cells were grown according to standard conditions. The 50 L cultures were grown for 10-15 hours at 30°C. Aeration was initially 5 L min^{-1} and increased to 10 L min^{-1} if it appeared the upper limit of agitation would be reached before the run was completed. Agitation was varied automatically to control dissolved O_2 at a partial pressure of 0.04 -

0.06 atm. The pH was controlled at 7.0 throughout and backpressure was maintained at 1.5 atm.

During the 50 L fermentation, *in vivo* acetylene reduction assays were carried out every 1-2 hours as required. Every two hours a 250 mL sample of cell broth was sampled and centrifuged at 4080 g x 5 minutes at 4°C. The supernatant was poured off and the pellet frozen in liquid nitrogen. The pellet was degassed and resuspended with 0.1 M reducing Tris, pH = 8.0 in a 1:8 weight to volume ratio. The cell paste was disrupted anaerobically in a French Pressure Cell at 4°C and 50 µL of this extract was assayed for MoFe protein both under saturating Fe protein concentrations and under non-saturating Fe protein concentrations using the standard *in vitro* acetylene reduction assay. Samples of the inlet and outlet gas streams from the fermenter were delivered continuously by metering pumps to the mass spectrometer.

D. Homocitrate Incorporation Experiments

Overnight cultures of *K. pneumoniae* UN and UN1990 were grown in LB medium at 37°C. The overnight liquid broth (LB) culture was added to 1L of Burk's + fixed nitrogen medium. The 1 L inoculum was grown for 8 hours at 37°C. For starter cultures and inoculum, the pH was monitored and adjusted to pH 7.4 as necessary. A 20 L fermenter containing Burk's + fixed nitrogen was inoculated and the OD monitored. When the cells had reached and OD of 3, they were anaerobically concentrated, using a Pelicon tangential flow membrane concentrator, and resuspended two times in a nitrogen free Burk's media with 1.0 g/L L-serine. During derepression, the *in vivo* activity and OD were monitored. At maximal activity (5-8 hours of derepression), the cells were concentrated and pelleted into liquid nitrogen for storage.

In the wild-type phenotype rescue experiments, synthesized homocitric acid lactone was converted to homocitric acid by adjusting the pH of the solution to 11-13 and allowing to stir overnight. The completion of this

reaction was monitored by HPLC. R,S-homocitric acid was added to derepressing cultures of UN 1990 to a final concentration of 166 mg/L. When maximal activity, as determined by *in vivo* activity, was reached (7-9 hours), the cells were harvested as above.

E. Optimization of UN106 and UW45 Growth and Derepression Experiments

Unless otherwise stated, all cultures were grown in Burk's + nitrogen media and derepressed in Burk's nitrogen free with the appropriate amino acid added. To estimate the amount of time required for UW45 derepression, 10 mL LB cultures were inoculated from single isolate colonies grown on LB plates. Two 250 mL cultures of Burk's + urea were inoculated with a 10 mL LB culture in each and grown up to a density of 2.2-2.7. The cultures were pelleted and resuspended in Burk's nitrogen free media with L-arginine. For the next 24 hours, 1 mL samples were taken every hour, spun down in an eppendorf tube @ 10,000 rpm for 5 minuets. The pellet was resuspended in 100 µL of Laemmli buffer and placed in a -20°C freezer.

Samples (10-20 µL) were loaded onto a SDS-PAGE gel with a MoFe/Fe protein standard sample. The gels were run at 10 mA current for 18 hours and then immunoblotted against an antibody for the structural polypeptides of the MoFe and Fe proteins. The immunoblots were developed using a horseradish peroxidase color development solution.

The optimal derepression time for both UN106 and UW45 was examined at the 20 L and 50 L scale. The standard procedure for growth in repressed cultures was followed. During derepression, 250 mL samples were collected, spun down and frozen into liquid nitrogen. The pellets were then anaerobically degassed and diluted two fold with 0.1 M Tris, pH 8.0, 10 mM $Na_2S_2O_4$ and cracked anaerobically in a French Pressure Cell. 50 µL of cell extract was assayed for the presence of Fe protein using standard conditions.

F. Iron Starvation Experiments:

The ability of *A. vinelandii* to sequester iron was examined by monitoring the absorbances of siderophore production. Cultures of *A. vinelandii* OP were sequentially grown from plates through 10 mL and 250 mL volumes. The 1 L media did not contain an iron source and the 250 mL culture was used as the innoculum. The cultures were monitored at 310 nm, 410 nm and 660 nm. At 12, 14, 16 hours, 250 mL of the iron starved cultures were placed into separate flasks and 25 µL of a 30 mg/mL iron citrate solution was added. The same absorbances were monitored over a period of four hours.

The ability of UW45 to survive during iron starvation conditions was also monitored using the same conditions as *A. vinelandii* OP. The derepression of UW45 without the presence of iron in the derepression media was also investigated.

III. Results and Discussion

A. Optimized Growth of wild-type *A. vinelandii* and *K. pneumoniae*

Bacteria which fix dinitrogen usually have a doubling time of 2-4 hours, which is long when compared to *E. coli*'s optimal doubling time of 20 minutes. Production of nitrogenase as well as its function are very energy intensive processes. For *Azotobacter,* which is an obligate aerobe, its high respiratory rate generates the ATP required for nitrogen fixation and reduces the oxygen tension in the cell to protect the oxygen sensitive nitrogenase. There is a balance between the cell density and the amount of active nitrogenase present. Through repetitive 1000 L fermentation runs, conditions were optimized for the production of active enzyme (Hamel et al., 1990). It was determined that on the 1000 L scale, the enzyme is quickly deactivated at high cellular densities. This ratio of active enzyme to cellular density can be monitored throughout the fermentation by performing acetylene reduction assays and measurement of the absorbances at 660 nm,

referred to as OD_{660} (figure 2.1). As the fermentation progresses, the bacteria are in their exponential growth phase, but this growth is affecting the overall activity of nitrogenase.

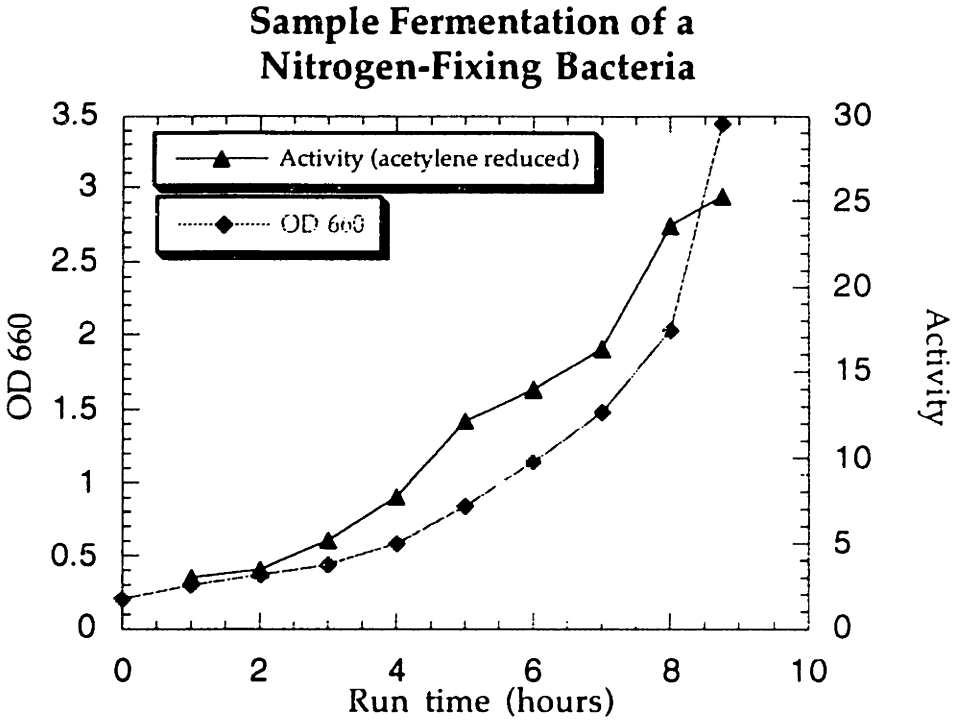


Figure 2.1: Fermentation of *A. vinelandii*.
This is a typical example of a 20 L fermentation of *A. vinelandii*. The bacteria continue to exhibit exponential growth, but the activity of the nitrogenase is beginning to level. This indicates that it is time to harvest.

Plotting the ratio of the enzyme activity, determined by the acetylene reduction assay, and the OD_{660} produces a number which can be used to determine an appropriate harvest time (figure 2.2).

When the cellular density reaches high levels, the amount of active nitrogenase decreases. The best time for harvest is when there is the most active enzyme per bacteria. For the fermentation in figure 2.1, the harvest time appears to have been after five hours of fermentation. It was decided to increase the cell density to determine if a higher activity could be obtained. At t = 8 hours, there is 2.5 times as much biomass as there was at t = 5 hours. Even though the nitrogenase present at t = 8 hours is 72 % as active than at t =

5 hours, because of the higher density, there was 2.5 times as much protein. Therefore, for an additional three hours of fermentation, the loss in activity is overcome by the increase in yield. In all the 20 L fermentations of *A. vinelandii* OP, it was very difficult to maintain activity of nitrogenase whenever the cellular density was above OD_{660}= 3.5. At these high densities, the dissolved oxygen levels were not able to be maintained high enough due to the technical limitations of the fermenter rotor and gas delivery system. The bacteria was consuming oxygen faster than it could be transferred to the fermenter, hence the bacteria was becoming oxygen stressed.

Fermentations of nitrogen fixing bacteria can be accomplished by monitoring the production of active nitrogenase. When *nif*- mutants are grown, they must be supplied with a nitrogen source, due to their inability to produce active nitrogenase. This complicates the fermentation process. Typical fermentations of these non-nitrogen fixing bacteria involve growth of the organism with a supplement of a fixed nitrogen source followed by harvesting five or six hours after the depletion of the ammonia source (for example see Shah et al., 1973). The strategy adopted in the Orme-Johnson laboratory was slightly different. The cells are grown with an initial nitrogen source to a predetermined OD. The nitrogen source is then removed, and the cells washed in nitrogen free buffer. In this nitrogen-free buffer, low levels of amino acids are added to support essential requirements for fixed nitrogen but not at high enough levels to repress nitrogenase synthesis. In this nearly nitrogen-free medium, the cells are then induced to produce their mutant nitrogenase system. Characterization of this derepression fermentation is an area investigated in the following experiments.

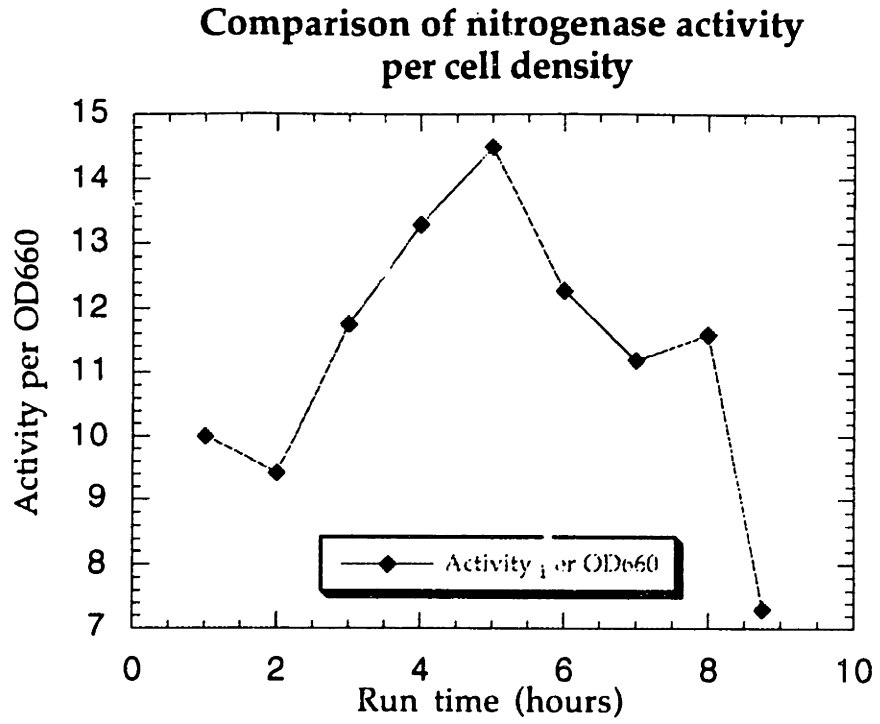


Figure 2.2: Enzyme Activity per OD660 for *A. vinelandii*.
The purpose of the fermentation is to produce as much active enzyme as possible. Even though the cellular density is increasing (see figure 2.1), the amount of active nitrogenase per cell is decreasing.

B. Estimated Nitrogen Uptake Rates (NUR)

The NUR can be modeled based on the maximum cellular density, the specific growth rate of an organism and estimating that 10% of the dry weight of cells is nitrogen. Using equations derived by Prior and coworkers (1995), the NUR can be estimated to have a maximum value of 0.9 -1.6 mmol L^{-1} h^{-1}. The NUR can be calculated from the following equations:

$$NUR = \frac{1}{V_L}\left(Q_{in} y_{N_2,in} - Q_{out} y_{N_2,out}\right) - \frac{d C_{N_2}}{dt}$$

where NUR is the N_2 uptake rate (mmol $L^{-1}h^{-1}$), V_L is the liquid volume (L), Q is the molal gas flow rate (mmol h^{-1}), C_N is the concentration of dissolved N_2 (mmol L^{-1}) and y_{N2} is the mole fraction of N_2 in the gas stream. Because use of a flow meter on the exit gas line is difficult, the flow (Q_{out}) must be calculated

based on a constant molal flow of an inert component. Using this approach to calculate Q_{out} and assuming the C_{N2} is constant, the NUR equation can be simplified to:

$$NUR = \frac{Q_{in}}{V_L}\left[y_{N_2,in} - y_{N_2,out}\left(\frac{y_{Ar,in}}{y_{Ar,out}}\right)\right]$$

An example of the measured NUR trend is in figure 2.3.

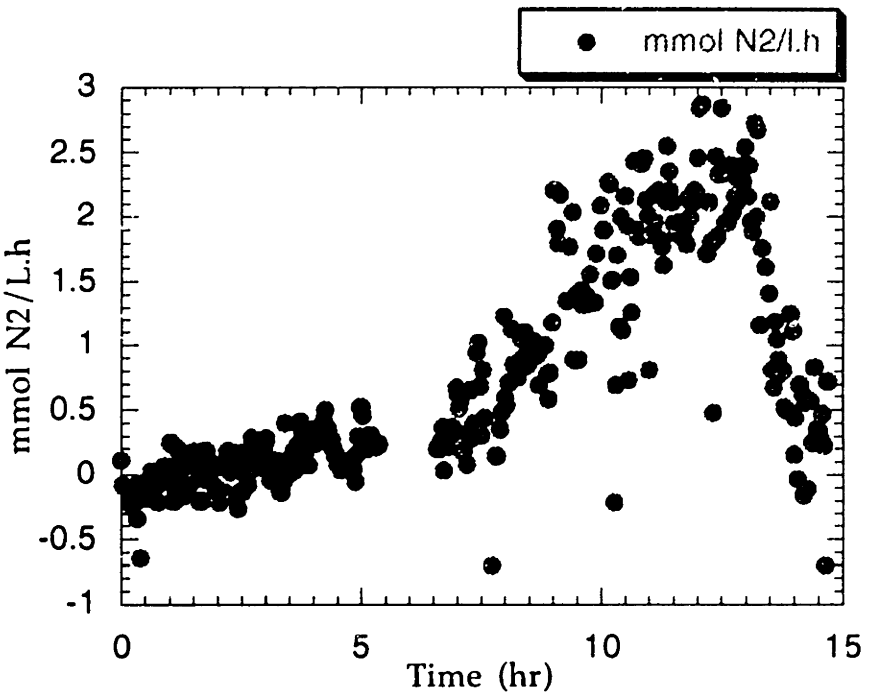
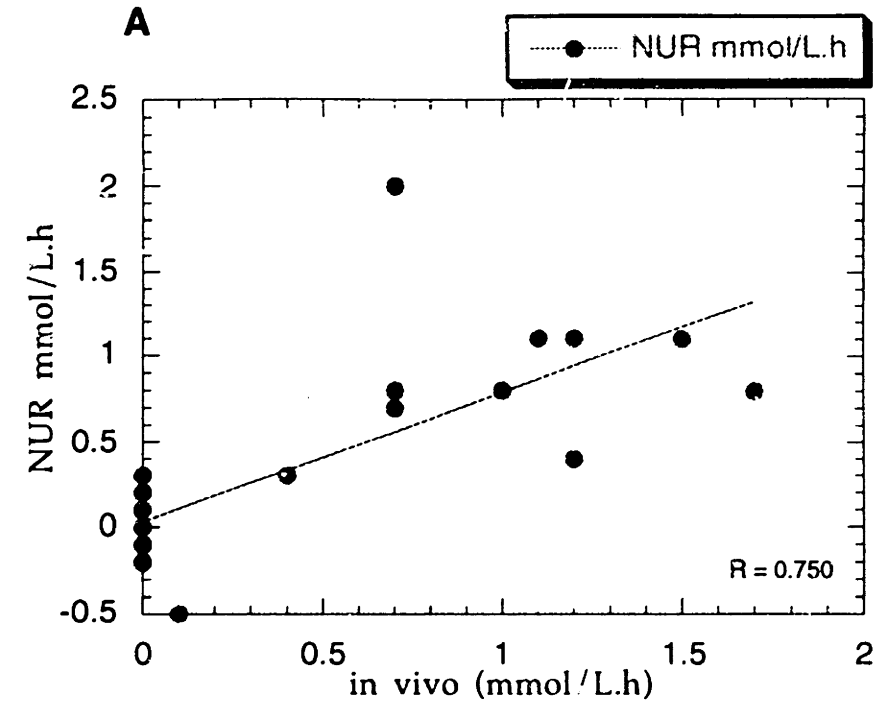
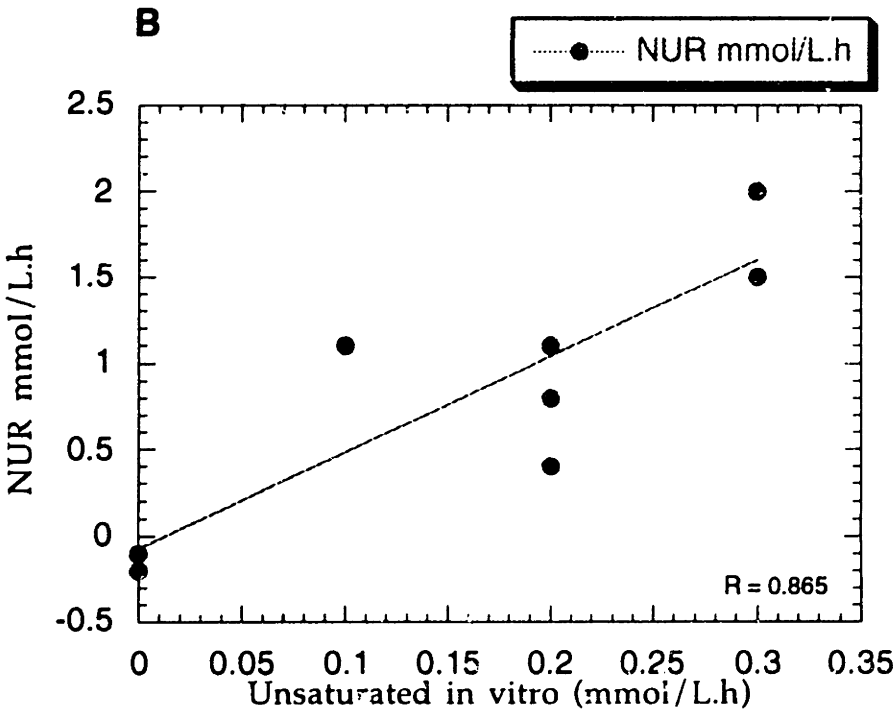


Figure 2.3: NUR trend during a fermentation of *A. vinelandii*.
This is the measured NUR trend for a fermentation experiment with the *A. vinelandii* wild type organism. The break in the data is due to a data communication error.

In this fermentation experiment, the bacteria were allowed to exhaust the carbon source, which led to the cessation of respiration and N_2 fixation. The NUR and standard acetylene reduction assays (both *in vivo* and *in vitro*


A
NUR mmol/L.h
2.5
2
1.5
1
0.5
0
-0.5
NUR mmol/L.h
R = 0.750
0
0.5
1
1.5
2
in vivo (mmol/L.h)


B
NUR mmol/L.h
2.5
2
1.5
1
0.5
0
-0.5
NUR mmol/L.h
R = 0.865
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
Unsaturated in vitro (mmol/L.h)

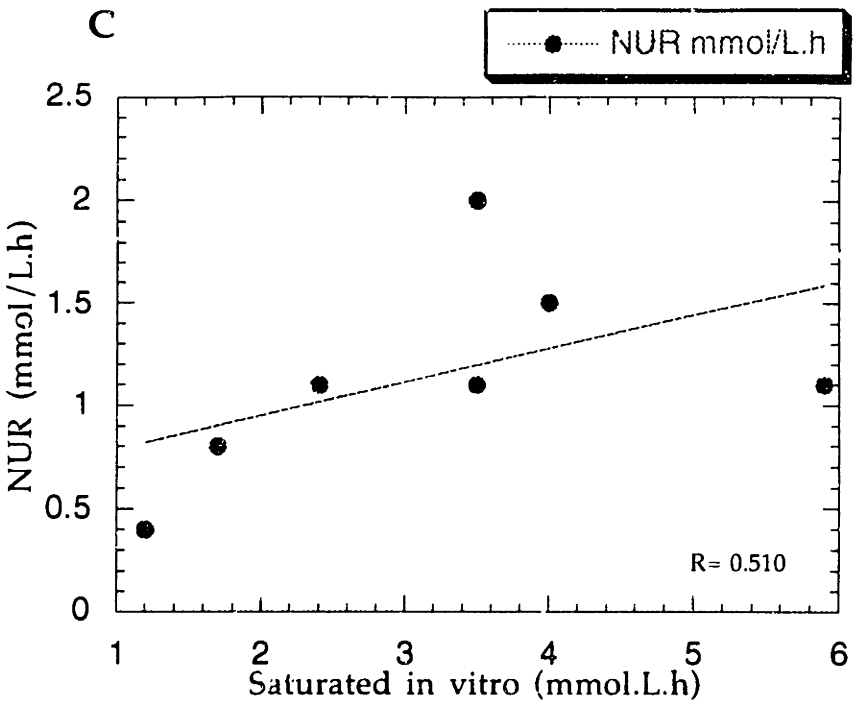


Figure 2.4: Comparison of acetylene reduction assays and NUR.
There is a linear relationship between the NUR and the acetylene reduction assay. NUR is compared to *in vivo* acetylene reduction activities (A), unsaturated *in vitro* acetylene reduction activities (B) and saturated *in vitro* acetylene reduction activities (C). For the *in vitro* activities, there is either additional Fe protein added to the cell extract (designated as saturated *in vitro*) or there is not any additional Fe protein added to the cell extract (designated as unsaturated *in vitro*)

with/without additional Fe protein) were shown to have a linear relationship (figure 2.4).

The measurement of the NUR trend during a fermentation can provide an on-line instantaneous measurement of the nitrogenase activity instead of the traditional methods of assaying nitrogenase activity which take between 30-45 minutes to derive an number. This ability to determine the harvest time instantaneously would directly impact the expected protein yields from the fermentation.

C. Homocitrate Incorporation Experiments:

Following the results of Hoover and coworkers which showed that the wild type phenotype of *nifV-* strains of *K. pneumonia* could be cured upon the addition of homocitric acid to derepressing cultures (Hoover et al., 1988), a similar experiment was performed on a *nifV-* strain of *A. vinelandii*, DJ71. Unlike strains of *K. pneumoniae*, the addition of homocitric acid in the open form did not restore diazotrophic growth to cultures of DJ71. Furthermore, a number of protected open forms of homocitric acid were also used in attempts to restore the wild type phenotype with a similar lack of success (Table 2.3).

Table 2.3: DJ 71 Rescue Experiment

Form of Homocitric Acid	DJ 71 nifV- Growth	UN wt Growth
No acid		growth
Open Form	No growth	*
Lactone Form	No growth	*
Tri-t-butyl ester	No growth	*
3,6-dimethyl-1-t-butyl ester	No growth	*
Dimethyl homocitric Acid lactone	No growth	*

* Not determined

Replication of the results of Hoover and coworkers (1988) was then attempted using *K. pneumoniae*. *K. pneumoniae* (UN, wild type) was used to characterize the growth and derepression fermentation. Since the wild-type

organism is able to produce active nitrogenase, its production of the enzyme can be followed by the acetylene reduction assay. The wild type organism is also able to fix dinitrogen and grow (figure 2.5).

If there is a mutation in one of the *nif* genes, a non-nitrogen fixing mutant (*nif*-) is usually produced. This occurs in UN 1990 (a *K. pneumoniae* *nifV*- mutant) (figure 2.5). When the mutant was being characterized, it was surprising to discover that the bacteria retained its ability to reduce acetylene (McLean and Dixon, 1981). This biochemical ability enables the production of the nitrogenase system to be monitored by utilizing the *in vivo* acetylene reduction assay (figure 2.6). When the open form of homocitric acid was added to the derepressing media of UN 1990, the wild type growth phenotype was restored (figure 2.5).

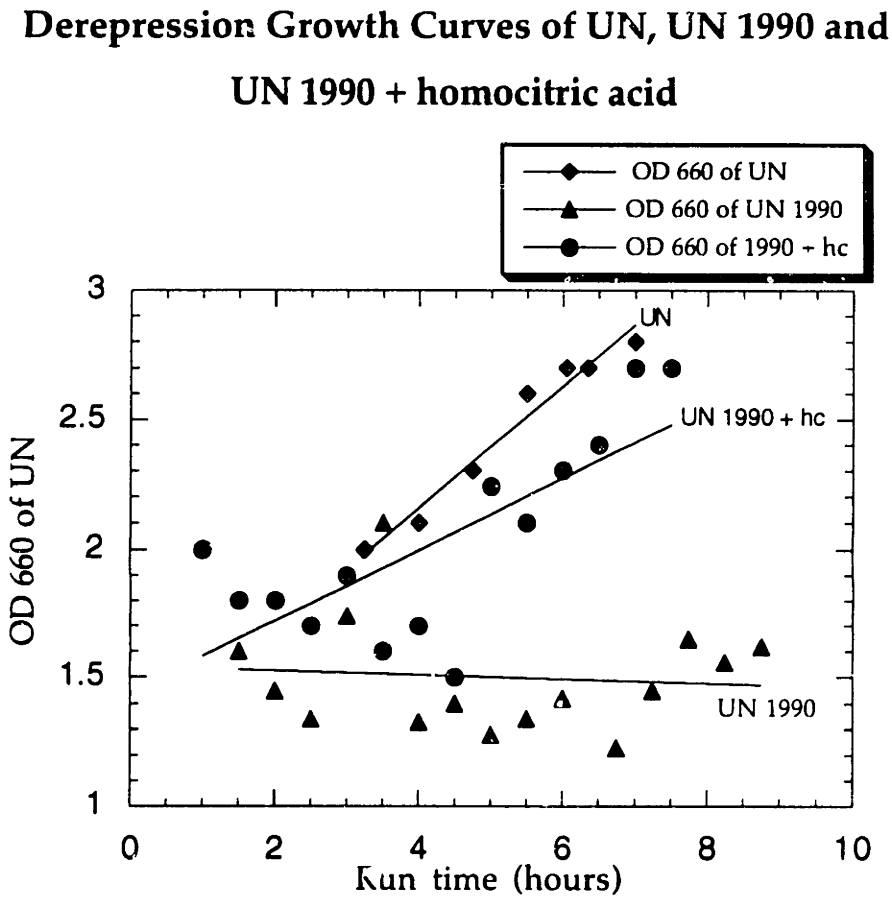


Figure 2.5: Depression Growth curves of UN, UN 1990 and UN 1990 + homocitric acid.

K. pneumoniae wild type is capable of sustaining growth during derepression. When a mutation in the *nif* genes is introduced, growth during derepression is not possible due to the inability to fix dinitrogen. The addition of homocitric acid to the fermentation of a *nifV*- overcame the mutation and produced wild type growth characteristics.

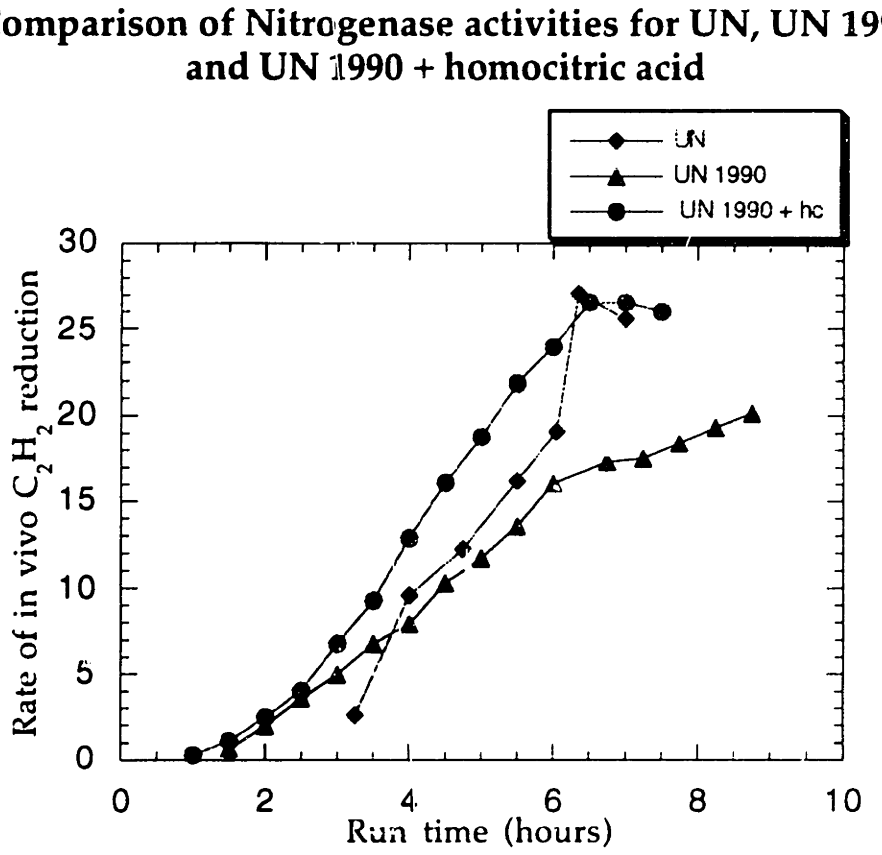


Figure 2.6: *In vivo* activities of UN, UN 1990 and UN 1990 + homocitric acid. The activities of the fermentations were monitored using the *in vivo* acetylene reduction assay. The cultures were harvested once the maximum activity was achieved.

Even though UN 1990 still has the ability to reduce acetylene, its overall rate of reduction is lower than for the wild type organism (figure 2.6). When the open form of homocitrate is added to the culture of UN 1990, the culture is quickly able to show the wild-type activity. The addition of the closed form of homocitric acid does not restore wild-type activity. It had been shown by NMR study (Wright, 1994), that once the homocitric acid has been opened, it is stable in the fermentation media. It can then be concluded that this is the active form which the organism transported across its cell membrane.

Comparison of UN, UN 1990 and UN 1990 + homocitric acid

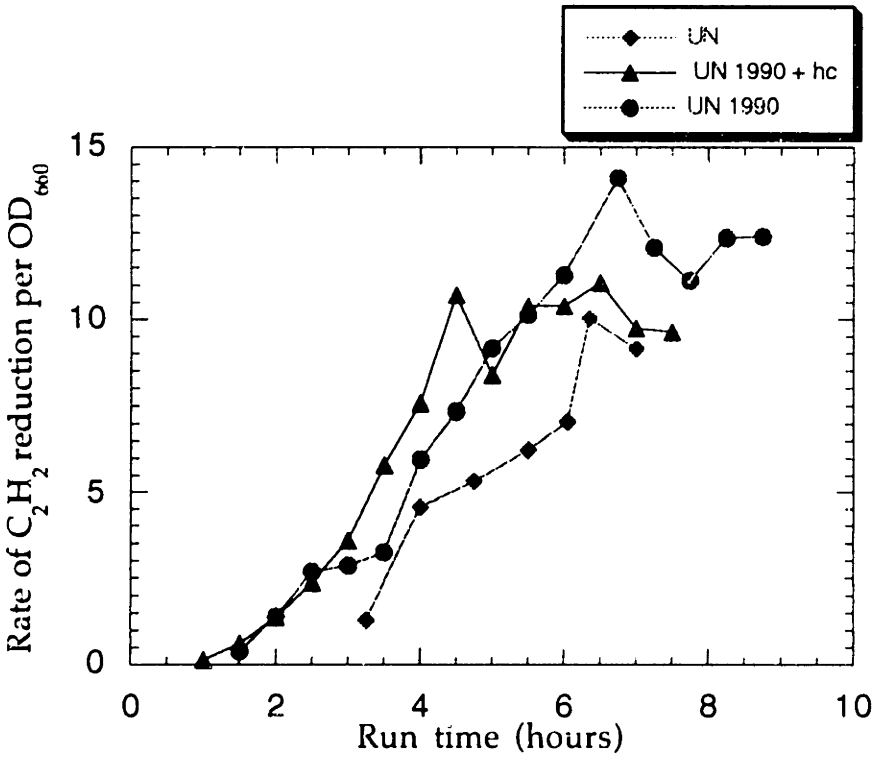


Figure 2.7: Specific activities of UN, UN 1990 and UN 1990 + homocitric acid. During the derepression the specific activities of the cultures (*in vivo* acetylene reduction assay divided by the OD_{660}) were monitored. The cultures were harvested when the highest specific activity was achieved.

UN 1990 was very good at producing its nitrogenase system relative to cell density (figure 2.7). In fact, it had the highest specific activity of all three

organisms during these experiments. An additional incorporation experiment using $^{2}H_{2}$-homocitrate acid deuterated in the methylene between the two carboxylic acids was done (figure 2.8). A similar growth profile established the positive incorporation of the homocitrate label into the MoFe protein. The deuterium incorporation experiment was designed to be used in an ESEEM experiment which will be discussed in detail in a later chapter.

Homocitric acid

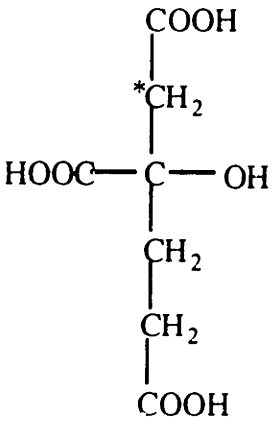


Figure 2.8: Homocitric Acid.
The deuterium label was incorporated into the methylene between the two carboxylic acids and is indicated on the figure by *.

D. Optimization of UN 106 and UW45 Growth and Derepression Experiments:

Both UN 106 and UW45 are *nifB-* mutants in *K. pneumoniae and A. vinelandii,* respectively. Both are a point mutation which was generated by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis and penicillin selection (St. John et al., 1975; Shah et al., 1973; Nagatani et al., 1974). Since both are mutations in the **nifB** gene, under nitrogen fixing conditions, the bacteria produce a fully functional Fe protein and apoMoFe protein that can be activated to holoMoFe protein by the addition of an extract containing intact FeMoco.

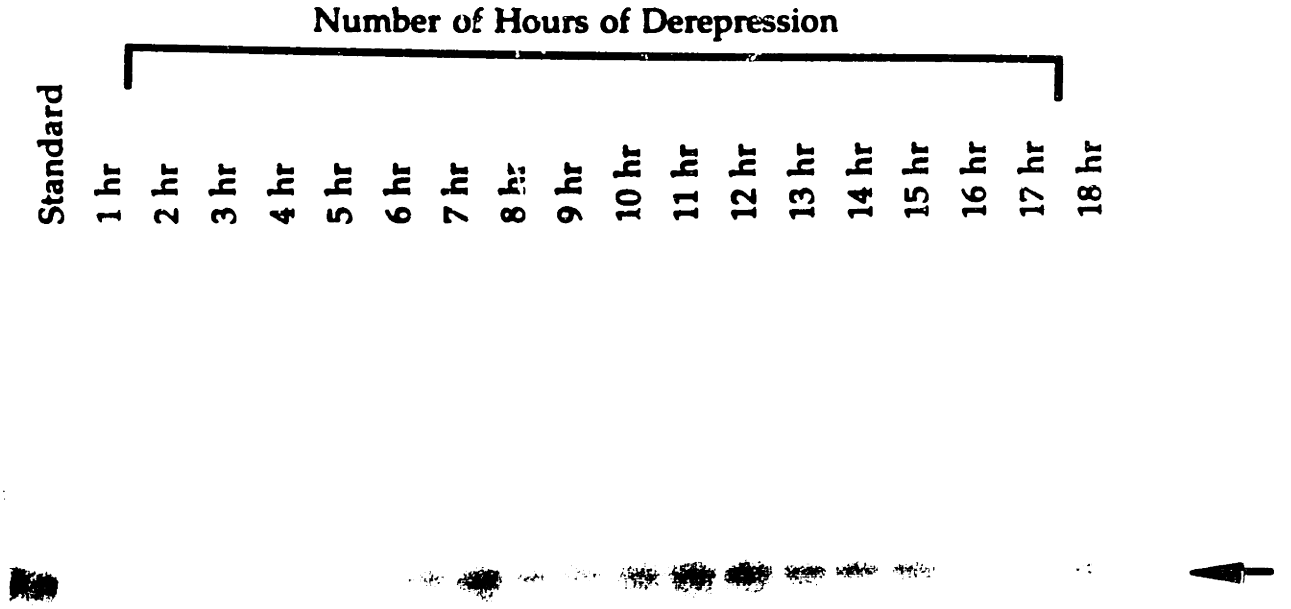


Figure 2.9: Derepression of UW45. UW45 was derepressed on the shaker table over a period of 24 hours. Every hour, 1 ml. samples were taken, spun down and resuspended in Laemmli buffer. Samples were loaded onto a SDS-PAGE gel with a MoFe/Fe protein standard sample. The gets were run at 10 mA current for 18 hours and then immunoblotted against an antibody for the structural polypeptides of the MoFe and Fe proteins. The immunoblots were developed using a horseradish peroxidase colour development solution. In the figure, the arrow indicates the MoFe protein in the immunoblot. Sampling loading for hours of derepression are as follows: hours 1-8, 20μL; hours 9-14, 15 μL and 16-18, 10 μL

Monitoring this derepression in these mutants is accomplished by either following the production of Fe protein or the apoMoFe protein. To look at the stability of the structural peptides of these proteins during derepression, samples from derepressed small scale cultures were developed on SDS-PAGE gels and immunoblotted with antibodies to **NIFHDK** (figure 2.9). The standard used for this gel is a mixture of purified MoFe and Fe proteins. There is a separation between the subunits of the MoFe protein. Over the course of 18 hours, it appears that the most optimal length of time for derepression is 12 hours. This experiment was performed on the shaker table without constant pH or dissolved oxygen control. This number however can be used as a rough guide for the large scale fermentations.

When the derepression of UN 106 on the shaker table was performed (figure 2.10), the amount of production and degradation of the nitrogenase Fe protein was examined. In the immunoblotting experiment, the presence of the structural polypeptides was examined. It was not determined if those polypeptides were catalytically active. The use of the *in vitro* assay looks specifically at the active Fe protein produced. After 18 hours of derepression, the nitrogenase proteins were no longer active.

For large scale fermentations, the derepression time course needs to be followed during the fermentation where there is constant pH and oxygen level control. During a fermentation of UN106, the derepression was monitored through the Fe protein activity in cracked cell extracts (figure 2.11).

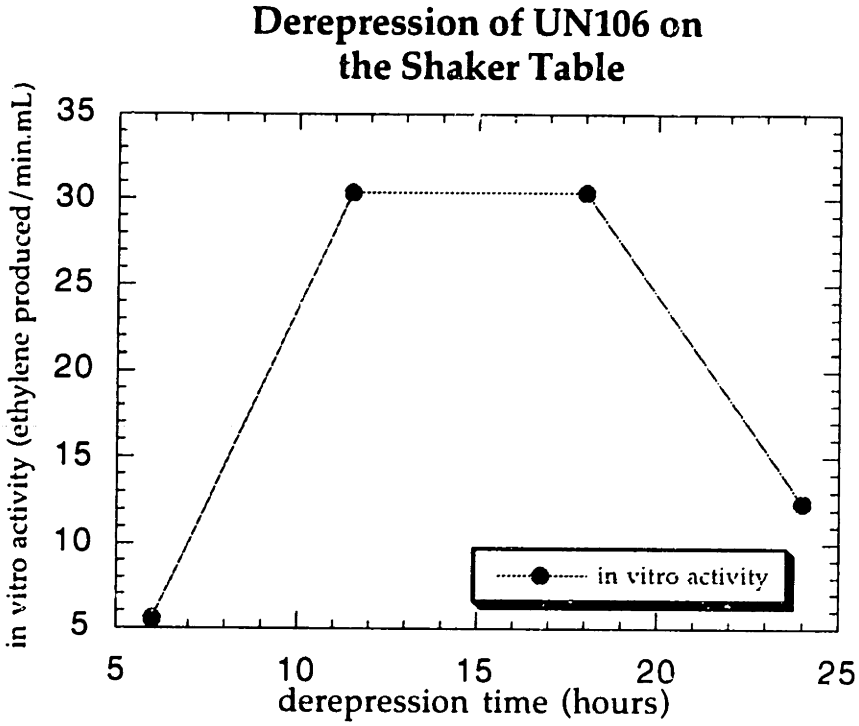


Figure 2.10: Determination of derepression time of UN106 on the shaker table.

UN106 was grown with a fixed nitrogen source overnight. The cultures were then spun down, the pellets washed and then resuspended in nitrogen free media. The derepression was monitored through the production of Fe protein using the acetylene reduction assay on the cracked cell extract. The optimal time for derepression was determined to be between 12 and 18 hours.

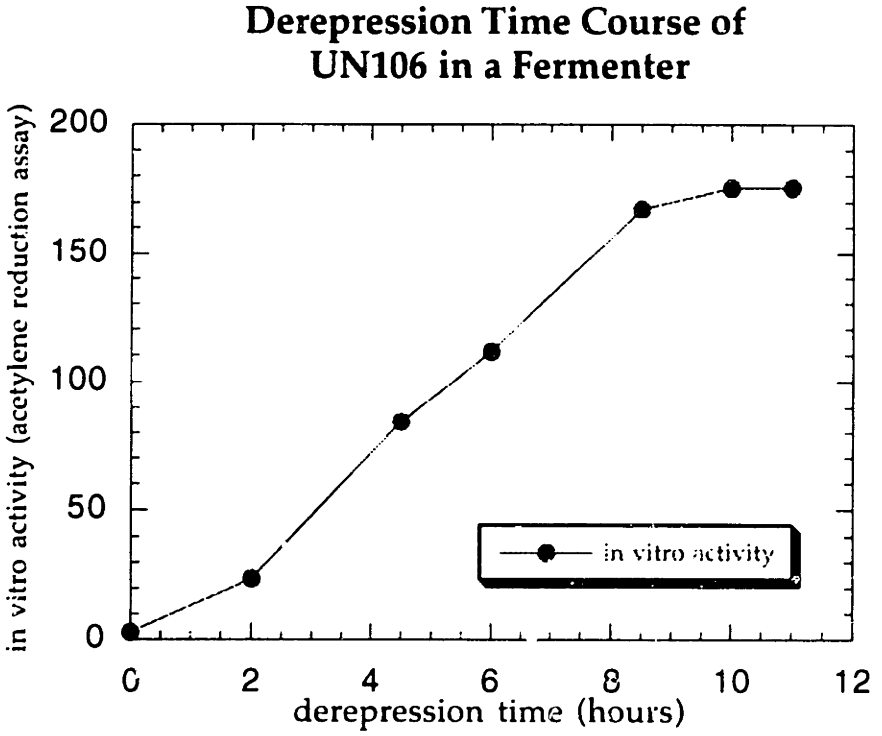


Figure 2.11: Derepression of UN106 in a 20L Fermenter.
The amount of production of Fe protein during the derepression of the nitrogenase system in UN106 was followed by assaying disrupted cell extracts in the in vitro acetylene reduction assay. The harvest of the culture was initiated once the Fe protein activities appeared to be leveling off.

The activity of the Fe protein steadily increased over nine hours and then started to level off. When the Fe protein remains active, the apoMoFe protein is present and in an activatable form. If the Fe protein activity begins to fall, the apoMoFe protein is also becoming inactivatable. In fact, when the Fe protein activity has fallen 30%, there is no longer any activatable apoMoFe protein present. This culture was harvested after 11.5 hours of derepression. The rate of derepression appears to occur faster in the fermenter than it does on the shaker table (compare with figure 2.10) and the Fe protein produced is five times more active. These rate increases can be attributed to the ability to control growth conditions (i.e. pH, temperature, oxygen concentration) more rigorously in the fermenter than on the shaker table. Within the fermenter it is possible to attain more active nitrogenase due to the maintenance of low oxygen tension within the media, which is also not possible with a shaker table.

The derepression of UW45 (*A. vinelandii nifB*- mutant) was followed in a 50 L fermentation experiment (figure 2.11). The amount of Fe protein produced by UW45 varies from batch to batch. The results of this experiment illustrate the variance in activity that can occur between fermentations. It is only through constant monitoring of the cultures can an intelligent decision on harvest time be made.

When the cell paste from the first UW45 batch was tested for apoMoFe protein, there was no apoMoFe remaining even though there was Fe protein activity. This is an indication of the lability of apoMoFe protein. In fact, with the second batch, a technical problem created a delay in the harvest by 2 hours. Even though the fermentation broth was kept on ice and purged with nitrogen, the destruction of all activatable apoMoFe protein resulted. There are several possible modes of inactivation. The apoMoFe protein complex could begin to dissociate. For instance, the γ subunit could separate.

It was then determined that the largest scale of UW45 production that we could successfully undertake was 20 L. It is important to harvest the

culture while the bacteria are still producing activatable apoMoFe protein. Restricting the volume to 20 L enabled the fermentation culture to be chilled, spun down and frozen quickly enough to prevent the inactivation of the apoMoFe. This is critical when one is trying to produce the apoMoFe protein for FeMoco insertion.

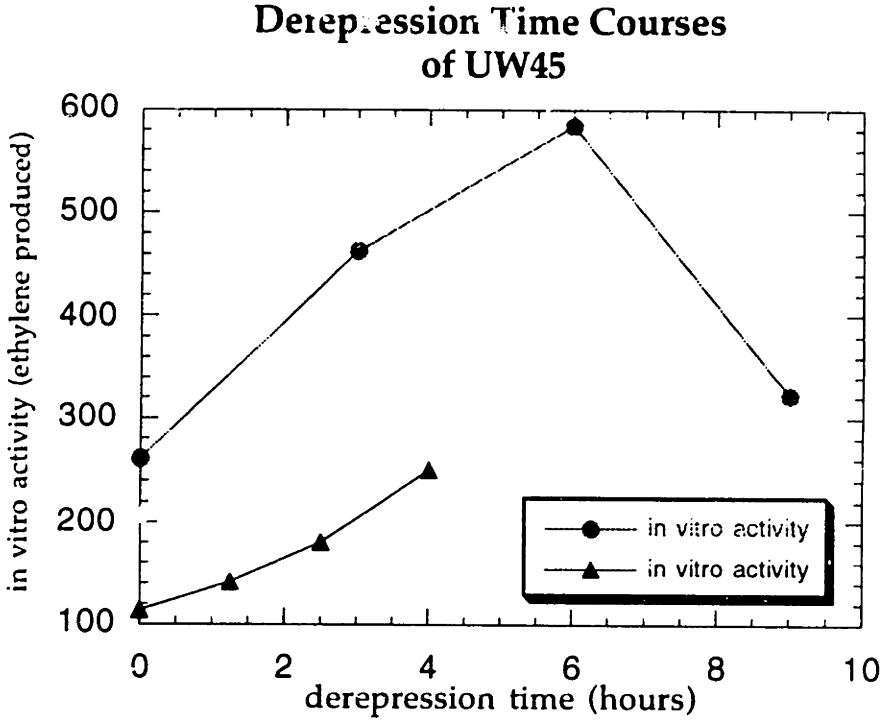


Figure 2.12: Derepression of UW45 in a 50 L fermenter.
In the first experiment (indicated by the circles), the culture was allowed to derepress for 9 hours and then was harvested. From the *in vitro* assays, the best time for harvest was after 6 hours. In the second experiment (indicated by the triangles), the *in vitro* activity was monitored during the fermentation and the decision to harvest was made after four hours of derepression.

E. Iron Starvation Experiments:

The purpose of examining the iron chelating and sequestering in *A. vinelandii* was to optimize the incorporation of iron isotopes into the bacteria. The cost of 100 mg of ^{57}Fe in the oxide form is $1000. This can be

used to create a stock solution of 17.1 mL of an 0.1 M iron citrate. During a typical 20L fermentation, the concentration of iron citrate added is 3.0 mg per liter. For wild-type fermentations, this utilizes about 2.15 mL of Fecitrate stock solution. This would consume only ~ $130 of the stock solution. The production of UW45 uses up to 63 L of media for each complete fermentation, which requires 6.3 mL of the stock solution. The addition of iron citrate to all of the media of a single UW45 fermentation would cost ~ $370. This will quickly devour the costly stock solution of ^{57}Fe citrate. Therefore, alternative means of incorporating the iron were investigated.

Azotobacter species produce siderophores which cause the cultures to fluoresce. The concentration of the siderophores can be monitored by examination of specific absorbances. The 250 mL cultures of *A. vinelandii* OP were grown overnight to an OD_{660} of between 1.14-1.30, and then batched into 1 L of iron free media. The production of the iron scavenging siderophores was followed at 410 nm and 310 nm (figure 2.13).

At various times, 250 mL of the iron starved culture had iron citrate added to it and the siderophore production was monitored (figure 2.14). The levels of siderophore production leveled off, whereas in the iron starved cultures (figure 2.13), the siderophore levels continued to increase. Over the four hours that the culture was monitored after the addition of iron citrate, it did not grow.

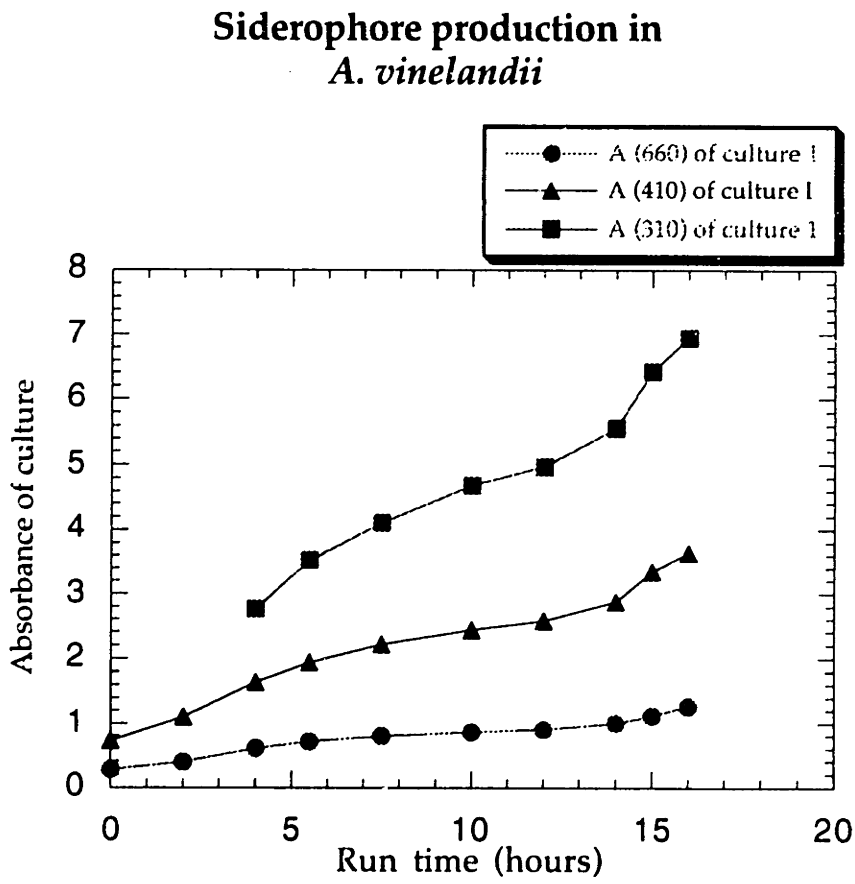


Figure 2.13: Siderophore production in *A. vinelandii*.
A. vinelandii was grown overnight in a 250 mL culture. This culture was then used to inoculate 1 L of iron free Burk's nitrogen free media. The production of siderophores was monitored through their characteristic absorbances at 310 nm and 410 nm with growth being monitored at 660 nm.

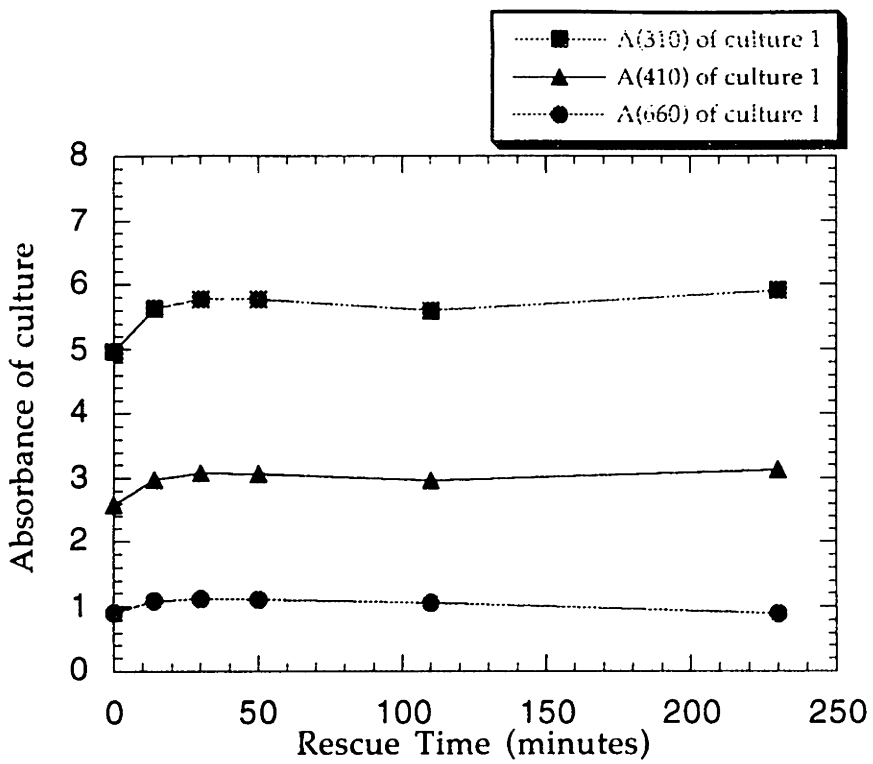


Figure 2.14: Siderophore Production after the addition of iron citrate. After 12 hours of exposure to iron free media, 250 mL of the culture was placed into a flask and an iron citrate solution was added to it. During the four hours the culture was monitored, there appeared to be no increase in growth or siderophore production.

The production of siderophores is a symptom of the culture being iron starved. For the production of active nitrogenase, the level of activity during this iron starvation is more important. When the nitrogenase activity is monitored after the addition of the iron citrate, it was discovered that the nitrogenase activity was very low (figure 2.15), probably due to the bacteria having to expend energy to scavenge iron and not having sufficient quantities to be able to dedicate to the nitrogenase system. It quickly became apparent that this methodology was not useful for producing nitrogenase proteins. When the experiment was done using UW45, the cultures did not

survive the iron starvation phase. Another strategy for efficient iron usage needed to be developed.

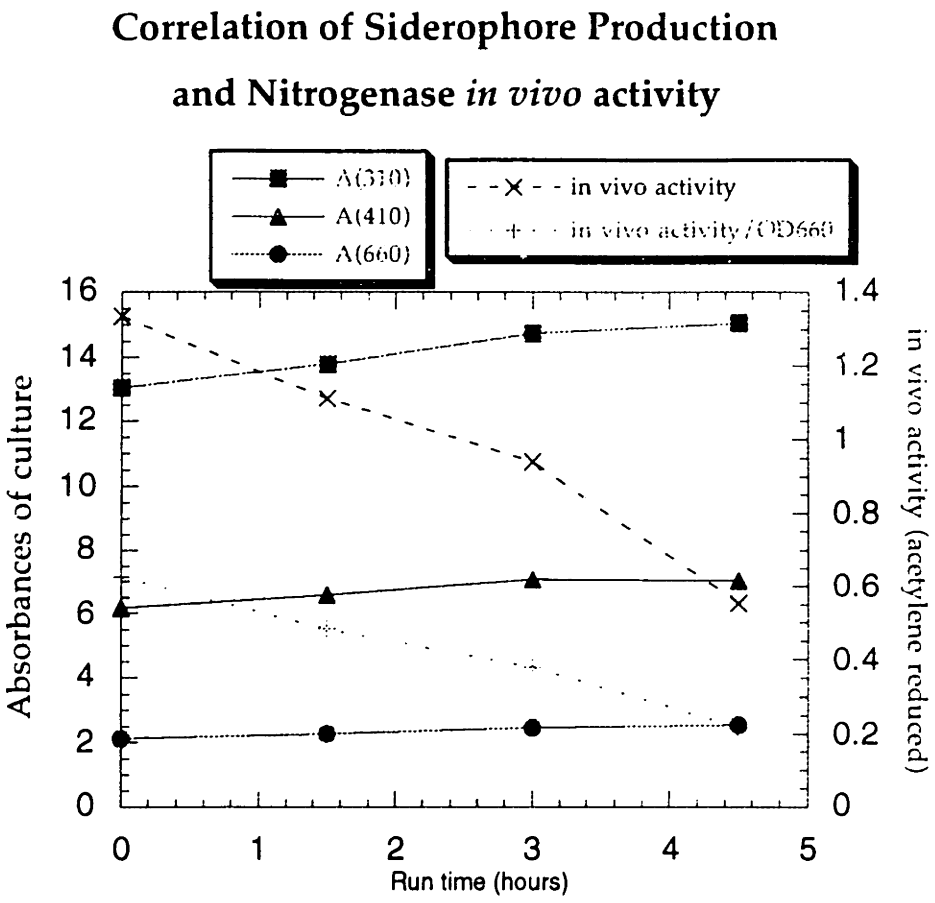


Figure 2.15: Correlation of siderophore production and nitrogenase in vivo activities.

After 12 hours of iron starvation, iron citrate was added to the culture (this is time point 0). The concentration of siderophores has not increased after the addition of the iron citrate. The level of nitrogenase activity has decreased dramatically.

Instead of using the iron siderophores that are produced by the bacteria, the next strategy made use of the iron storage proteins in *Azotobacter*. To test the ability of the bacteria to use its iron reserves for nitrogenase production, 500 mL cultures were grown for 19 hours with a fixed nitrogen source, then spun down and split into two 250 mL cultures for derepression. One of the cultures contained iron in the media, one did not. The results appear in table 2.4. The original cultures were split between A and E, B and F, C and G and D

and H. Both the iron protein activities and the cofactor reconstitution activities are the same. The elimination of iron from the derepression medium does not affect the production of either Fe protein or the apoMoFe protein. UW45 appears to have the ability to hoard iron. When the derepression medium is completed, sodium citrate is added in place of iron citrate. The iron in the stock solution is $FeCl_3$ because the stock solution is prepared by dissolving iron oxide in concentrated hydrochloric acid, and the bacteria would not grow without the additional sodium citrate added to the fermentation medium. This protocol led to successful derepression of UW45.

Table 2.4: Does UW45 require iron in the derepression media?

Culture #	added iron	Iron protein activity (nmoles C_2H_4/min/mL)	Reconstituted MoFe Protein activity (nmoles C_2H_4/min/mL)
A	yes	890	*
B	yes	1057	1702.1
C	yes	546	*
D	yes	839	*
E	no	1014	*
F	no	1021	1812.1
G	no	704	*
H	no	781	*

* = not determined

IV. Conclusions

These fermentation experiments were undertaken to determine the optimal conditions for biomass production using isotopes. How do these conditions and yields differ from those used in other research laboratories (table 2.5)? The *A. vinelandii* OP fermentation at the 50 L and 1000 L scale produces the largest yields. This is due to the fermenter being continuously monitored and conditions automatically adjusted through computer control. At the 20 L scale, the control and adjustments are made manually. Due to technical limitations of the fermenter, the final cell density is not as high as with the 50 L and 1000 L fermentations. Growth of *A. vinelandii* on ^{57}Fe was

much improved over the method used by Jacobson (1980). These growths were under taken in a metal-free fermenter and without dissolved oxygen control. The iron concentration was 1 μM for the fermentations by Jacobson and for all the other fermentations the concentration of iron isotope was 10 μM. The iron concentrations used by Jacobson appear to be too low to support high yields of nitrogenase. The use of a metal free fermenter is not necessary. When the MoFe protein grown on ^{57}Fe in the stainless steel 50 L fermenter was purified to homogeneity, the Mössbauer spectrum indicated that the level of enrichment of ^{57}Fe was the not affected by the fermenter. Examination of the iron content of the media after a simulated run, indicated no additional iron contaminants.

Table 2.5: Comparison of Cell Yields from Fermentations

Operator	Bacteria	Yield (per 20 L)	No. of Runs	Volume
P. McLean	*A. vinelandii* OP	35.6 - 92 g average = 70	9	100 L
P. Christie	*A. vinelandii* OP	62.5 - 92 g average = 74.3 g	3	50 L
	A. vinelandii OP	43.5 - 66.3 g average = 52.5 g	6	20 L
WHO-J Group	*A vinelandii* OP	40 - 120 g average = 100 g	12	1000 L
Burgess et al. (1980)	*A.vinelandii* OP	80 g	N A	400 L
Jacobson (1980)	*A. vinelandii* OP	1 g	N A	100 L
P. McLean	UW 45	Unable to grow		
P. Christie	UW 45	43.5 - 68.3 g average = 56.4	9	20 L
P. McLean	UN 106	30 - 35.4 g average = 30 g	3	100 L
P. Christie	UN 106	48.5 - 50 g average = 49.25 g	2	20 L

The growth of active UW45 was not possible in the Orme-Johnson laboratory until the fermenter was equipped with a dissolved oxygen probe. This is why Paul McLean chose to use UN 106 as his stock for the wild type apoMoFe protein. The use of a double phase fermentation for *nif*- for fermentations larger than 10 L in volume is not widely used. This fermentational approach produces higher cell yields in less time. Additionally, the activities of the proteins produced are higher. This is dramatically demonstrated with the comparison of the UN 106 fermentation of derepressing conditions (9 g) and the double phase (100 g).

The investigation of fermentation strategies for the production of isotopically labeled nitrogenase provided the optimized protocols for the growth of the required bacteria. The yields of the required fermentations were greatly increased for the mutant organisms.

V. Summary

Development of fermentation methodologies are discussed for growth of *A. vinelandii* OP, UW45, *K. pneumoniae* (UN), UN 1990, and UN 106. The wild type growth phenotype was restored to UN 1990 upon the addition of the open form of homocitric acid to the fermentation media. The optimal derepression times for UN 106 and UW45 were determined first on the shaker table and then in 50 L and 20 L fermenters. UW45 apoMoFe protein appears to be not as stable as UN106's apoMoFe protein. The apoMoFe protein is significantly less stable than the Fe protein. It was also discovered that the addition of iron to the derepression medium is not required. Elimination of iron citrate in the derepression medium does not affect the nitrogenase activity.

Chapter Three: Optimization of Large-Scale Extractions of FeMoco

I. Introduction

The active site of nitrogenase is the Molybdenum Iron cofactor (FeMoco) of the MoFe protein. Given the importance of dinitrogen in the biosphere, researchers have been intrigued for 75 years by the functions of this biological catalyst, which carries out a reaction that is done in industry at 100 atm and several hundred degrees Celsius. With the crystal structure of the MoFe solved for *C. pasteuranium* and *A. vinelandii* (Kim and Rees, 1992a, 1992b; Kim et al., 1993) and the proposed model of FeMoco within the protein, it was still not clear how this enzyme functions. However, having a model of the active site in the resting state of the MoFe protein created a new level of excitement to determine if that was the structure in the extracted form.

Researchers have been able to study the extracted FeMoco since 1977, when Shah and Brill (1977) reported the first isolation. There are many technical difficulties associated with examining FeMoco. FeMoco only constitutes 1% of the total weight of MoFe protein (see figure 3.1). To extract FeMoco in sufficient yields for study, every step has to be optimized. Even with each optimized step, a single 1000 L fermentation will yield only 80-120 mg of FeMoco. One strategy that is utilized frequently to overcome the problem of small quantities of proteins is to overexpress the desired protein in another organism, for instance *Escherichia coli*. The nitrogenase genes have been successfully transferred into *E. coli* (for instance see Howard et al., 1986 and references within), but the best overproducer remains *A. vinelandii* in which nitrogenase represents 7-10% of the soluble protein. Transferring the *nif* genes into *E. coli* produced a bacterium that is not as able to meet the intense energy demands made during the production of the nitrogenase proteins at levels anywhere comparable to *A. vinelandii*.

In addition to the difficulties of working with small quantities of sample, FeMoco is very easy to inactivate with compounds which are introduced at various times in the extraction (see table 3.1):

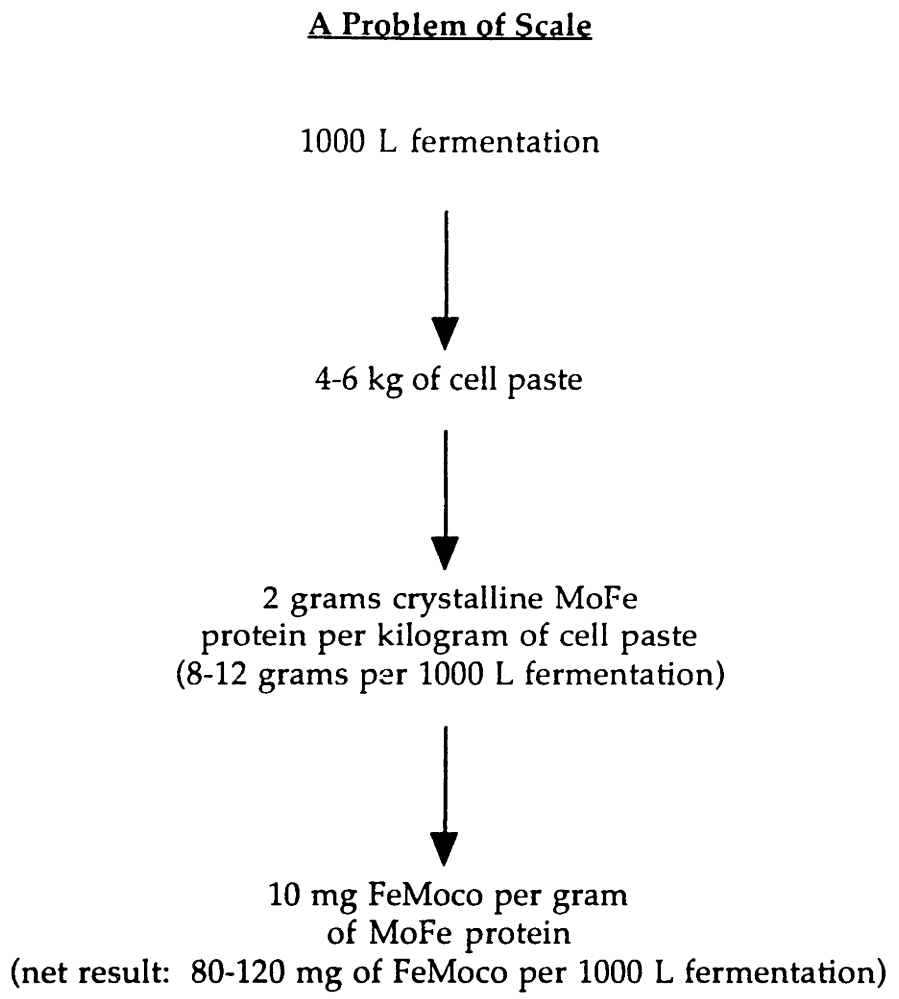


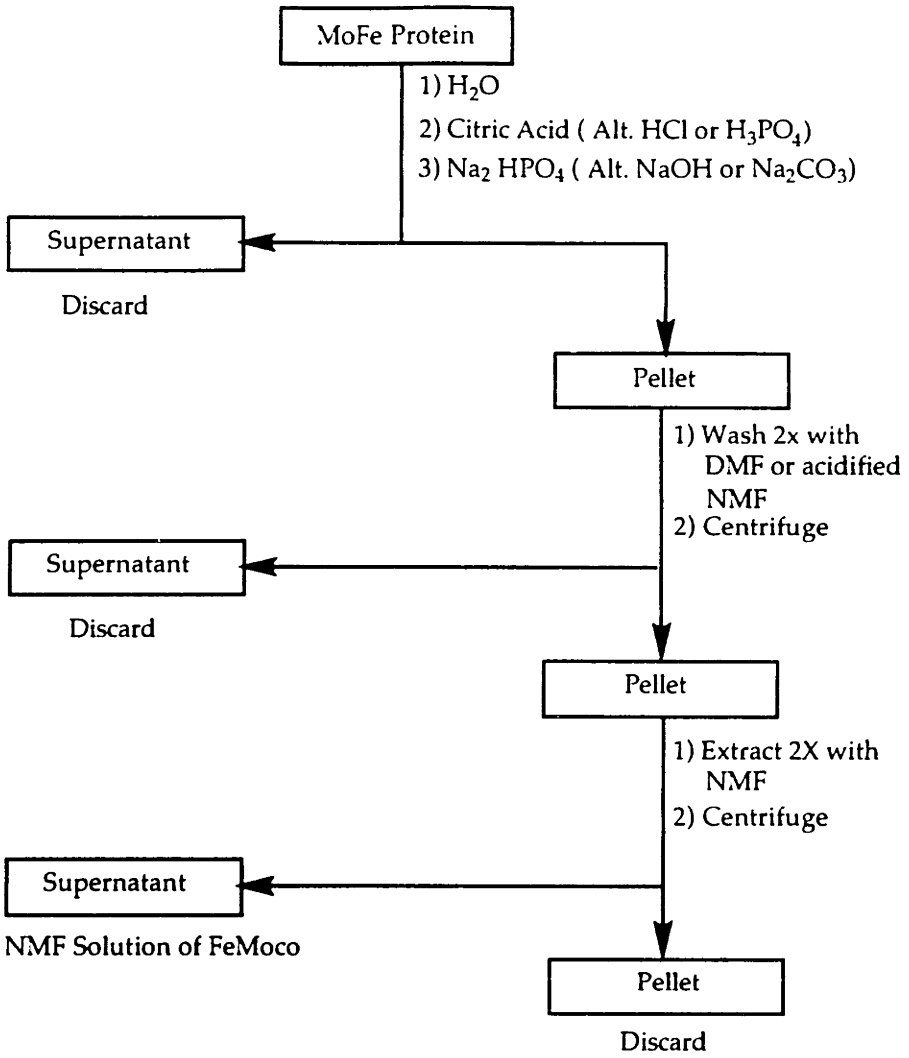
Figure 3.1: A Problem of Scale.
FeMoco constitutes only 1% of the MoFe protein. To enable sufficient quantities of the cofactor to be available for study, the production and purification of MoFe protein was optimized. Even with 1000 L fermentation, the total yield is still only 80 - 120 mg of FeMoco.

Table 3.1: Factors Affecting Stability of FeMoco

Inactivating Reagent	$t_{1/2}$
O_2	seconds
H^+	minutes
OH^-	minutes
H_2O	minutes-hours
High salt concentrations	hours-days
Degradation products of $S_2O_4^{2-}$	hours-days

Since these substances are present in the extraction process, this is a probable major source of inhomogeneity of extracts (Schutlz et al., 1988).

The standard methods of extraction of FeMoco from MoFe protein have been based on the procedure of Shah and Brill (Shah and Brill, 1977; Shah, 1980; Yang et al., 1982) (figure 3.2). This extraction method has many technical difficulties. The procedure was carried out under a hydrogen atmosphere at 0-4 °C using Schlenck lines (i.e. outside the controlled atmosphere of an anaerobic glove box). The first step of the extraction preparation was the unfolding of the protein using an acid treatment at ice bath temperatures. The P clusters were also degraded and released from the protein. The MoFe protein was first diluted with water with 1.2 mM sodium dithionite in order that the concentration of MoFe protein was 5 mg/mL (Shah and Brill, 1977, Shah, 1980; Burgess et al., 1980). In the original procedure (Shah and Brill, 1977) a citric acid solution was added until the final concentration was 15 mM. When the effect of pH on the cofactor extraction was examined by Smith (1980), the pH of the citric acid/protein solution used in the acid precipitation was estimated to be 2.8, with optimal yields of FeMoco in a pH range of 2.0 and 2.2. If the resulting pH was below 2.0, the yield decreased markedly. It was concluded that pH, not the type of acid, was important in the acid treatment step (Smith, 1980). In practice, phosphoric acid and hydrochloric acid were just as effective as citric acid. The degassed acid was made reducing , with a final concentration of 1.2 mM $Na_2S_2O_4$ just prior to use and the pH was adjusted to 2.2. The acidified



Disadvantages:

1) Extremely variable yields
2) Requires high boiling point solvents
3) Unfolded protein must be manipulated outside of glove box
4) Preparations contain contaminating amino acids, Na^+, K^+, and citric acid

Figure 3.2: The extraction procedure of Shah and Brill (1977).
This method of FeMoco extraction involves acid precipitation, DMF washes and NMF suspension of FeMoco. There are multiple centrifugation steps and the entire procedure is carried out on the lab bench using Schlenk lines and ice baths

solution was allowed to sit for 2-3 minutes. The solution was green-brown. The pH was then raised by the addition of base until the MoFe protein's isoelectric point of 5.0-5.5 was reached. The solution was allowed to incubate on ice for 25 minutes. After the protein precipitated, a cloudy, gray-brown solution was observed. Then the first of many centrifugation steps was performed.

After centrifugation, the colourless supernatant is discarded by drawing it out of the centrifuge tube with a gas-tight needle. This supernatant contains the iron from the denatured P clusters (Shah, 1980). Excess water is removed from the pellet through multiple washes with freshly distilled dimethlyformamide (DMF) followed by centrifugation. These washes must be done quickly to minimize the amount of possible exposure of FeMoco to water. The amount of FeMoco lost in the washes can be minimized by maintaining the temperature at 4°C (Burgess, 1990). The supernatants from these washes should be colourless and discarded; if they are brown, they contain FeMoco and should be saved.

The last step in the procedure involves the extraction of FeMoco into freshly distilled, reducing (1.2 mM $Na_2S_2O_4$) N-methlyformamide (NMF). The reducing NMF is added, the solution vigorously vortexed for 5 minutes and then centrifuged. This step can be done at room temperature. After centrifugation, the supernatant is removed and saved. The extraction is repeated until the pellet is light gray to white in colour. The extracted FeMoco in NMF can vary in colour from brown, green-brown to green in colour (Shah and Brill, 1977; Lough et al., 1988; Walters et al., 1986).

The NMF solution had the dithionite solution added from a water stock resulted in the cofactor solution containing 1% water. It was then discovered that the source of NMF greatly affected the ability to extract FeMoco. After a proton NMR was run on the solvent, it was determined that the NMF in solution differed depending on where it was purchased. It was determined that the ability of the NMF to extract the FeMoco depended on

the "pH" (pH was determined by diluting the organic solvent 1:5 with water). If the pH was higher than 8, ("basic"), it was able to extract FeMoco; below 5 ("acidic"), it was ineffective. This created an additional step in preparation since NMF needed to be stirred overnight with sodium bicarbonate followed by filtration and distillation to achieve the "basic" state (Schultz, 1985). Schultz and coworkers (1985) stated that this never fails to extract FeMoco. The yields from this extraction procedure vary from 87 % (Shah and Brill, 1977), to 60-70 % if not enough NMF washes were done but could be increased to 75-85 % if additional washes were included (Burgess et al., 1980; Yang et al., 1982).

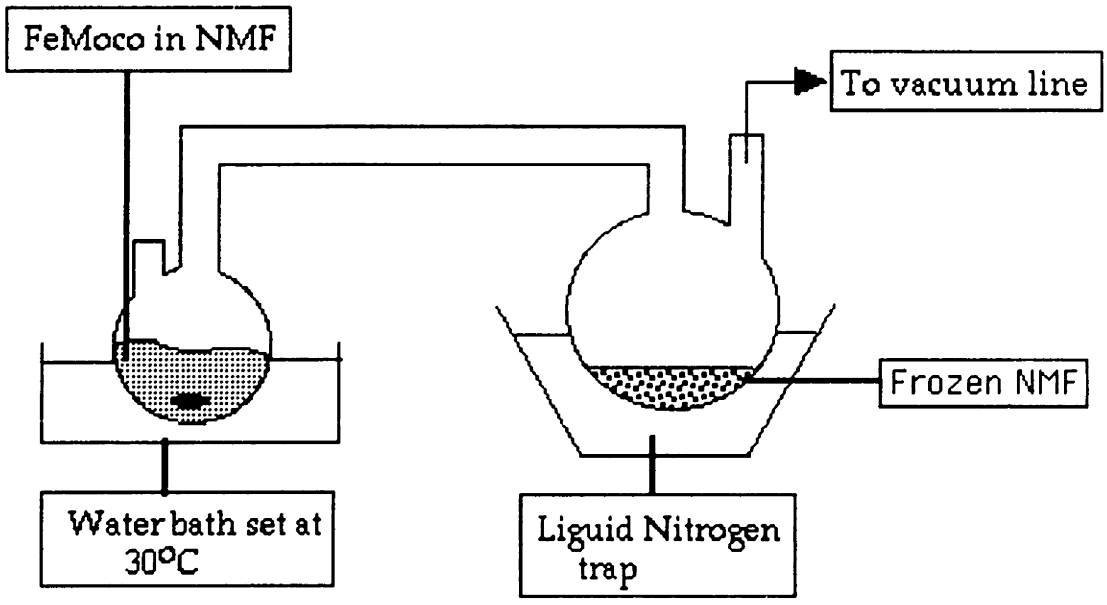


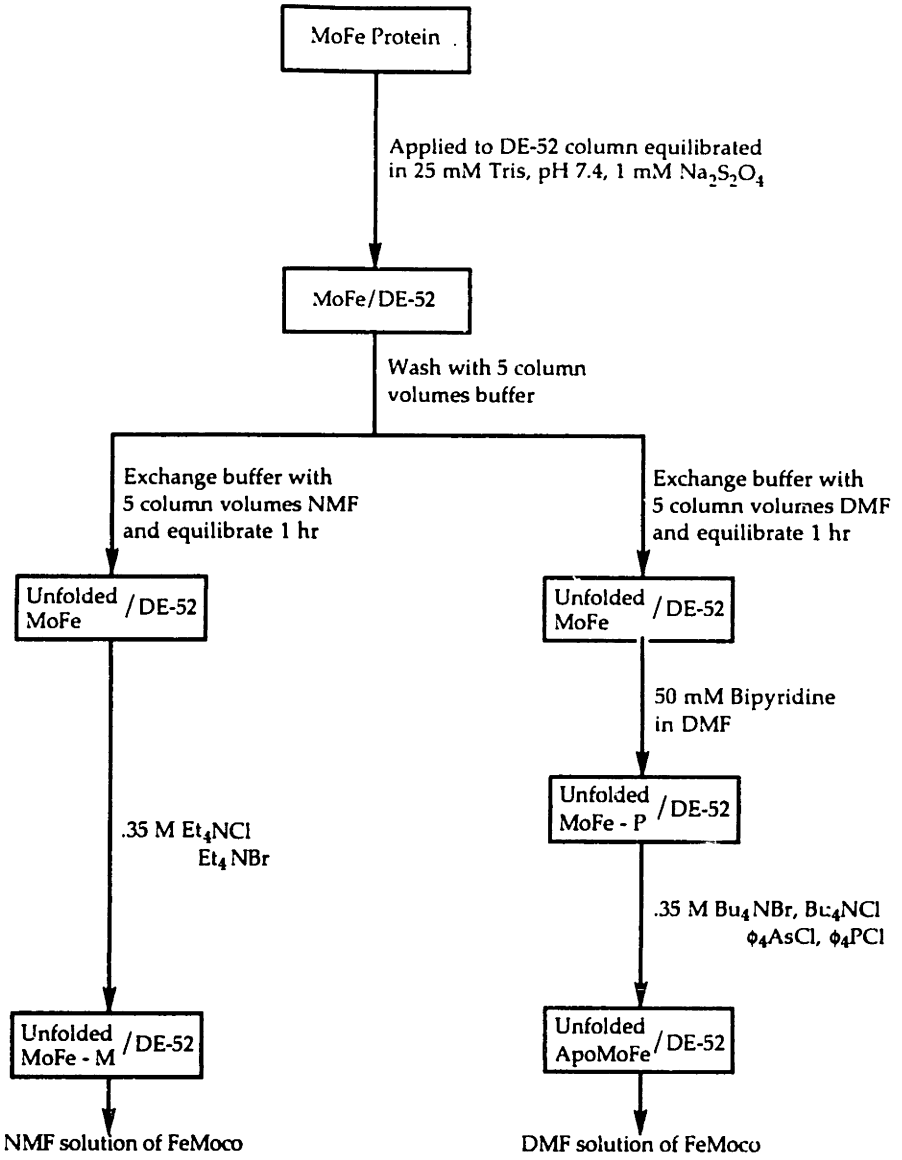
Figure 3.3: Vacuum distillation of excess solvent from FeMoco solutions in NMF.
To concentration the NMF solutions of FeMoco, the solvent is evaporated off with a 30°C water bath under vacuum. The solvent is then condensed in a liquid nitrogen trap.

For a typical scale of 30 mg MoFe protein, the extracted FeMoco in NMF has a concentration of approximately 50 μM. The excess solvent can be removed by using a vacuum distillation procedure developed by Burgess and

coworkers (Burgess et al., 1980). The temperature in the water bath cannot be raised above 30°C as higher temperatures degrade the FeMoco. It is reported that the FeMoco solution can be concentrated 10 fold in 1 hour (Burgess et al., 1980).

This method of extraction is extremely fickle. All the extraction manipulations are accomplished outside of an anaerobic dry glove box. Most of the procedures have to be done on ice and under hydrogen. There are multiple centrifugation steps, followed by removal of the supernatant using a syringe and then additional solvents are added through this septum. All of the centrifuge tubes must be resistant to NMF and be airtight. The solvent of choice, NMF is very difficult to remove due to its high boiling point and low vapor pressure. This extraction procedure has the potential to have many other contaminating 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 (Yang et al., 1982; McLean et al., 1989). Furthermore, scale up with this procedure would require multiple centrifuge tubes, increasing the chance that pellets would be dissolving while other pellets were being precipitated. To solve these technically demanding problems, a new procedure for the extraction of FeMoco was developed in the lab of Orme-Johnson (McLean et al., 1989; Wink et al., 1989). This was a procedure which could be done entirely within in the confines of an anaerobic dry glove box and eliminated the cold acid precipitation step. A summary of the procedure is contained in figure 3.4.

The extraction procedure uses a column method for extraction. The MoFe protein is first bound to a pre-swollen, reducing DEAE column poured and packed into an BioRad Econo Column. The column is washed with five column volumes of Tris buffer with 2 mM Na_2S2O4 (reducing Tris) to ensure that all the protein is adsorbed onto the column and the material is sufficiently packed. The protein is then unfolded in an organic solvent, either DMF or NMF. The organic solvent is made reducing by the addition of dithionite. Sodium dithionite is slightly soluble in NMF, but not soluble in



Disadvantages

1) FeMoco is unstable in high salt concentrations
2) Extraction results in fairly large volumes
3) FeMoco trails off of the column
4) Scale up is technically non-trivial

Figure 3.4: The extraction method developed in the Orme-Johnson laboratory.

This is a procedure which is done entirely inside the controllable environment of an anaerobic glove box. The MoFe protein is bound to a DEAE column, the protein is unfolded with an organic solvent and the cofactor is extracted with a salt solution in an organic solvent

DMF. To make DMF reducing, tetrabutylammonium dithionite, made by the method of Lough and McDonald (1987) is used. To ensure that all of the water is removed from the column material, at least five column volumes of organic solvent are eluted through the column, being careful to ensure that the water is eliminated from the top of the column. The organic solvent exchanged column material is then allowed to sit for 60 minutes to complete unfolding.

If DMF is used, this exposes not only FeMoco but also the P clusters to the solvent phase, thus the P clusters must be removed before the extraction of the FeMoco. The iron from the P clusters can be removed by eluting a 50 mM bipyridine (bpy) in reducing DMF solution through the column. Once one column volume of this solution has passed through the column, the column flow is stopped by plugging the bottom of the column and allowed to equilibrate for 30 minutes. The column will gain a slight pink tinge from the resulting $Fe(bpy)_3^{2+}$ complex. This complex is removed by elution with reducing DMF. The FeMoco is now extracted using 0.2-0.5 M solutions of organic salts in reducing DMF. The salts commonly used are $[Et_4N]Br$, $[Bu_4N]Br$, $[Ph_4As]Cl$ or $[Ph_4As]Cl$.

If NMF is used, the unfolding of the protein only exposes the cofactor. FeMoco can be directly extracted after exchange into the organic solvent. Organic salts commonly used are 0.2-0.5 M $[Et_4N]Br$ or $[Et_4N]Cl$ in NMF. Wink and coworkers (1989) were also able to demonstrate than after protein unfolding with DMF, FeMoco can be efficiently eluted with a variety of organic salts in acetonitrile, acetone, dichloromethane, benzene or THF. Such procedures do require that the DMF phase be completely exchanged with the solvent of choice before the elution of the cofactor.

The extracted cofactor is now effectively a contaminant in an organic salt solution in an organic solvent (Table 3.2)(Wright, 1994).

Table 3.2: Common Contaminants Found in FeMoco Preparations

Contaminant	Concentration	Ratio of Mo
Organic Salt	0.35 M	8500:1
Organic S2O4	2 mM	50:1
Na^+, K^+	2- 0.05 mM	50-1:1
Excess $Fe^{2+/3+}$	0.37 mM	8:1

Wink and coworkers developed a method to remove the excess salt using an LH-20 column in an organic solvent. The column is poured in the organic solvent of choice, for instance, acetonitrile, DMF, or NMF, and then the oxidizing equivalents in the stationary phase are removed by eluting a small volume of 0.1 M organic dithionite solution through the column. LH-20 has a molecular weight cut off of ~2,000, so the FeMoco with its organic counterion elutes in the void volume and the contaminating salts are held up in the column material. Even though after a single pass through the desalting column 99% of all the contaminants are removed, the salt to FeMoco ratio is still ~200:1. To reach stoichiometric levels of salt to FeMoco, additional sizing columns must be used.

This column method of FeMoco extraction is a vast improvement on the original FeMoco extraction. The yields are comparable to the original method and it takes less time. A number of potential contaminants such as citrate, phosphate and water are removed. The greatest improvement is that the procedure is done entirely within the confines of a glove box where the oxygen levels can be strictly maintained. This method is not perfect. Using the column method, several unexpected difficulties have been reported when an increased scale is used. The most problematic of these difficulties is that FeMoco trails off the column resulting in large volumes of eluent with lots of destabilizing salt that needs to be removed (Burgess, B.K. personal communication). This excess salt has to be removed quickly before it deactivates the cofactor.

The extracted cofactor will be utilized in the production of isotopically labeled hybrids of the MoFe protein. If 30 mg of MoFe protein is used as the starting material for the extraction, only 0.2-0.3 mg of FeMoco will be produced. For a single fermentation of UW45, there could be as much as 150 mg of activatable apoMoFe protein produced. Using the 30 mg MoFe protein scale, this would require multiple extractions, with subsequent pooling of extractions, concentrations and reassaying the activities of the pooled cofactor. This is a long and tedious process which could result in variable activities of FeMoco. The current attempts at up scaling produced lower yields in large volumes. Since the starting material is expensive to produce and very time consuming in its production, unnecessary waste is not desirable. It is for these reasons that an investigation of the dynamics of FeMoco extraction was undertaken. One of the goals of this study was to reveal the optimal conditions required for efficient extraction of FeMoco using at least 200 mg of MoFe protein as starting material. Another goal is to quickly put FeMoco into a state where it was stable for extended periods of time.

II. Materials and Methods

All manipulations of air sensitive materials were carried out either in a Vacuum Atmospheres Model M040-1 glove box with a He atmosphere at <1 ppm O_2 or in serum stoppered vials using Schlenck techniques. Tris-HCl buffers at pH 7.4 contained 2-10 mM NaS_2O_4 to ensure the removal of oxidizing equivalents. The presence of dithionite was confirmed when strips of filter paper, soaked in 2 mM methyviologen dihydrochloride and then dried, turned blue upon exposure to the solution. Solvents were vacuum distilled after drying over Linde 4 Å molecular sieves for at least 24 hours. Organic solvents were 2 mM in $X_2S_2O_4$, where X is $[Et_4N]^+$ or Na^+ depending on solubility. DE-52 was purchased from Whatman and used as received. Weights of DE-52 are for the preswollen material from the bottle. Bu_4NBr,

Ph_4AsCl and $[Ru(bpy)_3]Cl_2$ were purchased from Aldrich or Lancaster Chemicals. All solvents were Aldrich HPLC "Sure Seal" grade solvents.

The MoFe and Fe proteins were purified from cell extracts according to Burgess et al, (1980) with modifications made for the bulk rapid processing of multi-kilogram quantities of cell paste (see Appendix, Guide to Protein Purification). The protein was homogeneous as determined by MoFe protein acetylene activity assays, modified Lowry assays and Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Stock protein solutions were 16-100 mg/mL with a specific activity of 2200-2400 nmole of C_2H_4produced.$min^{-1}mg^{-1}$ of protein in 0.25 M NaCl, 25 mM Tris-HCl buffer with 2 mM $Na_2S_2O_4$.

A. Preparation of Organic Dithionite:

The organic dithionite is prepared by the method of Lough and McDonald (Lough and McDonald, 1987). A 2.5 X 1.5 cm column (Econocolumns, Bio-Rad, new or base bathed) of Bio-Rex 5 anion exchange resin (Cl^- form) is made anaerobic by elution of 1 L of rigorously degassed H_2O. The column is then converted to the dithionite form by the slow elution of 600 mL of 50 mM $Na_2S_2O_4$. The column is washed with degassed H_2O until the dithionite is not longer detectable with methyl viologen paper. The resin is treated with degassed 0.5 M $[Bu_4N]OH$ in H_2O. The pH and reducing ability of the fractions are monitored with pH paper and methyl viologen paper, respectively. When the pH suddenly increases, the elution is discontinued. The stoichiometric organic dithionite is in the fractions which are reducing to methyl viologen paper and the pH is constant. The eluent is evaporated on a liquid nitrogen chilled rotoevaporator at 30°C to give a pale oily residue. The material is then heated to 80°C under vacuum for an additional 6 hours causing the solidification of a well-formed product and a color change to an intense canary yellow.

B. Analysis of the MoFe cofactor:

FeMoco is assayed by a reconstitution assay using a cofactorless mutant, *NifB-*. The common strains used are *K. pneumoniae* UN 106 and *A. vinelandii* UW 45. Conditions for growth and harvesting of these strains may be found in the appendix A, Guide to Fermentations. The cell paste of the derepressed *nifB*⁻ mutant was diluted 2 times in 0.1 M Tris, pH 8.0, 5 mM $Na_2S_2O_4$. After the cells are thawed at 4°C, the cell suspension is disrupted in the French pressure cell and aliquoted into degassed vials (St. John et al., 1975; Roberts et al., 1978). For titration of the extracted cofactor, 0.1 mL of the cracked cell extract is diluted with 0.3 mL of 25 mM Tris, pH 7.4, 5 mM $Na_2S_2O_4$ in a stoppered Ar-filled vial. To this diluted extract, aliquots (2-10 μL) of diluted FeMoco were added and incubated at 4°C for 5-30 minutes. A 50 μL aliquot of FeMoco was removed from the incubation vial and assayed. The activity of FeMoco is reported as nmole of C_2H_4 produced.min^{-1}(nmole Mo)$^{-1}$. Reported recovery of an extraction is based on the total activity (units defined as nmole C_2H_4/min) of the MoFe protein used in the extraction, while the recovery of FeMoco after various manipulation is based on the total activity (units) of the cofactor used in the experiment.

Metal analysis was determined with a number of techniques. Atomic absorption analyses of Molybdenum were obtained with a Perkin-Elmer Model 2380 spectrometer, using an HGA-400 graphite furnace (McLean et al., 1989). FeMoco samples for Mo determination were digested in 2% HNO_3 and the concentration calculated against a linear standard curve. An additional method for iron concentration was the spectrophotmetrical ferrozine assay (Carter, 1971). Ru in the form of $Ru(bpy)_3^{2+}$ was quantitated spectrophotometrically based on a λ_{max} at 452 (ε = 8000-12000 depending on solvent).

C. FeMoco Extracted from the MoFe protein bound to DEAE Cellulose by the Batch-Column Method:

An outline of the procedure is in Figure 3.5. The desired amount of MoFe protein is diluted five fold with 25 mM Tris, 2 mM $Na_2S_2O_4$ (reducing Tris). The DE-52 (0.04 g preswollen weight DE-52/mg of MoFe to be loaded) is swirled in a small amount of reducing Tris, checking to see if the supernatant is reducing. While the solution is stirring, the dilute MoFe protein solution is slowly added. After the MoFe protein has been successfully batch loaded (stirred for 1-1.5 hours), the DEAE cake is collected on a filter paper in a Buchner funnel. The filter cake is washed with 3-5 volumes of reducing Tris and then poured into a 2.5 cm diameter BioRad Econo Column. The column is packed with at least one column volume of reducing Tris.

The column material is then exchanged into reducing DMF (DMF with 2 mM organic dithionite). At least five column volumes are eluted through the column, making sure all the water is removed. When the column has been completely exchanged, the column is stoppered and allowed to stand for at least 30 minutes. After this equilibration time, the column material is stirred and packed with one column volume of reducing DMF. The protein is now completely unfolded.

The P clusters are extracted by elution with at least one column volume of 50 mM Bipyridine (bpy) in reducing DMF. After elution of one void volume, the column flow is stopped and the column is allowed to equilibrate for at least 30 minutes. The column is then eluted with reducing DMF until the bpy solution is eluted, evident by the clear flow through.

FeMoco is then extracted with 0.4 M $(Ph)_4AsCl$, tetraphenylarsonium chloride or 0.17-0.22 M $Ru(bpy)_3Cl_2$. Upon the first drop of FeMoco at the bottom of the column, the flow through is stopped and the column is allowed to equilibrate for at least one hour. After one hour, the cofactor is eluted off the column with another void volume of salt solution. FeMoco elutes off as a dark green/black band if $[(Ph)_4As]^{1+}$ was used and as a blood red solution if the counter ion was $Ru(bpy)_3^{2+}$.

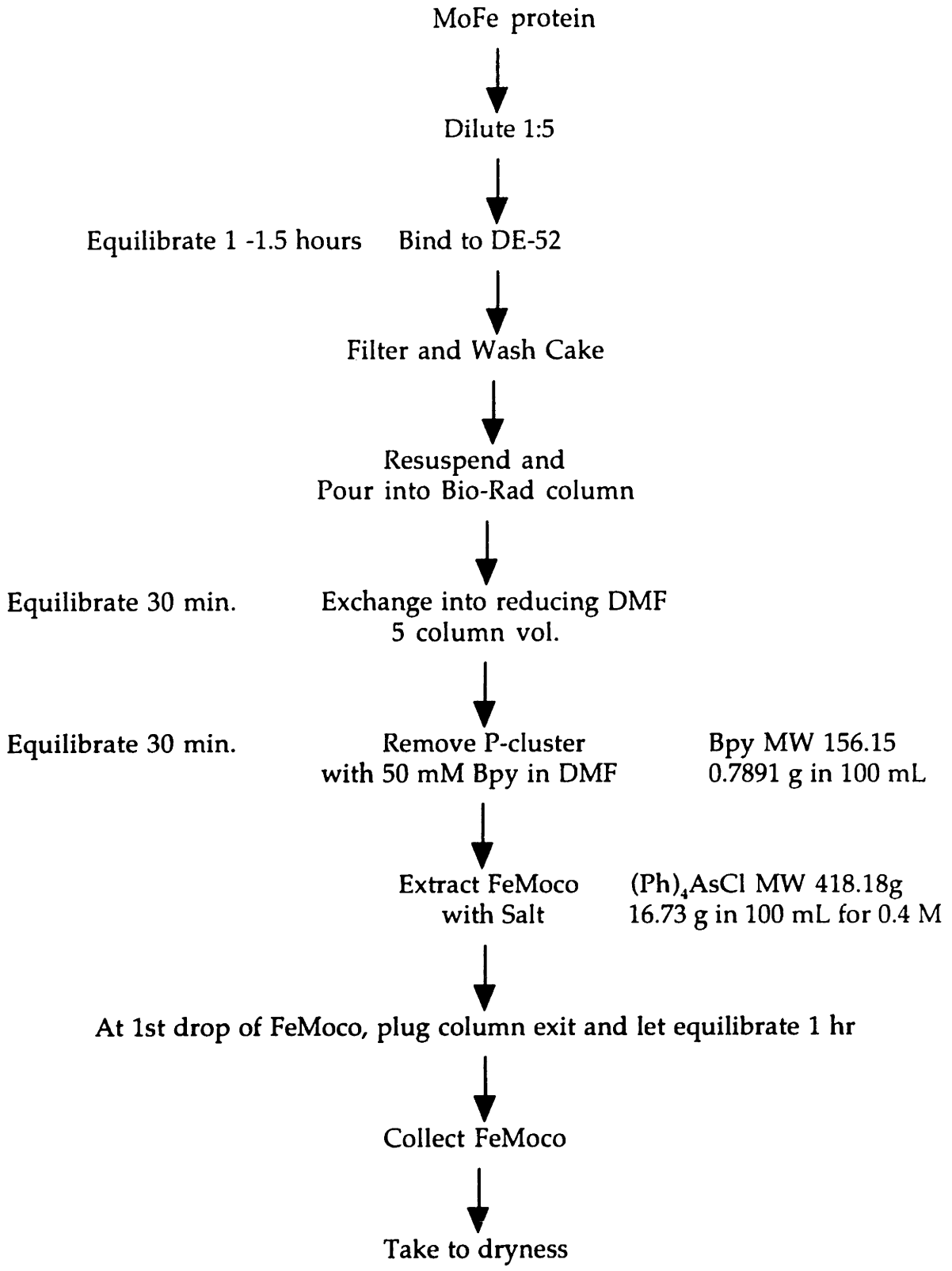


Figure 3.5: Outline for the extraction of FeMoco from MoFe protein using the Batch-column method.

D. Storage of FeMoco inside the Glove Box:

The 0.2-0.5 M salt solution containing FeMoco was taken to dryness by rotoevaporation at 30°C using a wide bore, high vacuum, liquid nitrogen cooled Brinkman rotoevaporator. When the solution was completely dry, the solid was finely ground in the collection flask. It was possible to store the FeMoco in this dry state for weeks at a time.

When FeMoco was required for an experiment, the dry product was triturated with half the volume of the original sample. The non-dissolved salt was collected in medium glass fritted filters by vacuum. The required FeMoco could be removed from the stock solution and the rest taken to dryness once again.

All of the techniques and preparations required for successful FeMoco extractions are included in the Appendix C, Guide to FeMoco Extractions at the end of this thesis.

III. Results and Discussion

FeMoco is attached to the MoFe protein through a thiol cysteine and an imidazole histadine (see figure 1.5). It is these bonds that are broken when the cofactor is extracted from the protein. Once in solution, FeMoco has a negative charge and is associated with a positive counter ion. It has been suggested that in its extracted form, FeMoco exists as a dimer (Huang et al., 1993). Huang and coworkers purified FeMoco using calibrated columns with various molecular sizing matrixes to suggest that in NMF and DMF solutions that FeMoco's molecular weight was twice the value expected from the minimal stoichiometry (Huang et al., 1993). Spectroscopic examination of extracted FeMoco indicates that it maintains its overall structure and symmetry. The ability to extract active FeMoco is a technically challenging procedure.

Since the column method of FeMoco extraction proved superior to the original acid treatment extraction of FeMoco, a major effort was undertaken

to upscale the extraction process from 30 mg of starting material to 100 mg and ultimately to 1-5 grams. When it was initially upscaled, the yields from the extraction were very disappointing. This led to the careful examination of the methodology of the cofactor extraction. Several potential problem areas were examined.

When the column method was developed by Wink and coworkers, the purity of the starting MoFe protein compared to the total protein in solution ranged from 15-75% (Wink et al., 1989). On the 30 mg scale, the purity of the MoFe protein did not affect the extraction process. When larger quantities of inhomogenous solutions of MoFe protein are bound to columns, the column size quickly becomes unmanageable. This results in larger volumes of solvents, and more importantly larger volumes of salt solutions, which must be evaporated off to preserve stability. Use of larger volumes of salt solutions resulted in the cofactor being exposed to high salt concentrations for longer periods of time. It became difficult to completely remove the water from the column material, which also increased the probability of cofactor inactivation. The majority of these problems were minimized by incorporating a crystallization procedure in the purification of the MoFe protein (see Guide to Protein Purification in the Appendix 2).

When the MoFe protein is bound to the top of a pre-poured DE-52 column, there is uneven loading which creates a gradient of MoFe protein. The DEAE is designed so that the anionic protein will bind to the quaternary amine sites in a uniform manner. As the sites become filled in one level, the sites below should then be filled. This is not what happens in the experimental setting. The ionic strength and concentration of the protein solution, flow rate, temperature and other factors create a slight gradient of protein concentration biased toward the top of the column. When Wink and coworkers were designing the column method, the amount column material was increased by 10-25% as a safety margin. This presented the cofactor with a range of effective concentrations of unbound anionic sites during the various phases of extraction. When the process was upscaled, the effective removal of

the P clusters with the bipyridyl treatment was decreased as well as the overall extraction yields of the cofactor. The excess polycationic DE-52 introduced another complication. Even though the salt concentrations and volumes were high enough to displace the FeMoco from the column material, the additional interaction of the empty binding sites on the column material with the cofactor resulted in the trailings of FeMoco as it equilibrated with the various sites on the stationary phase.

Most of these difficulties were addressed by adopting a batch load method of adsorbing the crystalline MoFe protein to the DE-52. The first step was the determination of the minimum amount of DE-52 required to bind the desired quantities of MoFe protein. This was accomplished by titrating a solution of 100 mg of dilute MoFe protein with 0.4 g aliquots of Whatman DE-52 (figure 3.6). It can be concluded from the activity assays of this experiment that 4.0 grams of DEAE binds 100 mg of MoFe protein. With each addition of DEAE, the colour of the supernatant decreased. Upon addition of a total of 4.0 grams of the column material, the supernatant appeared to be colourless. The addition of DEAE to a stirring solution of MoFe protein in small aliquots until the supernatant becomes colourless is not an efficient way to bind the protein to the column matrix. A more efficient procedure is to have a given quantity of column material and bind MoFe protein to the DEAE, but there is a question as to how long should this procedure take. To find out how quickly the MoFe protein binds to DEAE, 4.5 grams of DE52 was suspended in reducing Tris, and a diluted solution of 100 mg of MoFe protein was added to it. The solution was stirred for a period of time and samples were taken. The results are in figure 3.7.

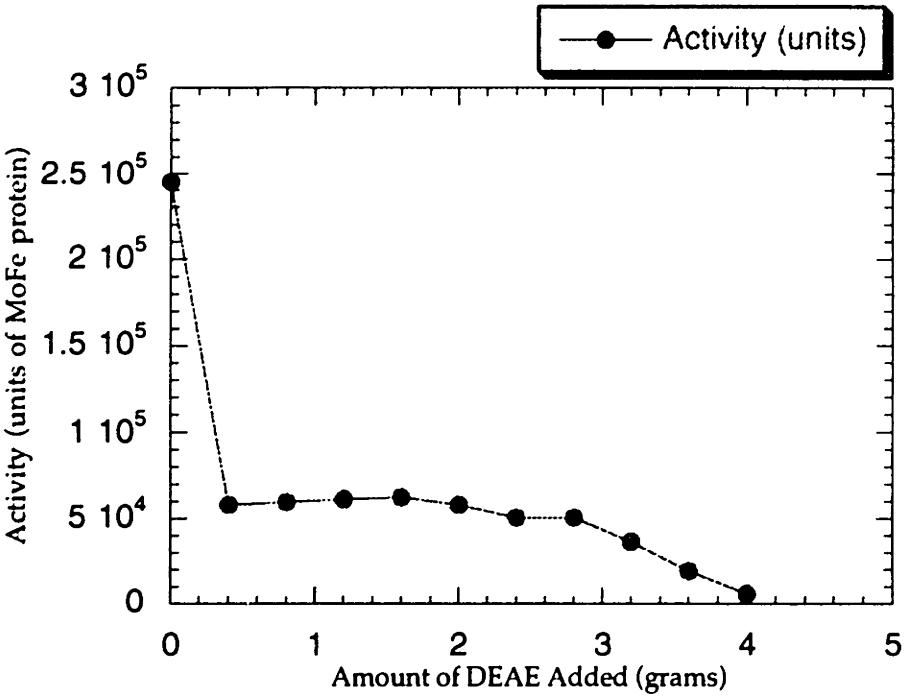


Figure 3.6: The titration of 100 mg of MoFe protein with DEAE.
After the initial addition of the slurry of DEAE, the solution was stirred for 5 minutes. The stirring was stopped and the DEAE was allowed to settle. The supernatant was then sampled for MoFe protein activity.

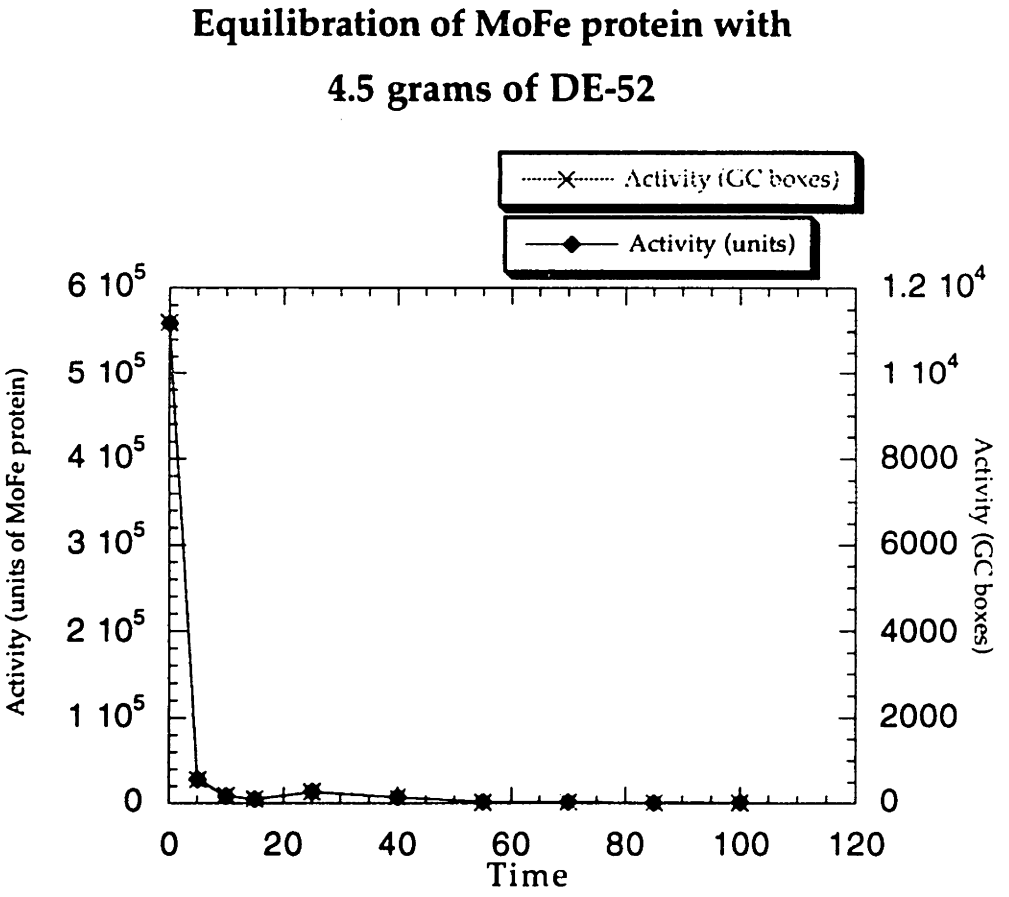


Figure 3.7: The equilibration of 100 mg of MoFe protein with 4.6 grams of DEAE.

To a stirred slurry of DE-52, 100 mg solution of MoFe protein was added. Over a period of 100 minutes, the supernatant was sampled for MoFe protein activity.

After 40 minutes, 88% of all of the MoFe protein was bound to the column matrix. The time points were stopped after 100 minutes upon which 99.9% of all MoFe protein activity was eliminated from the supernatant. Through batch loading, an even loading of MoFe protein was obtained within the column material.

MoFe protein is loaded onto the column matrix via a batch process occurring within 60 - 90 minutes. The next step in the extraction process is unfolding of the protein. Although a batch process was efficient for loading, it was determined that a batch unfolding of the protein was not easily accomplished. The column material becomes clumpy when exposed to organic solvents and efficient exchange was not possible. It also was very difficult to pour a column with material that was exchanged into an organic solvent, so if the column material is batch exchanged, the usage of a column for salt extraction was completely eliminated. The salt extraction of FeMoco using a batch process is in Table 3.3.

Table 3.3: Batch Extraction of FeMoco

Time of sample	Activity ($nmole C_2H_4$ produced.min^{-1}.mL^{-1})	Units of Activity
19	565.8	29,422
30	762.3	39,640
40	679.6	35,339
60	568.8	29,578
75	515.6	26,811

The solution was stirred for 75 minutes. Samples were being taken at the times indicated. The units of activity calculated represent the number of units that would be present if the extraction process was stopped at the given time. The units of activity are not additive. The instability of FeMoco in high salt solutions is evident from this experiment. The number of recoverable units is at its highest value after 30 minutes of contact with the salt solution, the values drop off quickly after that time point. Beginning with around 250,000 units of MoFe protein, batch extraction resulted in a maximum yield

of ~16%. This is obviously not the best way to extract FeMoco from MoFe protein. Based on these experiments, the best procedure for extracting the cofactor was determined to be a batch-loading method in combination with the previously detailed column method to unfold the protein and extract the cofactor. Because less DEAE was being utilized, the scale-up of this step was feasible.

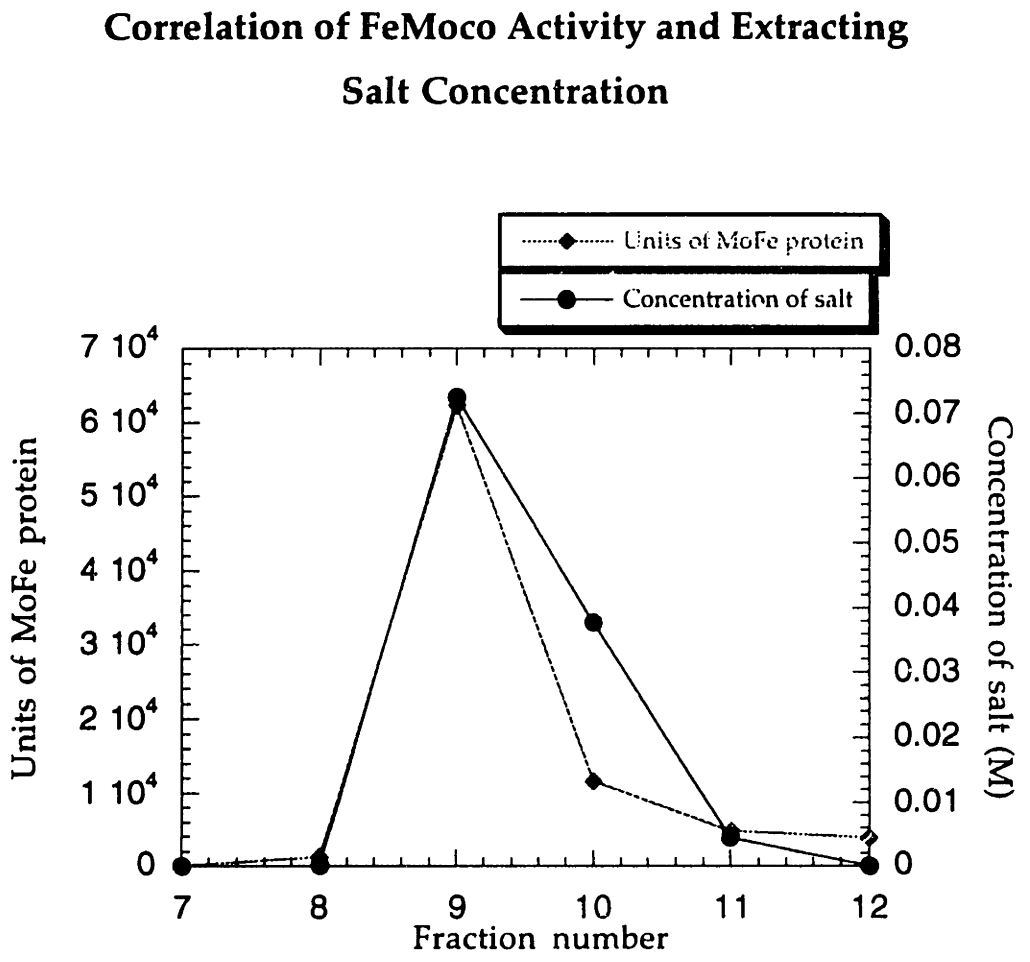


Figure 3.8: Comparison of FeMoco activity and concentration of $Ru(bpy)_3^{2+}$. The column was continuously eluted with 0.25 M $Ru(bpy)_3 Cl_2$ in NMF. The number of units of MoFe protein was determined for the fractions 7 through 12. For the same fractions, the concentration of $Ru(bpy)_3^{2+}$ was determined by the absorbance at 452nm.

Elution of FeMoco from DEAE

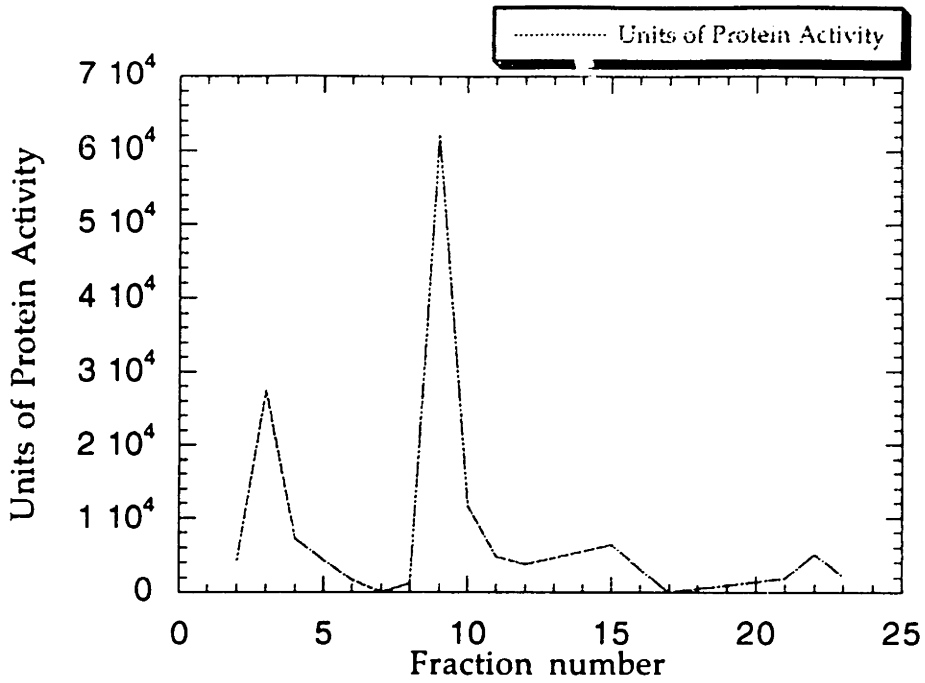


Figure 3.9: Elution of FeMoco from DEAE with continuous exposure to extracting salt.
After the column was exchanged into NMF, the column was eluted with 0.22 M $Ru(bpy)_3Cl_2$ with a intervening elution of NMF to wash off the salt. This was repeated a total of four times over a period of 2.5 hours.

In the original procedure, McLean and coworkers (McLean et al., 1989) used organic cations with a single charge. To show that the salt was extracting the cofactor, a solution of 0.25 M ruthenium tris(2,2'-bipyridyl)dichloride ($Rubpy_3Cl_2$) in NMF was used. $Rubpy_3^{2+}$ has an intense absorption at 452 nm due to a metal-to-ligand charge transfer transition. When the comparison of the units of MoFe protein activity to the concentration of the $Rubpy_3^{2+}$ was examined, it was determined that the two activities correlated. The fraction with the greatest activity of MoFe protein also contained the highest concentration of the extracting salt.

To examine the interaction of the extracting salt with the cofactor, a column was eluted with four aliquots of 0.22 M $Ru(bpy)_3Cl_2$ in NMF followed by NMF. The first volume of salt solution was 1.5 mL; the subsequent volumes were 3.0 mL. The results are shown in figure 3.9. There appeared to be four distinct peaks due to the salt solution initially interacting with the cofactor. The majority (75%) of the cofactor was within the first two peaks. This suggested that there is an equilibrium occurring in the column. Several probable scenarios included binding process between FeMoco and the MoFe protein active site, FeMoco and available anion binding sites on DE-52, FeMoco and random side chains of the unfolded MoFe protein or a combination of all three. Any of these factors could retard the elution of FeMoco from the column. The total contact time of the salt solution with the column was estimated to be 30-45 minutes and an equilibrium time of 30-60 minutes depending on the scale of the extraction has been incorporated into the procedure. For instance, when extracting FeMoco from 100 mg of MoFe protein, 30 minutes is sufficient time for the anionic component of the salt to displace the cofactor, but on the 1 gram scale, more time (60 minutes) is required. The yield from this procedure ranges from 70-100 %.

Cofactor can be stored in the glove box in the dry state. When the cofactor is required, the dry solid can be ground up into a powder and resuspended in half the original volume in which it was extracted. After filtration, approximately half of the contaminating salt is removed. After one trituration, the specific activity (nmole C_2H_4produced.min^{-1}.Mo^{-1}) increased from 121.3 to 207.8. It is also possible to remove the solvent, triturate and resuspend a number of times for a single preparation of FeMoco without loss of activity, which is demonstrated in the sequential production of isotopically labeled MoFe proteins in Chapter four of this thesis.

It was now possible to extract FeMoco in large enough quantities and yields to be able to produce the isotopically labeled hybrids of MoFe protein using the batch load-column method of extraction, leaving the cofactor in a dry, stable state at the end of the day.

IV. Summary

The optimal amount of DE-52 required to bind 100 mg of homogeneous MoFe protein is 4.0 grams. The binding of the protein to the DE-52 is an equilibrium process that takes about 60 minutes. The best way to extract FeMoco is to use a batch-load, column extract method. It can be demonstrated through the use of the spectroscopically active compound $Ru(bpy)_3Cl_2$ that the salt extracts the cofactor from the unfolded protein. The interaction of the salt and FeMoco is also an equilibrium process. The time required for the process to reach equilibrium varies from 30-60 minutes depending on the size of the column poured.

Chapter Four: Production of Isotopically Labeled MoFe Protein

I. Introduction

The mechanism of nitrogenase has not been completely described on a molecular level. Until the crystal structure provided a model for FeMoco and the P cluster, not even the elemental composition of these clusters had been completely agreed upon. It is the presence of these clusters which provides a spectroscopic tool to study the mechanism of nitrogenase.

The determination of R-homocitric acid as a component of the cofactor was the climax of years of research. In the examination of *K. pneumoniae nifV-* mutants, McLean and Dixon, (1981) found that these mutants produced a fully functional Fe protein and a MoFe protein that was able to reduce acetylene to ethylene but unable to reduce dinitrogen. Researchers up to this point had been using the acetylene reduction assay as indication of N_2-reducing ability. The ability of *nifV-* of *K. pneumoniae* to reduce acetylene but not dinitrogen invited further biochemical characterization (Table 4.1).

Table 4.1 Comparison of Wild type and nifV- phenotype.

Reactivity	Wild type Homocitrate	*nifV-* Citrate
Nitrogen Reduction	+	-
Acetylene Reduction	+	+
H/D Formation*	+	-
H_2 Evolution Inhibited by CO	-	+
Relative Efficiency for HCN Reduction	1	1.15

*$D_2(g) + H_2O(l) \rightarrow HD(g) + HOD(l)$

When the cofactor from the *nifV-* MoFe protein was extracted and inserted into wild-type apoMoFe protein, the reconstituted MoFe protein exhibited the **NIFV** phenotype (Hawkes et al., 1984). This data strongly suggested that FeMoco was the site of dinitrogen reduction in nitrogenase, and that a mutation in **NIFV** created an altered form of the cofactor.

As a result, the differences between the wild-type and *nifV*- cofactors were examined by a variety of spectroscopies. Using EPR, EXAFS and ENDOR spectroscopies, McLean and coworkers demonstrated that although the cofactor in *nifV*- MoFe is functionally different from wild type, the metal composition and EPR signals are the identical (McLean et al., 1987). The EXAFS data also did not indicate any differences in the local S and Fe environment of molybdenum. The only discernible differences were observed in ENDOR measurements in which a perturbation in the molybdenum site in the *nifV*- cofactor was observed. The molybdenum site of the wild-type FeMoco cluster had an appreciable quadrupole interaction, $P_3^{Mo} \sim 1.6$ MHz, and was of rhombic symmetry, $\eta \sim 1$ (Venters et al., 1986). The data for *nifV*- indicated that P_3^{Mo} was unchanged. Differences were observed in the ^{95}Mo coupling at g'_3 and g'_1. The wild-type A_3^{Mo} was 8.3 MHz and A_1^{Mo} was 4.65 MHz. The value for *nifV*- at A_3^{Mo} was 7.3 and at A_1^{Mo} was 5.15 MHz. The authors suggested that this indicated a change in non-sulfur ligands weakly bound at or near molybdenum (McLean et al., 1987).

At the same time, while attempting to purify the **NIFEN** protein complex for their research on the biosynthesis of FeMoco, Hoover and coworkers discovered that all FeMoco biosynthesis activity was lost during purification of the **NIFEN** on a gel filtration column (Hoover et al., 1986). The activity was restored when all of the fractions were recombined. The mystery factor was determined to have a molecular weight less than 500 by Amicon filtration and to be stable to heat, oxygen and a wide range of pH. By examining an exhaustive profile of *nif*- mutants, it was shown that the factor was present in the media only under derepressing conditions in those cells containing a functional *nifV* gene (Hoover et al., 1986).

The V factor was purified from fermentation media. NMR and high resolution mass spectrometry showed the metabolite to be homocitrate (2-hydroxy-1,2,4-butanetricarboxylic acid) (Hoover et al., 1987). The conformation of the tricarboxylic acid was determined through polarimetry to

be the R isomer. The only other known physiological role of this acid is as an intermediate in the biosynthesis of lysine in some yeasts and fungi. Homocitrate, the first intermediate in the lysine biosynthetic pathway, is formed by the condensation of acetyl-CoA with α-ketoglutarate. In these cases, it is the L-isomer rather than the R-isomer which is formed (Tucci and Ceci, 1972). Further analysis of derepressing cultures of *K. pneumoniae nifV*$^+$ showed that 80-90% of the total homocitrate produced was excreted into the medium to final levels of 0.4 mM (Hoover et al., 1988c). Hoover and coworkers were able to cure the *nifV*- phenotype by the addition of homocitric acid to derepressing cultures of a nifV- mutant (UN 1990). *nifV* was postulated to be a homocitrate synthase (Hoover et al., 1987).

Hoover and coworkers then attempted to show that homocitrate was a component of FeMoco (Hoover et al., 1989). Using their *in vitro* FeMoco biosynthesis system, they incorporated [^{3}H]homocitrate into FeMoco, and purified the reconstituted MoFe protein. The cofactor from this reconstituted MoFe protein was then extracted. Quantitation of the tritium to Mo showed 80% of the expected homocitrate was recovered. The radioactivity was associated with the fractions containing the cofactor activity. When the model of FeMoco was developed by Kim and Rees (1992) homocitrate was found to be coordinated through a hydroxyl group and a carboxylic acid group to the Mo in FeMoco.

It was still not known how the *nifV*- cofactor was different than the wild type FeMoco. When the organic acid was eliminated in the *in vitro* FeMoco biosynthesis system, only 5% of the wild type enzymatic activity was observed (Hoover and coworkers, 1988b). From acid denaturing experiments of *nifV*- MoFe protein, citrate was detected by NMR and a standard citrate lyase assay (Liang et al., 1990). When quantitation of the citrate to Mo was done, only 12% of the expected citrate was recovered. The authors suggested that much of the citrate was in the form of an Fe-citrate complex which

presented an unsuitable substrate to citrate lyase, thereby rationalizing the low yields.

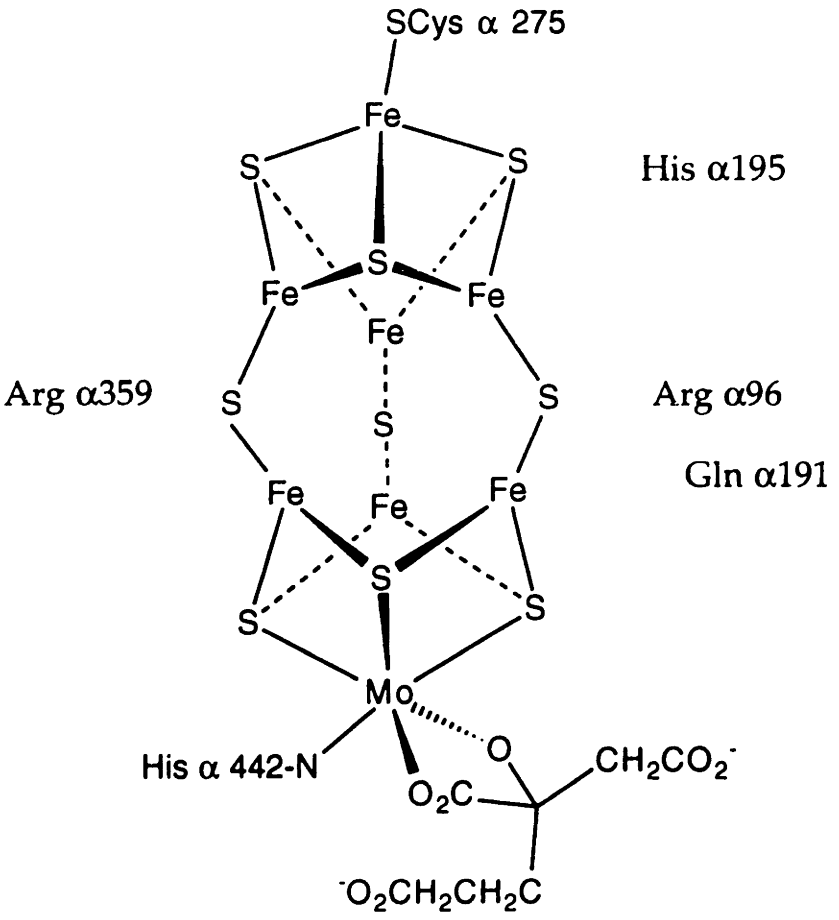
Using the *in vitro* biosynthesis system, Imperial and coworkers undertook an exhaustive study of the incorporation of organic acids into FeMoco (Imperial et al., 1989, Ludden et al., 1993). From the earlier publications of this study, it was shown that the *nifV*- biochemical characteristics can be induced upon the addition of citrate instead of homocitrate to the *in vitro* system (Hoover et al., 1988b). It is striking that the elimination of a methylene group in an organic acid coordinated to FeMoco can have such an effect on the catalytic properties of an enzyme.

Examination of the model of FeMoco by Kim and Rees (figure 4.1) shows some unique elements upon which spectroscopic handles can be incorporated.

The fact that homocitric acid is readily incorporated *in vivo* at a site near/on FeMoco provides an experimental ability to label the active site. As discussed above, homocitric acid can be added to the *K. pneumoniae nifV*- mutant during fermentation to restore wild type growth phenotype. If an isotopically enriched homocitric acid was incorporated, then a spectroscopic handle can be placed on the active site.

The metal ions present (Mo and Fe) can also be isotopically enriched. Isotopically labeling the sulphur in the active site of FeMoco has already been reported (Venters et al., 1986). Iron is present in both the P cluster and FeMoco. Mo is only found in the cofactor. The use of isotopic labels on Mo have been previously studied (for instance see Venters, 1986). One of the problems of using Mo labels to look at interactions is that the overall spin is either very delocalized or centered far enough away that Mo does not produce a large hyperfine interaction.

Structural Model for FeMoco as Proposed by Kim and Rees



The Iron Molybdenum Cofactor of Nitrogenase

Figure 4.1: The Kim and Rees Model of FeMoco.

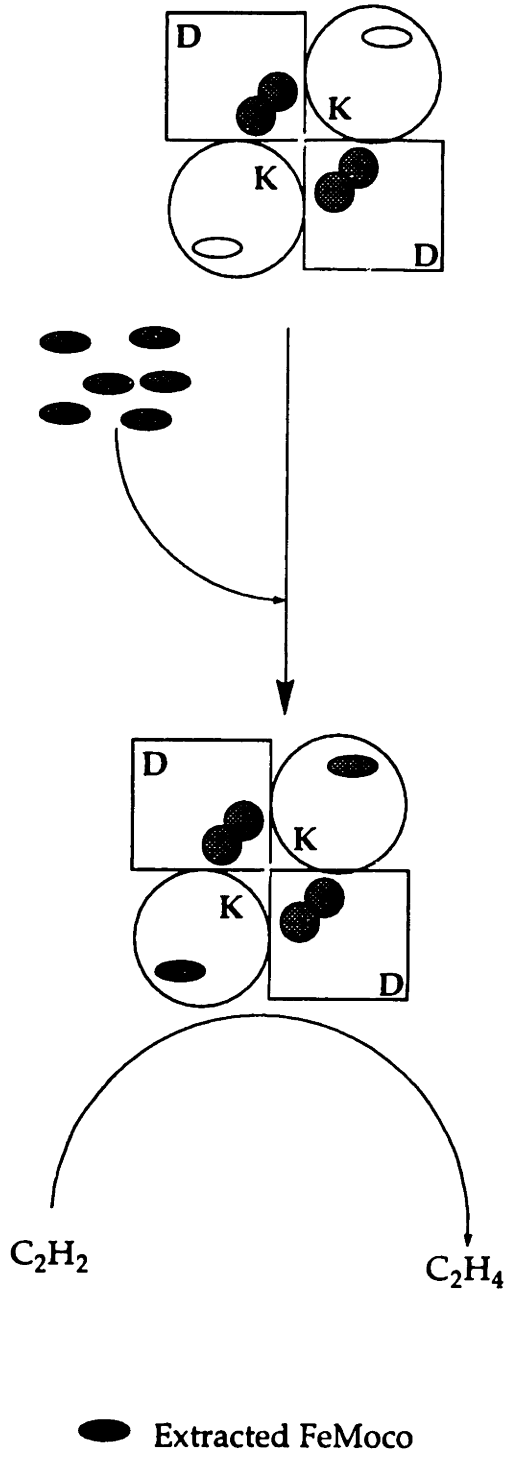


Figure 4.2: Assay for FeMoco Activity.
Extracted FeMoco is titrated to cell extracts of cofactorless mutants, usually *nifB*- mutants. The amount of reconstituted MoFe protein is then assayed in the *in vitro* acetylene reduction assay.

The method of labeling the P cluster and FeMoco with different isotopes of iron was pioneered by McLean and workers (McLean et al., 1987, McLean et al., 1988). Using the reconstitution assay for FeMoco (figure 4.2), apoMoFe grown on ^{57}Fe was titrated with cofactor extracted from MoFe protein purified from cells grown on ^{56}Fe. The first hybrids were made with apoMoFe protein from *K. pneumoniae* and FeMoco from *A. vinelandii*. The next logical step in the making of isotopically labeled hybrids would be to made a single species hybrid to ensure that there were not any observable interspecies differences. These isotopic hybrids of MoFe protein could be used to probe the mechanism of nitrogenase.

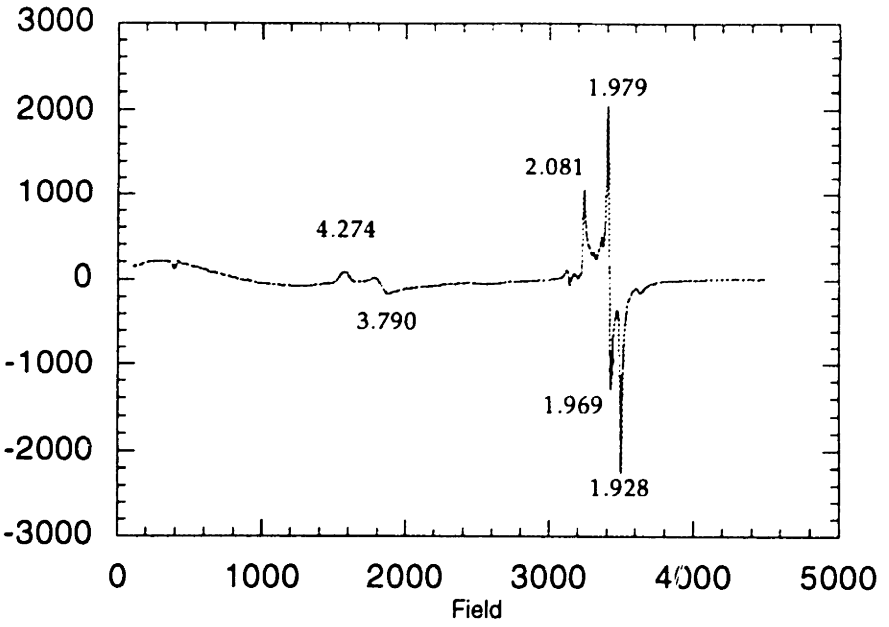
Carbon monoxide is a noncompetitive inhibitor of all substrates but not the evolution of H_2 with wild-type nitrogenase (Davis and Wang, 1980; Hwang et al., 1973; Rivera-Ortiz and Burris, 1975). Under CO-inhibited conditions, neither reductant utilization (Ljones, 1973) nor ATP hydrolysis (Hardy et al., 1965; Hwang et al., 1973; Davis and Wang, 1980) is affected and CO itself is not reduced (Ljones, 1973). What is not known is where on the enzyme CO binds. Using a dithionite reduction assay, Davis and coworkers (1973) did not observe any differences with either 0.9 atm N_2 plus 0.1 atm CO or 0.9 atm of Ar plus 0.1 atm of CO in the gas phase. Davis and coworkers concluded that CO interrupted the electron flow on the site at which N_2 is reduced, while the site which H_2 is evolved from remains unaffected. They also suggest that the site to which CO binds could be the N_2 binding site if CO binds to the enzyme in a different redox state than that which binds N_2. When both the Fe and MoFe proteins in an ATP-regenerating solution are allowed to turn over under CO, new signals are observed which have been attributed to CO binding (figure 4.3).

When MoFe protein is allowed to turnover under low CO concentrations, usually when there is less than 0.1 atm CO present, the signal has an average g value of less than 2.0 and is similar to an iron-sulfur cluster

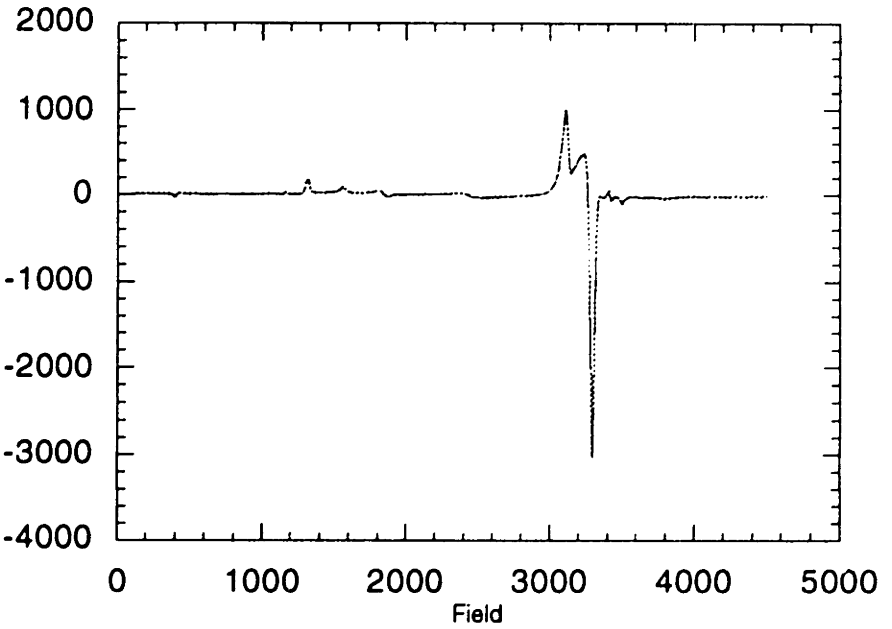
Figure 4.3: EPR Spectra of the signals of the CO-inhibited state of the MoFe protein of nitrogenase.

The EPR conditions are Center Field (CF) = 2300 G, Sweep Width (SW)= 4400 G, Modulation Frequency (MF) = 100 KHz, Modulation Amplitude (MA) = 6.44 G, Receiver Gain (RG) = 1.0×10^4, Sweep time (ST)= 335.54 seconds, Time Constant (TC) = 163.84 msec, microwave power = 10 mW, Temperature = 5 K. (A) the protein concentration is 50 mg/mL of MoFe protein and the ratio of MoFe protein to Fe protein is 2:1. The concentration of CO is 0.08 atm. (B) the protein concentration is 43 mg/mL of MoFe protein and the ratio of MoFe protein to Fe protein is 2:1. The ATP-regenerating solution is described in the materials and methods.

A. Example of a Low CO Signal from MoFe protein



B. Example of a High CO Signal from MoFe protein



in the same oxidation state as a reduced ferredoxin. The high CO signal, usually when there is about 0.5 atm CO present, has a g average above 2, and is resembles the signal from the oxidized from of the high-potential iron-sulfur proteins (Davis et al., 1979). Lowe and coworkers examined the EPR signal intensity as a function of substrates and various concentrations of inhibitors, designate the signal formed under low pressures of CO as a tight CO binding site and the signal formed under high pressures of CO as a weak CO binding site (Lowe et al., 1978).

Through ^{57}Fe labeling of the MoFe and Fe proteins and turning them over under CO, it was shown that the EPR signal was associated with an iron containing center of the MoFe protein (Davis et al., 1979). MoFe protein has two iron containing clusters, the P cluster or FeMoco. It is through the use of the isotopically labeled hybrids of the MoFe protein that it will be determined to which of these two clusters does the CO bind. ENDOR results suggest the low CO-inhibited MoFe protein has one CO molecule bound at the metal center and that the high CO-inhibitor MoFe protein has at least one and probably two CO molecules bound at the metal center (Pollock et al., 1995). It is currently thought that the high and low CO-inhibited MoFe protein signals arise from a single cluster and that under high pressures of CO, the CO molecule bound to the low CO form is kinetically labile in the high CO form (Pollock et al., 1995).

II. Materials and Methods

A. Homocitric acid Incorporation Experiments

The growth of cells and incorporation of homocitric acid have been previously described in Chapter two materials and methods. MoFe proteins from UN, UN 1990, UN1990 + homocitric acid and UN 1990 + deuterated homocitric acid cells were purified according to Burgess et al., (1980). The samples of purified MoFe protein were concentrated to ~50 mg/mL and their

EPR spectra taken at X-band wavelengths. The samples were then transferred to a Q-band EPR tube in which they were frozen.

Spectroscopic Measurements and Analysis

EPR Experiments: EPR experiments were performed on a Bruker ESP 310 spectrometer fitted with a variable temperature Oxford Cryostat. Samples were prepared in 4 mm OD quartz tubes and frozen in liquid nitrogen or isopentane baths. The specific conditions of each spectrum are provided in the relevant figure captions.

ESEEM (Electron Spin Echo Envelope Modulation) Spectroscopy: ^{1}H and ^{14}N ESEEM were performed on the isolated MoFe proteins from the homocitric acid incorporation experiments. Samples included *K. pneumoniae* MoFe wild type, MoFe V-, MoFe V- + ^{1}H-homocitrate and ^{2}H-homocitrate. The ^{2}H label was in the methylene arm of homocitrate. All samples were ~50 mg/mL with a minimum specific activity of 2200 units/mg.

ESEEM was performed at Northwestern University on a locally constructed spectrometer by Dr. Vickie DeRose. Experimental conditions were similar to those employed by Thomann and coworkers (Thomann et al., 1991) in their examination of whole cell of *A. vinelandii*. For three-pulse ESEEM (figure 4.4), the time-domain ESEEM waveforms were collected by recording the stimulated electron spin echo intensity, S(T, t), as a function of the evolution time, T, at a fixed time, τ, after the third microwave pulse. The pulse sequence was $\pi/2$-τ-$\pi/2$-T-$\pi/2$-τ-S(T,t). The stimulated echo decay function was digitally filtered and then Fourier transformed. The spectra were phase corrected to compensate for the "ringdown" time of the spectrometer. All of the ESEEM waveforms were recorded under identical experimental conditions at $g = 3.770 \pm 0.001$ in the EPR spectrum. The temperature was 2 K, the pulse sequence repetition rate was 500 MHz, and the τ was 132 ns.

B. Production of Isotopically Labeled MoFe Protein

All glassware was acid washed. Metal-Free pipette tips and sterile plastic pipettes were used when volumes were less than 50 mL. For larger volumes, acid washed and sterilized graduated cylinders were used. The source of the water for all these experiments was a Millipore Water filtration system. The 20L Chemap fermenter was washed with a 1% HNO_3 solution for at least 1 hour, rinsed with water at least twice and allowed to stir overnight full of water. The dissolved oxygen probe was placed in the fermenter after the nitric acid wash. The pH probe was nitric acid washed. The next day, the water was drained, the fermenter rinsed twice and then a sterilization loop was run with only water in the fermenter. After rinsing twice with water, the water filled fermenter was stirred overnight. The fermenter was now ready for use. The iron isotopes were purchased from US Services Inc. The iron stock solution was made by dissolving 100 mg of enriched ^{57}Fe or de-enriched ^{56}Fe in the oxide form in 17.4 mL of concentrated hydrochloric acid. Solid citric acid was added so that the final concentration was 0.1 M citric acid. The final iron concentration was 0.1 M.

A. vinelandii OP and UW 45 were grown under the optimized conditions discussed in Chapter two. The MoFe protein was purified according to the methods in the Appendix B entitled "Guide to Protein Purification". It was not necessary to crystallize every preparation of MoFe protein because of the small quantities of FeMoco extracted. FeMoco was extracted following the Batch-Column method described in chapter three. Each batch of UW 45 was titrated separately. Using the extracted cofactor, 1 gram of cell paste from each batch was titrated to determine the volume of cofactor required to titrate the entire batch.

Each batch of UW45 was diluted 1:4 with reducing 0.1 M Tris, pH 8.0, 10 mM NaS_2O_4 and allowed to thaw while stirring in a 4°C room. The diluted cell paste was disrupted anaerobically in 35 mL aliquots in a French Pressure Cell operating at 15,000 - 20,000 psi. The cell-free extract was collected

anaerobically in a round bottom flask containing 0.2 mM PMSF, 1.9 μg/mL leupeptin and 3.5 μg/mL pepstain.

After bringing the extract into the anaerobic glove box, the extract was diluted an additional four fold with reducing 25 mM Tris (25 mM Tris with 2 mM $Na_2S_2O_4$). The required volume of triturated cofactor solution was added while the extract was stirred. After all the cofactor solution was added, the cell extract solution was stirred for 15 minutes. The extract was then transferred to centrifuge bottles and the bottles were closed with o-ring containing stoppers and spun at 4080 g X 5.5 hours. After centrifugation, the centrifuge bottles were brought back into the anaerobic glove box and the supernatant was decanted into a reservoir. The supernatant was then pumped using a Masterflex pump system onto an prepared anaerobic DEAE column. The column was washed with at least 2 column volumes of 0.07 M NaCl in reducing Tris (25 mM Tris, 2 mM $Na_2S_2O_4$) and then the hybrid MoFe and Fe proteins were eluted with a 0.10 - 0.50 NaCl in reducing Tris salt gradient. Fractions containing MoFe protein activity were pooled and concentrated 10 fold. The concentrated fraction was then frozen in liquid nitrogen and stored in liquid nitrogen. This procedure was repeated until all of the apoMoFe protein had been titrated with FeMoco.

The similar hybrid samples were pooled and further purified by additional DEAE columns, Source 15Q columns and S-200 HR gel filtration columns. After each column, the fractions were characterized by MoFe activity assays, and precipitated Lowry assays. When the samples showed a pure EPR spectrum, the purification was halted.

C. Production of the CO-inhibited State of MoFe protein

To produce the CO-inhibited state of the MoFe protein, it must be allowed to turnover in the presence of CO. This is accomplished by placing the MoFe protein into a concentrated ATP-regenerating system. MoFe protein is exchanged into a solution containing MgATP and a MgATP-

regenerating system. The composition of the turnover mix is described in table 4.1. On ice, the first solution of $MgCl_2$, Na_2ATP, sodium phosphocreatin and HEPES (solution A) are diluted to the required volume. The pH is adjusted to 7.4 with NaOH and the solution is then degassed on a Schlenck line. The required weights of sodium dithionite (solution B) and creatine kinase (solution C) were degassed in separate vials. When the solution A was degassed, it was cannulated anaerobically into the vial containing the sodium dithionite. When the solid was dissolved, the solution B was cannulated into the vial containing the creatine kinase (solution C). This completed the turnover mix.

Table 4.1: Turnover Mix

$MgCl_2$	50 mM
Na_2ATP	100 mM
Naphosphocreatine	300 mM
HEPES	100 mM
$Na_2S_2O_4$	100 mM
creatine kinase	2 mg/mL

Once the MoFe protein was diluted with the turnover mix six fold, the protein solution was once again concentrated to its original volume.

Using a capillary monometer to measure the final pressure, the CO was distributed outside of the glove box into 4.5 mL Wheaten vials. Once in the glove box, the CO was scrubbed by injecting 1.0 mL of a 0.1 M sodium dithionite solution. After stirring for 5 minutes, the dithionite solution was removed and checked against methyl violagen paper for reducing equivalents. If it was not reducing, the scrubbing procedure was repeated.

Inside the glove box, the MoFe protein in turnover mix was added to the vial with the scrubbed CO and allowed to equilibrate for at least thirty minutes. The production of the CO-inhibited state was initiated by the addition of the Fe protein so that the ratio of Fe protein to MoFe protein was 1:2. The mixture was allowed to turnover for the required period of time. At

the proper intervals, 100 µl samples were taken out of the vial and placed into a Q band EPR tube. The Q-band EPR tube was placed inside of an X-band EPR tube immersed in an isopentane bath chilled with blowoff gas from LN_2 (temperature ranged from -30 to -70 °C). The samples were removed from the glove box and stored in plastic labeled tubes under liquid nitrogen. The EPR spectroscopy was performed as previously described.

The isotopically labeled MoFe proteins were exchanged into turnover mix using an Amicon Microcon 100. Because of volume limitations, the hybrids were exchanged into turnover mix by a 1:2 dilution and then a 1:3 dilution. The iron protein used to produce the CO-inhibited state was also diluted into turnover mix. A 100 mg/mL Fe protein solution was diluted tenfold and frozen in 100 µL aliquots. For each hybrid sample, one of these aliquots was thawed and used. The presence of the CO-inhibited state in each sample was confirmed by EPR spectroscopy.

ENDOR Spectroscopy: The ENDOR Spectroscopy was performed by Dr. Hong In Lee at Northwestern University. The ^{57}Fe ENDOR data was collected on a Varian Associated E-109 EPR spectrometer equipped with a E-110 35 GHz microwave bridge using 100 KHz field modulation as described elsewhere (Werst et al., 1991). For a single oriented paramagnetic center coupled with a Fe (I=1/2) nucleus, a doublet of ENDOR transitions ($V\pm$) are given by;

$$v(+\ -) = |\, v(^{57}Fe) + -A(^{57}Fe)/2 \,|$$

Here, $v(^{57}Fe)$ are the Larmor frequency of ^{57}Fe and the orientation dependent hyperfine coupling constant, respectively. In case of $A(^{57}Fe)/2 > v(^{57}Fe)$ which is valid for ^{57}Fe hyperfine coupling in CO-MoFe protein, the doublet is centered at $A(^{57}Fe/2)$ and split by $2v(^{57}Fe)$.

The experimental conditions are as follows: microwave frequency, ~ 35 GHz; modulation amplitude , 67G; receiver frequency (rf) power, 30 W, scan speed, 1 MHz/sec; time constant, 32 ms; temperature, 2 K; g values for hi-CO,

2.169, for lo-CO 1.931. All data was collected using 100 KHz rf modulation frequency (Hoffman et al., 1994)

III. Results

A. Rescue and Label Incorporation Experiments of NifV- Strains of K. pneumoniae with Homocitric Acid.

The proteins from these fermentation experiments were purified according to standard nitrogenase purification techniques (see Appendix B for complete description). After two anion exchange columns, the proteins were concentrated separately (Table 4.2) and their EPR spectra were examined for impurities (figure 4.4).

Table 4.2: Concentration of MoFe proteins

MoFe protein Source	Concentration
UN	52 mg/mL
UN 1990	48 mg/mL
UN 1990 + ^{1}H-homocitric acid	50 mg/mL
UN 1990 + ^{2}H-homocitric acid	48 mg/mL

ESEEM Studies. The time domain stimulated echo ESEEM waveforms are shown in figure 4.5 for MoFe wild type, MoFe from UN 1990 + ^{1}H-homocitric acid, and MoFe from UN 1990 + ^{2}H-homocitric acid. The waveforms appear to be qualitatively identical. This is confirmed by examining the Fourier transform of the waveforms which produce the ^{14}N ESEEM spectra shown in figure 4.6. Figure 4.7 shows a second set of time domain simulated echo ESEEM waveforms for MoFe wild type and the MoFe UN 1990 and their Fourier transformed ESEEM spectra (figure 4.8).

Comparison of *K. pneumoniae* MoFe protein of wild type protein and *nifV*- with deuterated homocitric acid incorporated.

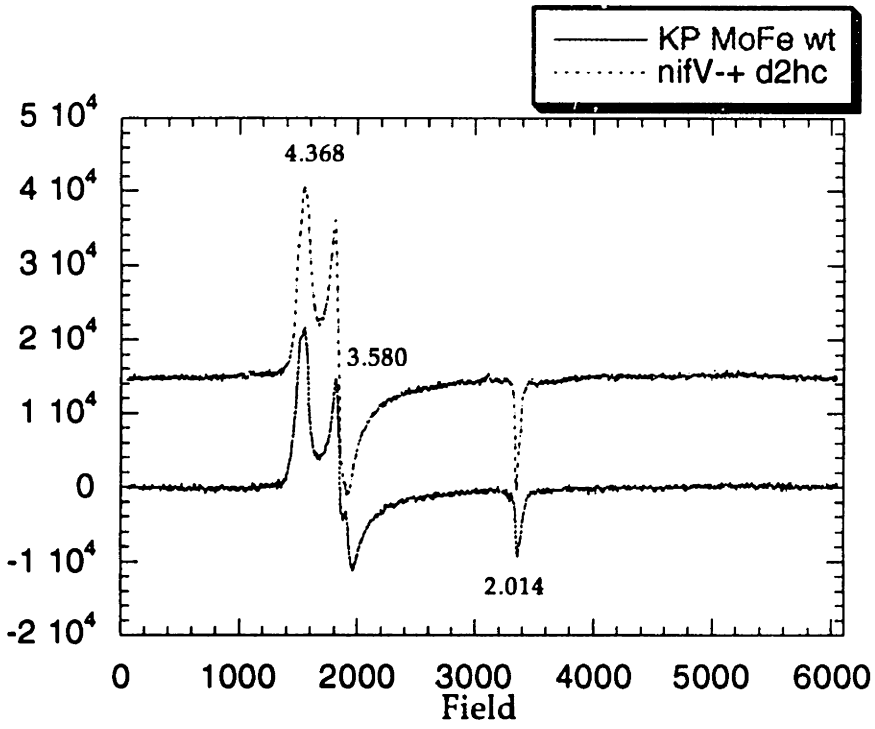


Figure 4.4: EPR Spectra of *K. pneumoniae* wild type MoFe protein and *nifV*- with deuterated homocitric acid incorporated.

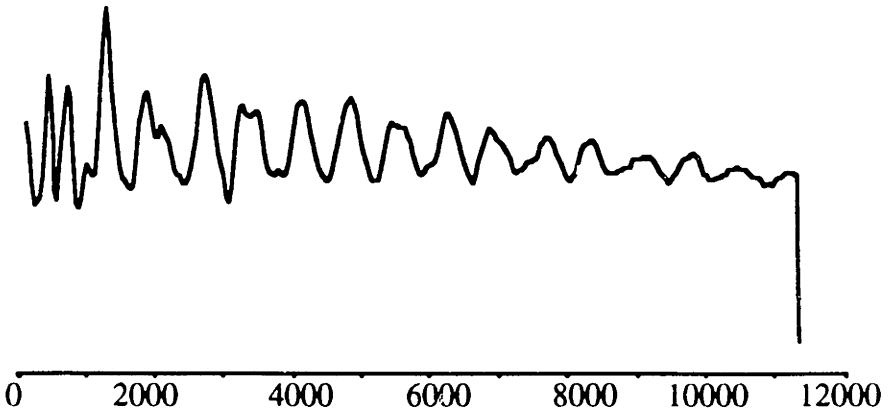
The EPR conditions are CF = 3050, SW = 6000 G, microwave frequency = 9.44 GHz, MF = 100 KHz, MA = 1.05 G, TC = 1.28 msec, SW = 671 sec, microwave power is 10 mW, temp = 5.0 K

Figure 4.5: Time Domain Stimulated ESEEM Waveforms.

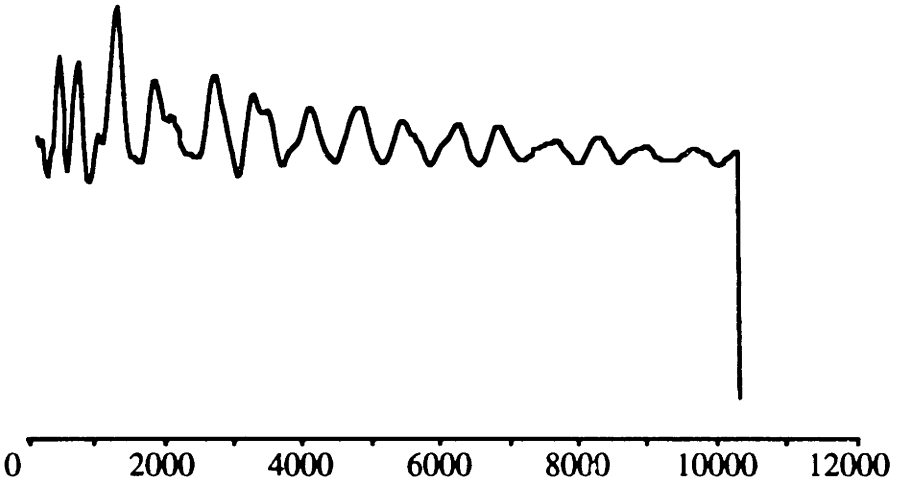
A. Purified MoFe wild type protein from UN, 52 mg/mL
B. Purified MoFe from UN 1990 + ^{1}H-homocitric acid, 50 mg/mL
C. Purified MoFe from UN 1990 + ^{2}H-homocitric acid, 48 mg/mL

Experimental conditions: Temp = 2 K; Frequency 9.449 GHz; Field 1800 G; τ 132 ns; number of scans 4-15. All time-domain ESEEM is scaled to show echo height.

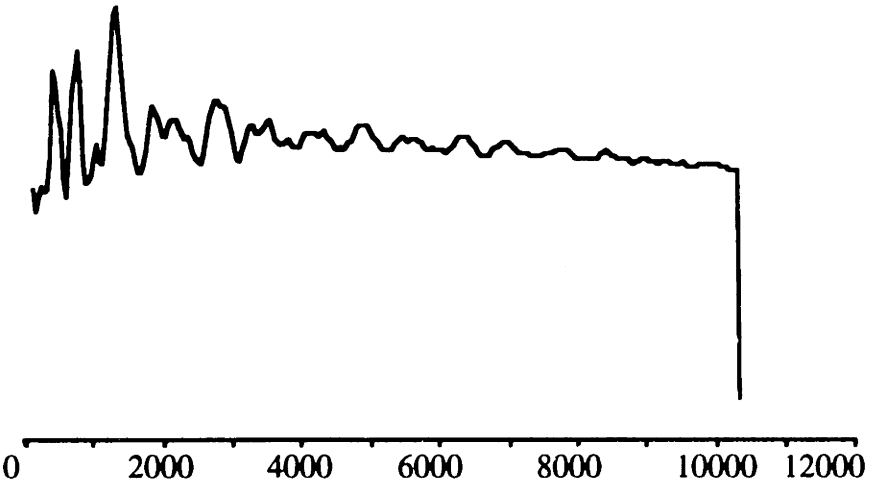
A. WT



B. V- + ^{1}H-homocitrate



C. V- + ^{2}H-homocitrate

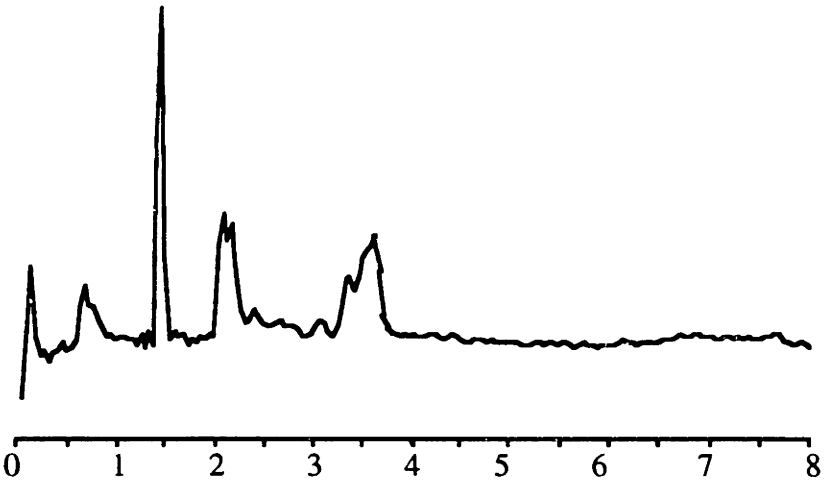


T + τ (ns)

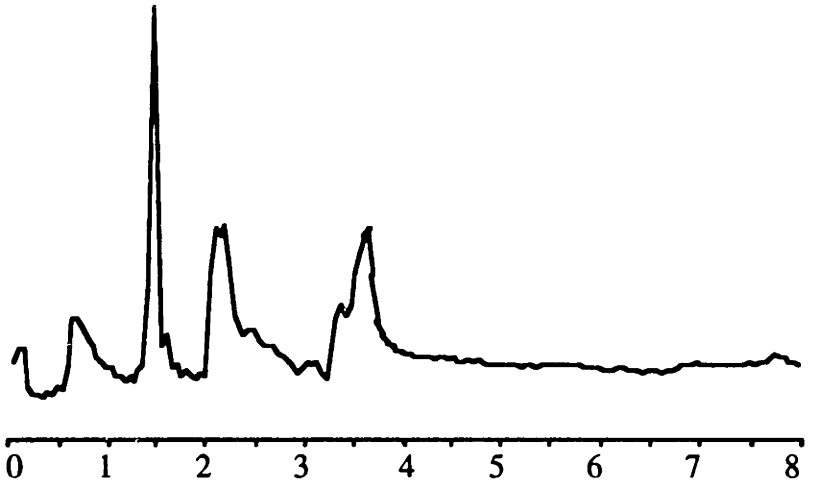
Figure 4.6. ESEEM Spectra.

A. Purified MoFe wild type protein from UN, 52 mg/mL
B. Purified MoFe from UN 1990 + ^{1}H-homocitric acid, 50 mg/mL
C. Purified MoFe from UN 1990 + ^{2}H-homocitric acid, 48 mg/mL
The ESEEM spectra are the cosine Fourier transforms of the corresponding time-domain spectra in figure 4.5.

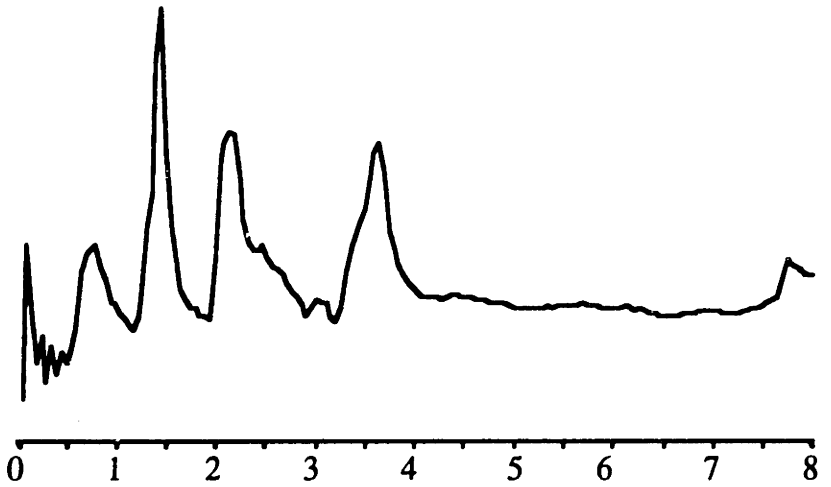
A. WT



B. V- + ^{1}H-homocitrate



C. V- + ^{2}H-homocitrate



Frequency (MHz)

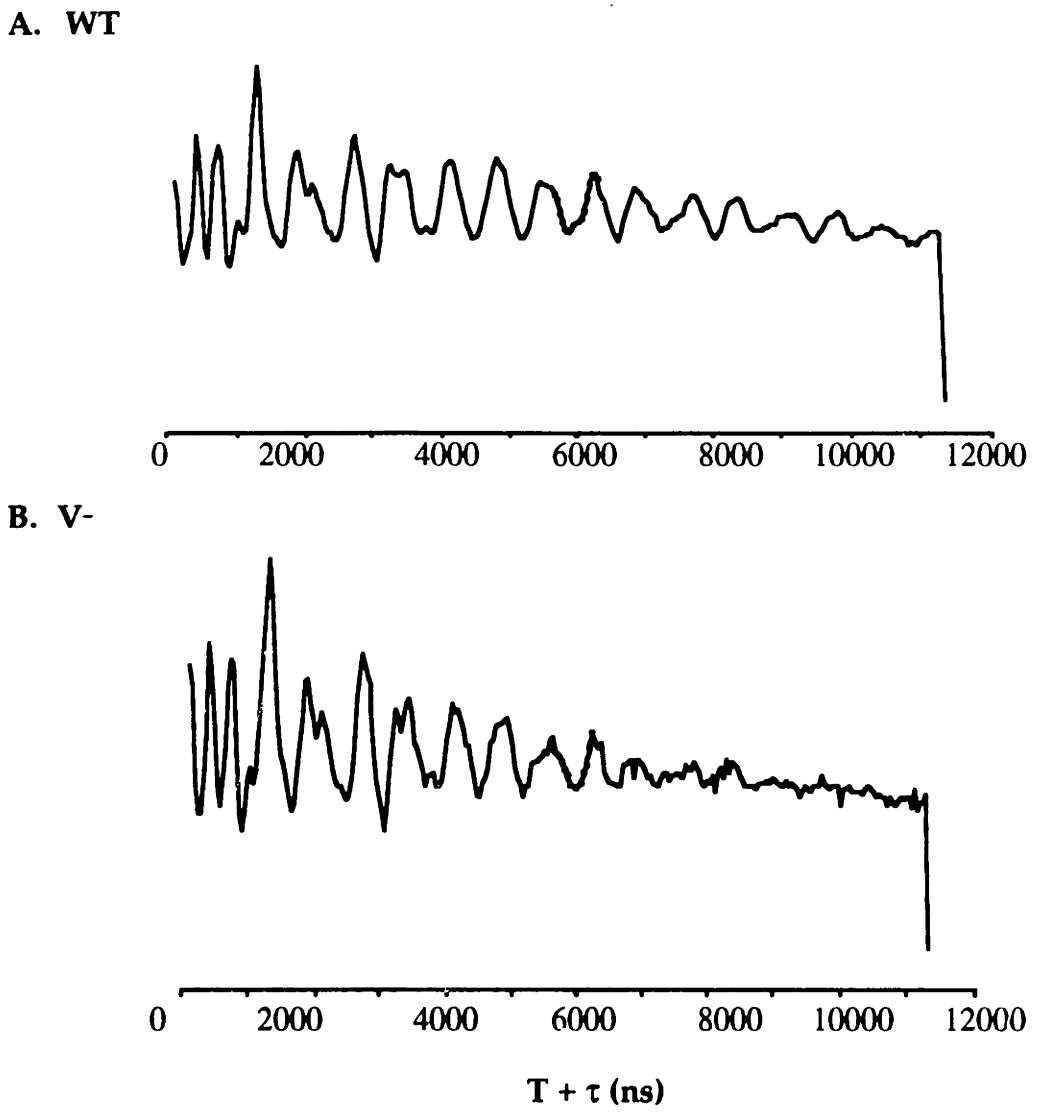


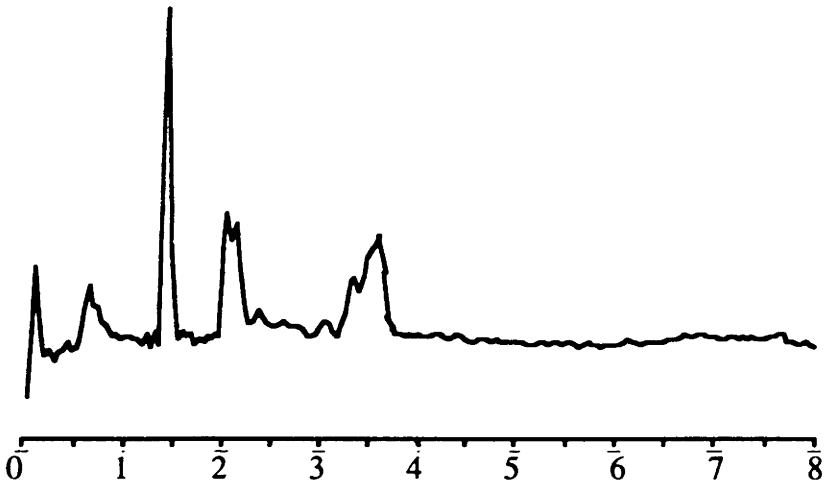
Figure 4.7. Time Domain Stimulated ESEEM Waveforms

A. Purified MoFe wild type protein from UN, 52 mg/mL

B. Purified MoFe *nifV*- protein from UN 1990, 48 mg/mL

Experimental conditions: Temp = 2 K; Frequency 9.449 GHz; Field 1800 G; τ 132 ns; number of scans 4-15. All time-domain ESEEM is scaled to show echo height.

A. WT



B. V-

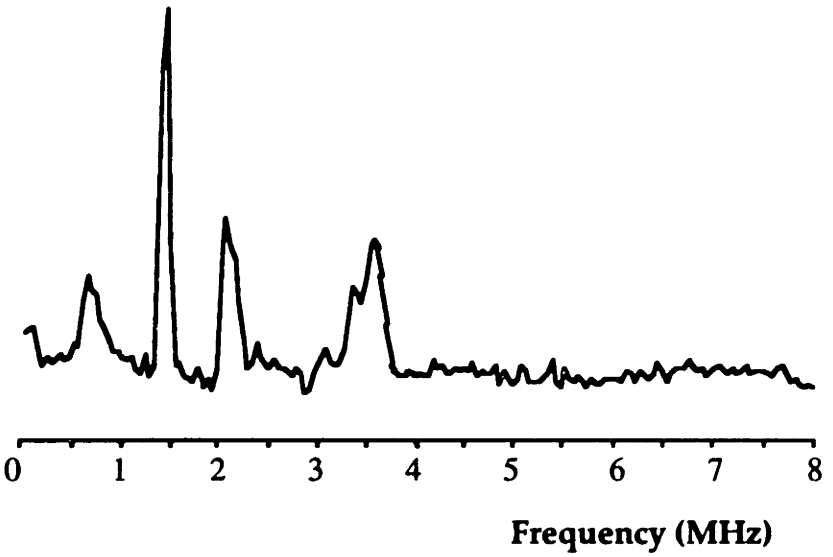


Figure 4.8. ESEEM Spectra.

A. Purified MoFe wild type protein from UN, 52 mg/mL
B. Purified MoFe *nifV-* protein from UN 1990, 48 mg/mL
The ESEEM spectra are the cosine Fourier transforms of the corresponding time-domain spectra in figure 4.7.

These spectra are analyzed assuming the so-called "exact cancellation" conditions. Such conditions arise in cases where the electron nuclear interactions contain a Fermi contact term of the same magnitude as the nuclear Zeeman term. The Fermi contact term creates an internal local magnetic field which can enhance or reduce the applied magnetic field depending on the spin state of the electron. When the magnetic fields are exactly canceled in the electron spin manifold, the pure nuclear quadrupole resonance, ν_0, ν_-, ν_+, and the double quantum transition are the observed ESEEM peaks. The magnitude of the nuclear quadrupole parameter, $K = e^2qQ/4h$, and the asymmetry parameter, η, can be calculated directly from the ESEEM. Since the nuclear quadrupole is determined by the electric field gradient, which in turn is defined by the total electron density, which itself is a function of the local geometry and electronegativity of the bonded atoms, under exact cancellation conditions, the observed ESEEM frequencies are akin the chemical shift of NMR.

The observed frequencies of the peaks are not only related to:

$$K = e^2qQ/4h$$

but are also related to the nuclear Zeeman and hyperfine coupling A by:

$$\nu n = g_n\beta_n H/h$$

Under the exact cancellation conditions, this simplifies to:

$$\nu_0 = (2n)K = \text{peak } 1$$

$$\nu_- = (3\text{-}n)K = \text{peak } 2$$

$$\nu_+ = (3 + n)K = \text{peak } 3$$

$$\nu dq \text{ or } (\nu_- \text{ or } \nu_+) = 2[(\nu + A/2)^2 = K^2(3 + n^2)]^{1/2}$$

At $g = 3.77$, the observed frequencies of the ESEEM peaks were 0.8, 1.4, 2.2 and 3.6 MHz with $\nu_n(^{14}N) = 0.55$ MHz. solving the above equations, $K = 0.6$ which corresponds to $e^2qQ/4h = 2.4$ MHz and an asymmetry parameter, η=0.67. Using these parameters, the hyperfine coupling constant A = 1.72 MHz.

B. Production of Isotopically Labeled MoFe proteins.

The nomenclature that is used to describe the isotopically labeled MoFe proteins refers to the isotope of iron used in the production of each cluster. For instance, M56P57 indicates that the cofactor was extracted from cells grown on ^{56}Fe and the apoMoFe protein with its incorporated P clusters was grown on ^{57}Fe. The hybrid M57P56 have the isotopes switched between the cofactor and the P cluster. MoFe57 indicates that both the cofactor and the P cluster was labeled with ^{57}Fe and this protein was isolated from *A. vinelandii* cells grown on ^{57}Fe.

In the initial hybrid production experiment, multiple batches of UW 45 were used as the source of apoMoFe protein. Because the production of hybrid protein represented a yield of only 50% after the first anion exchange column, subsequent batches of UW 45 were titrated individually with FeMoco as they were produced. All of the similar hybrids were pooled and purified on an additional DE52 column. The results are in table 4.3. The first pooled fraction, designated as I, contained the middle peak fractions of the anion exchange column. The II fraction contained the pooled side fraction and as such contained more impurities.

Table 4.3: Hybrid Protein Summary after the Second DE 52 Column

Hybrid Protein	mg/mL MoFe	% MoFe
M56P57 - I	4.09	2.90%
- II	0.11	0.37%
M57P56 - I	6.40	3.50%
- II	0.52	0.75%

Table 4.4: Hybrid Protein Summary

Column used	M56P57	M57P56
	% of total protein	% of total protein
DE-52	2.90	3.50
Source 15Q	7.92	7.87
S-200 HR	10.17	14.59

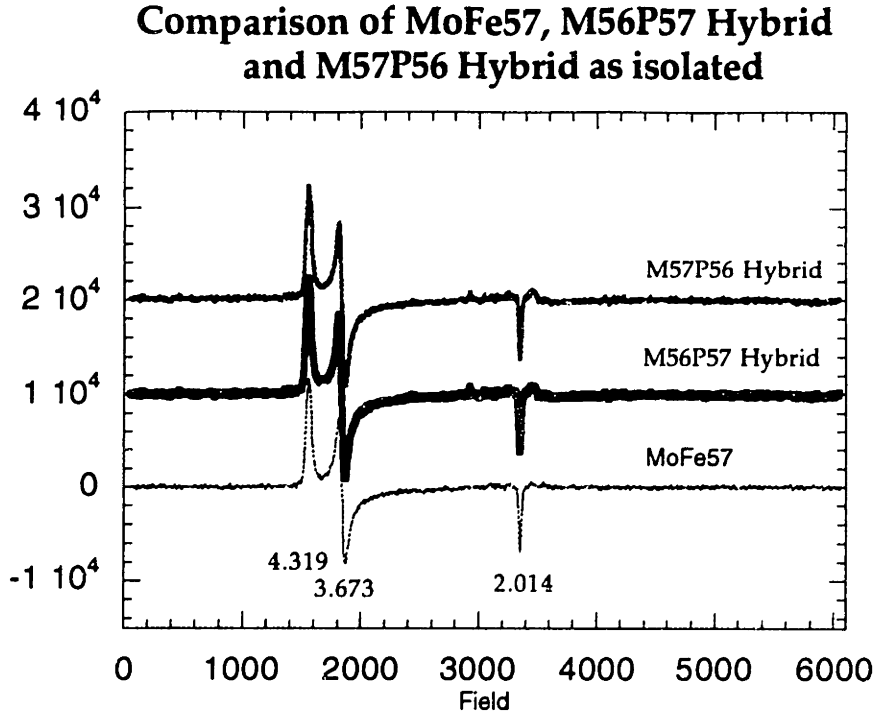


Figure 4.9: Comparison of MoFe57, M56P57 Hybrid and M57P56 Hybrid as isolated.

EPR conditions as follows: Temp = 5 K, CF = 3050 G, SW = 6000 G, RG = 6.30 X 10^5 , MF = 100 KHz, MA = 1.05 G TC = 1.28 msec, ST = 671 sec, Freq. = 9.44 GHz.

The hybrid proteins with the highest purity (M56P57 -I and M57P56 - I) were further purified using a HR 16/10 column filled with Source 15 Q from Pharmacia, and a 2.5 x 43 cm S-200 HR column (Table 4.4)

The proteins were then checked for EPR purity (figure 4.9). The high g region is examined in figure 4.10 and the g=2 region is examined in figure 4.11. Even though the hybrids represent less than 15% of the total protein in solution, they appear to be free of EPR active contaminants. Once purity had been verified, these samples were used for the CO inhibition experiments. The hyperfine broadening present in MoFe protein is only 20-40 gauss and since the EPR signals are very broad to begin with, it is difficult to observe the hyperfine broadening due to the ^{57}Fe isotopic enrichment. The hyperfine

broadening is also a function of the number of irons present and since the EPR signal is due to the presence of 7 irons in the cofactor, each with a different hyperfine contribution (Venters et al., 1986), this also contributes to the difficulty. There do appear to be slight differences in the EPR signals between the M56P57 hybrid and the MoFe57 and M57P56 hybrid seen in the expansion of the high field (figure 4.10) and the low field. It is easier to determine the hyperfine values using ENDOR spectroscopy.

C. CO inhibited State of the MoFe protein.

Before the isotopically enriched proteins could be CO-inhibited, characterization of the system used to produce the CO inhibited state of MoFe protein was required. The first set of experiments involved the characterization of the system using homogenous MoFe protein at 13.5 mg/mL, 26 mg/mL, and 50 mg/mL. The samples were all frozen in Q-band tube inserts within X-band tubes. At each of these concentrations, time course experiments were undertaken to determine the time required for the production of the various CO-inhibited state. The results for the 50 mg/mL MoFe protein under low and high concentrations of CO are in figure 4.12 and 4.13, respectively.

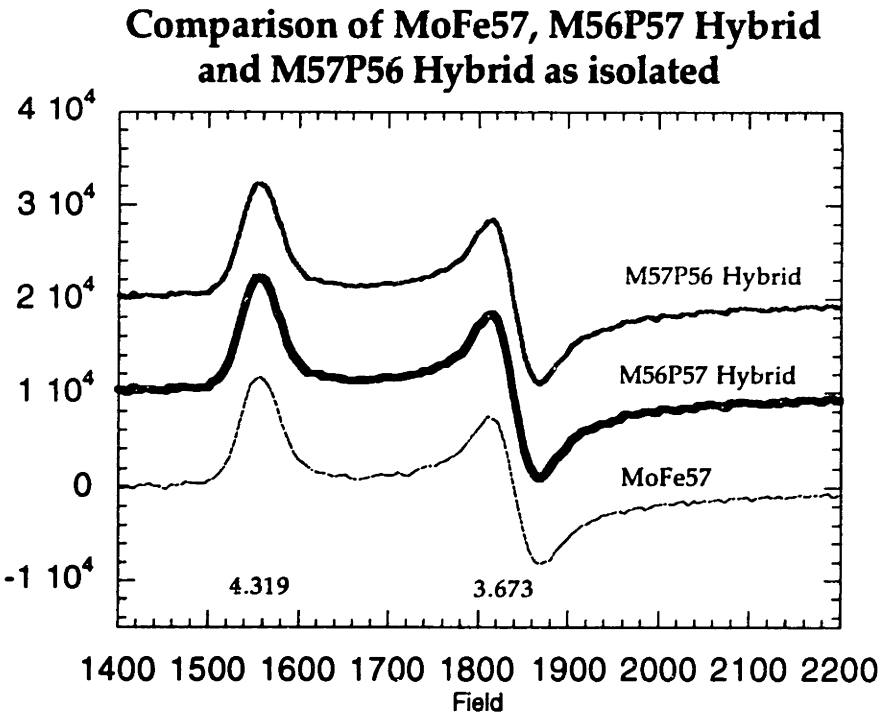


Figure 4.10: Examination of the high *g* values for the MoFe57, M56P57 and M57P56 Hybrids.

This is an expansion of the data in figure 4.9

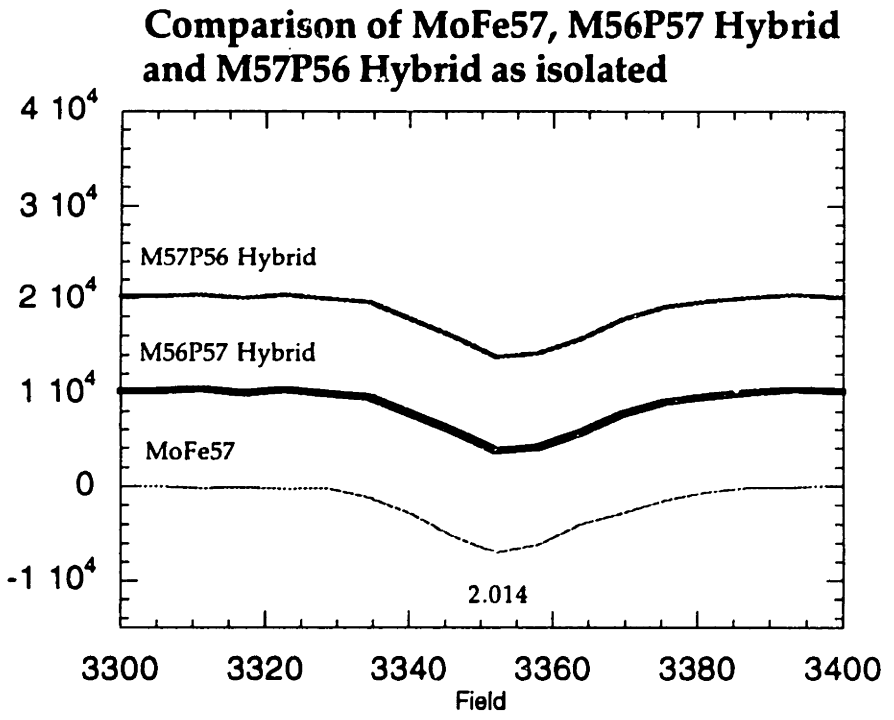


Figure 4.11: Examination of the *g*=2 region of MoFe57, M56P57 and M57P56 Hybrids.

This is an expansion of the data in figure 4.9.

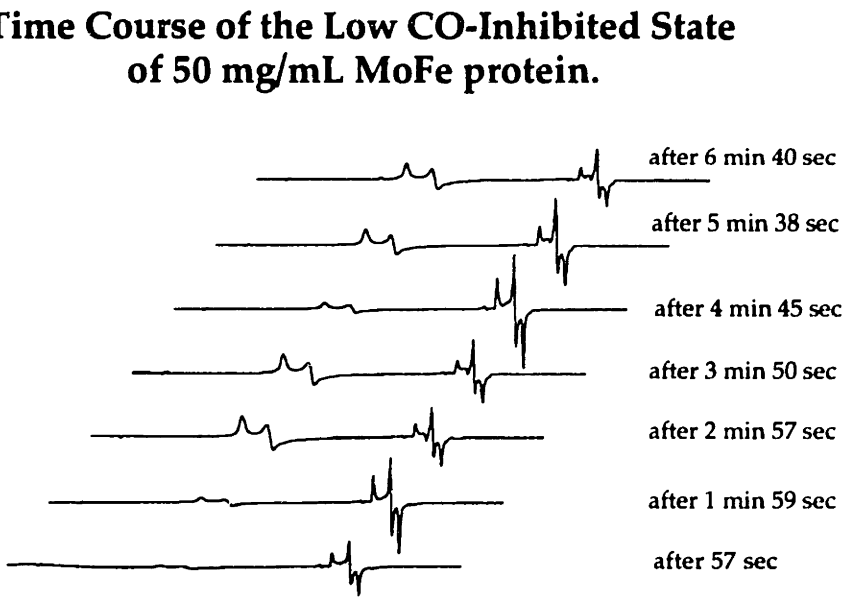


Figure 4.12: Time course of the Low CO-inhibited State of 50 mg/mL MoFe protein.

700 μL of MoFe protein in turnover mix was equilibrated under 0.08 atm CO for 30 minutes. Then Fe protein was added so that the ration of MoFe to Fe proteins was 2:1. At the times indicated in the figure, 100 μL samples were removed, put into Q-band tubes and froze in a chilled isopentane bath. The EPR conditions are as follows: CF = 2300 G, SW 4400 G, MF = 100 KHz, MA = 6.44 G, RG = 1.0 X 10^4, TC = 163.84 msec, ST = 335.54 sec, microwave power = 10 mW and temp = 5 K. The best low CO-inhibited signal occurred after 4 minutes 45 seconds of turnover.

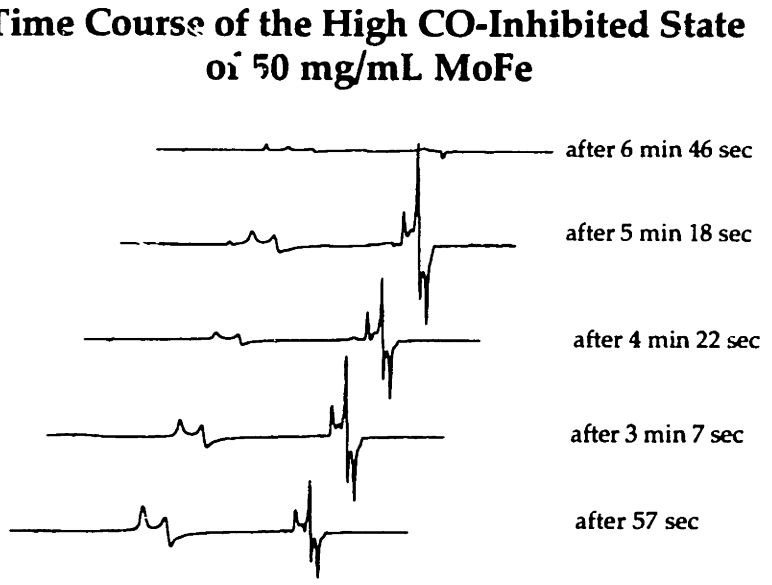


Figure 4.13: Time course of the High CO-inhibited State of 50 mg/mL MoFe protein

700 µL of MoFe protein in turnover mix was equilibrated under 0.50 atm CO for 30 minutes. Then Fe protein was added so that the ratio of MoFe to Fe proteins was 2:1. At the times indicated in the figure, 100 µL samples were removed, put into Q-band tubes and froze in a chilled isopentane bath. The EPR conditions are as follows: CF = 2300 G, SW 4400 G, MF = 100 KHz, MA = 6.44 G, RG = 1.0 X 10^4, TC =168.84 msec, ST = 335.54 sec, microwave power = 10 mW and temp = 5 K. The best high CO-inhibited signal occurred after 6 minutes 46 seconds of turnover, once all of the low signal had been converted to the high signal.

The best signals have the least amount of the as isolated S=3/2 signal of MoFe protein. The easiest signals in the EPR to follow are the g=4.3 and g =3.6. The results from all the time course experiments are summarized in table 4.5.

Table 4.5: Summary of the time required to produce the CO-inhibited state of the MoFe protein at various concentrations.

Protein concentration	Time required for High CO-inhibited State	Time required for Low CO-inhibited State
13.56 mg/mL	2 min 14 sec	43 sec
26 mg/mL	at least 5 minutes	4 min 48 sec
52.88 mg/mL	over 8 minutes	4 min 45 sec

At the higher protein concentrations and under high pressures of CO, the low CO-inhibited signal formed first and was then converted over time into the high CO-inhibited signal. This is consistent with the model proposed by Lowe and coworkers (1978) in which the "low" CO-inhibited state is produced from a tight binding site and would be kinetically formed first. It is only at "high" CO concentrations that the low binding site is occupied.

An examination of the temperature effect on the formation of the CO-binding states was also performed. Davis and coworkers (1979) observed a variation in EPR signal in the g =2.0 region at various temperatures. Their experiments were carried out using Schlenck adapted anaerobic X-band EPR tubes. A comparison between the production of the CO-inhibited state at 20°C and 33°C is shown in Table 4.6.

Table 4.6: Summary of the time required to produce the CO-inhibited state of the MoFe protein at 20°C and 33°C.

Protein Concentration	Time required for High CO-inhibited State	Time required for Low CO-inhibited State
26 mg/mL @ 20°C	4 min 11 sec*	4 min 48 sec
@ 30°C	4 min 48 sec	2 min 54 sec

* This was a mixture of high and low CO-inhibited states

The low temperature produced the most intense low CO-inhibited state of the MoFe protein, whereas the higher temperature produced the most intense high CO-inhibited state of the MoFe protein. In fact, over the course of 5 minutes at 20°C, a mixture of high and low CO-inhibited states was produced.

The final concentrations of the MoFe protein in turnover mix were as follows: MoFe57 was 20.61 mg/mL, M56P57 hybrid was 7.3 mg/mL and the M57P56 hybrid was 9.94 mg/mL. The initial attempt at turnover of the all the proteins resulted in only the MoFe57 producing both the low and high CO-inhibited state (figures 4.14 and 4.15).

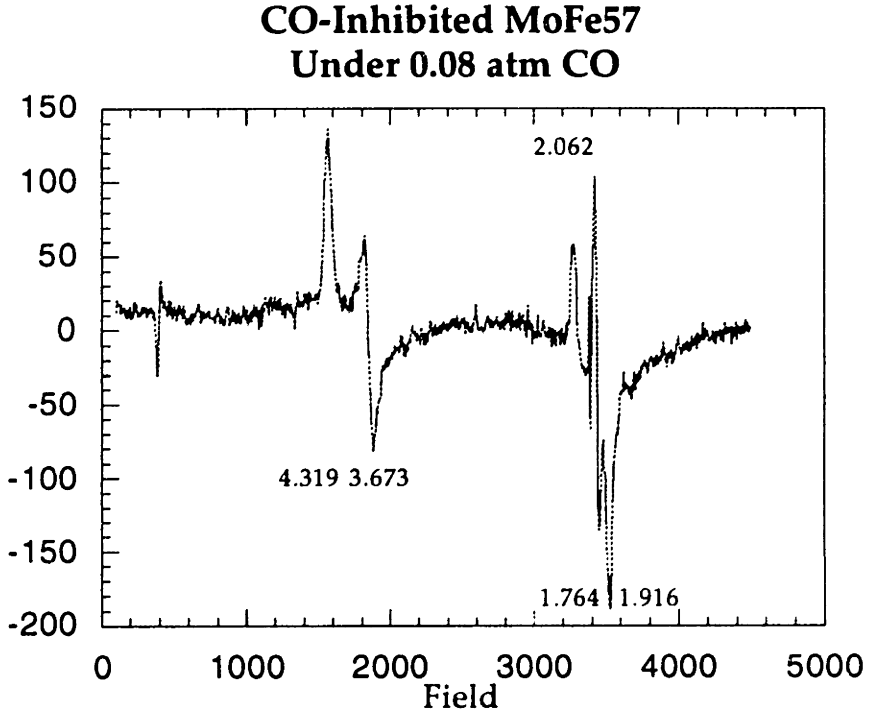


Figure 4.14: EPR Spectrum of the low CO-Inhibited form of MoFe-57 protein. The protein was allowed to turnover for 1 minute and 10 seconds before it was put into a Q-band EPR tube, inserted into an X-band EPR tube and froze in an isopentane bath. EPR conditions are the same as for figure 4.3, except the microwave power was 10 mW.

The low CO-inhibited MoFe57 protein was allowed to turnover for 1 minute and 10 seconds before it was frozen and the high CO-inhibited MoFe protein sample was frozen after 2 minutes and 50 seconds.

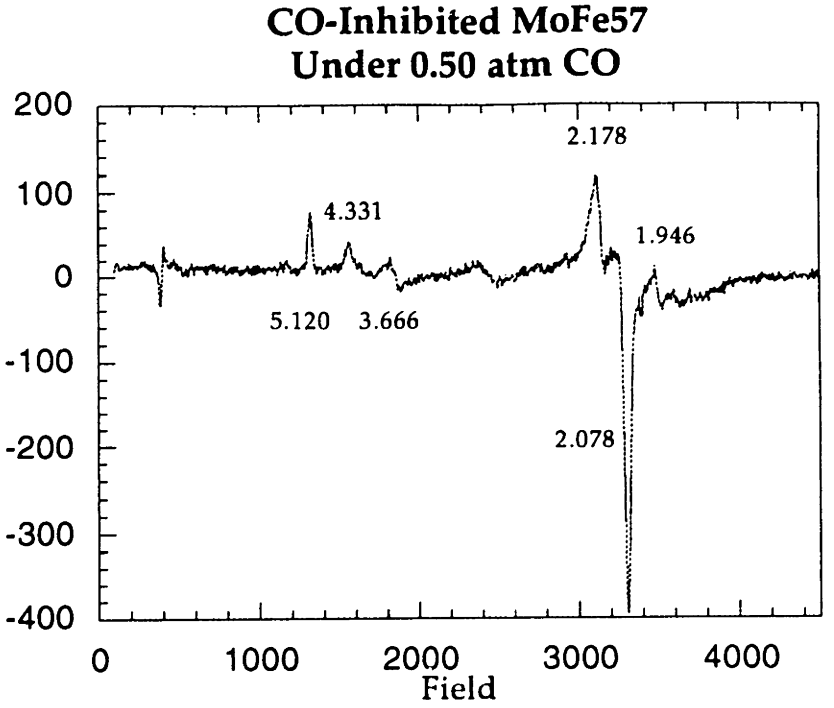


Figure 4.15: EPR Spectrum of the high CO-inhibited form of MoFe57 protein. The protein was allowed to turnover for 2 minutes 50 seconds before it was put into a Q-band tube, inserted into an X-band tube and froze in an isopentane bath. EPR conditions are the same as for figure 4.3, except the microwave power was 10 mW.

The initial times for turnover of the hybrid proteins were based on the time required to produce the most intense signals of homogeneous MoFe protein at the same concentration. The hybrid proteins, while containing comparable levels of MoFe protein, also contained an excess of contaminating proteins. If the total protein concentration was taken into account, the hybrid proteins could take longer to produce the CO-inhibited state. To model this type of interaction, an investigation using a wild type protein sample of the same level of purity was done. A 13% pure MoFe protein solution was exchanged into turnover mix. When this sample was allowed to turnover under 0.08 atm CO for 8 minutes, the low CO-inhibited form of the protein failed to appear. Under 0.50 atm CO, the signal formed in 4-7 minutes (figure 4.16).

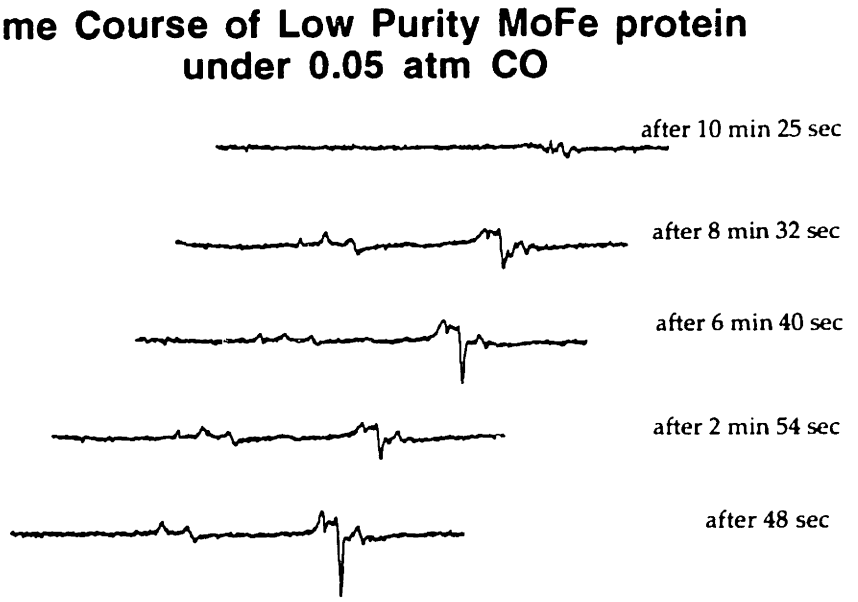


Figure 4.16: Time Course of the High CO-inhibition form of a low purity MoFe protein.
A protein solution containing 13% MoFe protein was exchanged into turnover mix and over 11 minutes allowed to turnover to produce the high CO-inhibited MoFe protein signal. All the spectra are on the same scale. The EPR conditions are the same as in figure 4.13, except the microwave power was 1 μW.

The time course for the production of the high CO-inhibited MoFe protein signal correlated well with protein solutions of similar concentration. The time required to develop the most intense signal depended on the total protein concentration and not simply the MoFe protein present. It was surprising, however, to observe the inability to produce the low CO-inhibited MoFe protein signal. It is possible that the contaminating proteins are non-specifically binding the CO. This would in turn greatly diminish the EPR signal intensity. This suggestion can be examined by turning over the impure

MoFe protein solution under various concentrations of CO. Hopefully, a concentration of CO could be found at which the "low" CO-inhibited EPR signal intensity would be large enough for observation.

Modeling the hybrid solution after the impure MoFe protein solution suggested longer turnover times could produce the required EPR signals. The M56P57 hybrid was allowed to turnover for 6 minutes and 15 seconds. The EPR spectrum is in figure 4.17. The M57P56 hybrid was allowed to turnover for 5 minutes and 9 seconds. The EPR spectrum is in figure 4.18. A comparison of MoFe57 protein and M56P57 hybrid protein high CO-inhibited states are in figure 4.19. A comparison of MoFe57 protein and M57P56 hybrid protein is in figure 4.20.

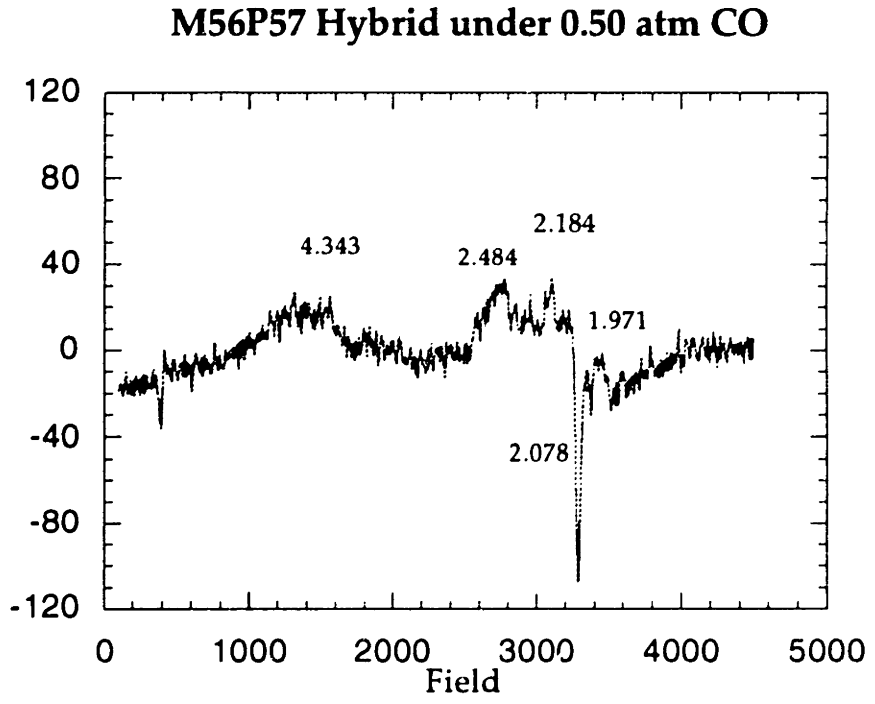


Figure 4.17: EPR spectrum of M56P57 hybrid under 0.50 atm CO.
The total turnover time was 6 minutes and 15 seconds. EPR parameters are the same as for figure 4.13 except the microwave power was 10 mW.

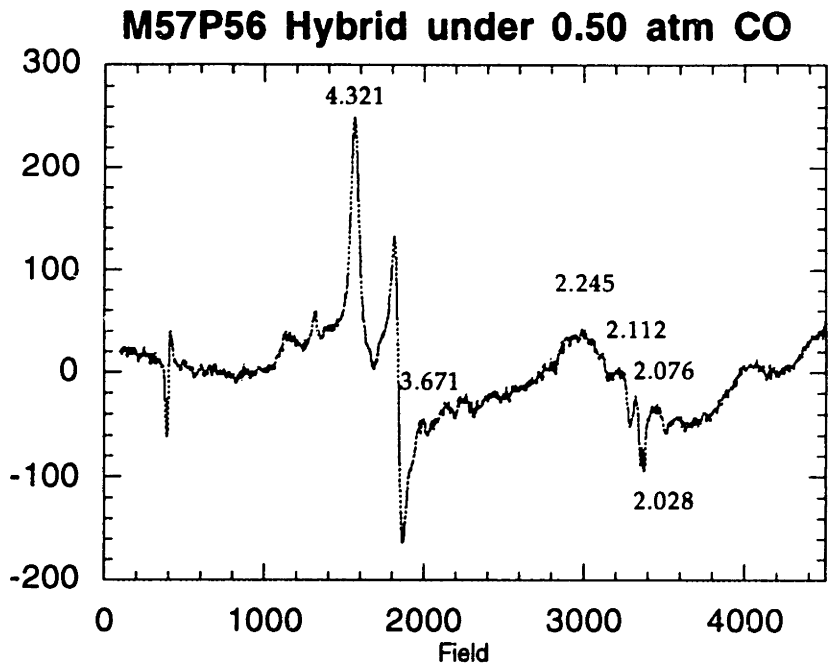


Figure 4.18: EPR spectrum of M57P56 hybrid under 0.05 atm CO.
The total turnover time was 5 minutes 9 seconds. EPR parameters are the same as for figure 4.13 except the microwave power was 10 mW.

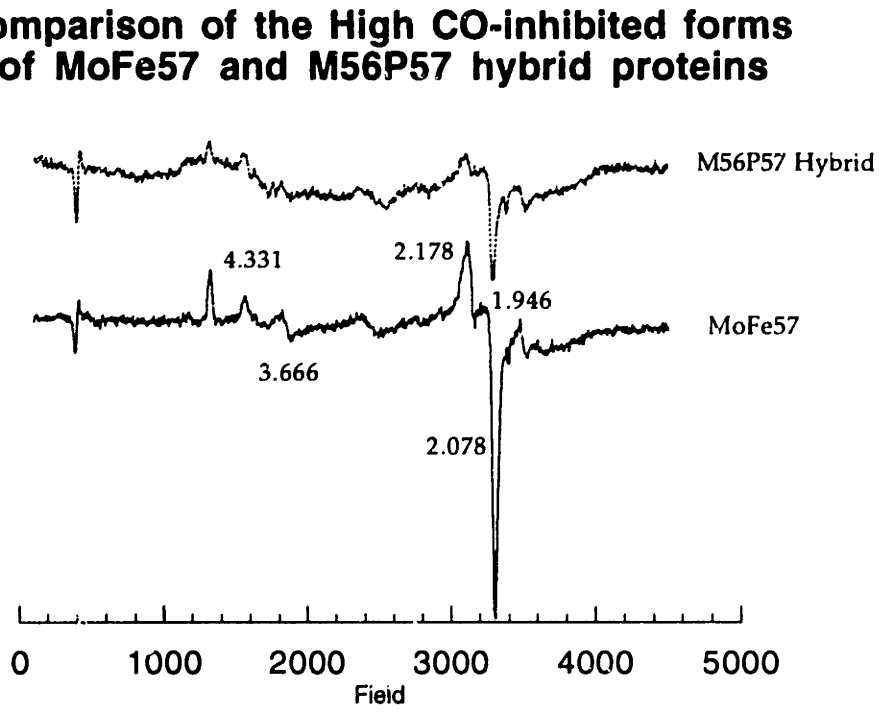


Figure 4.19: Comparison of the high CO-inhibited forms of MoFe57 and M56P57 Hybrid proteins.

EPR conditions have been described in figures 4.15 and 4.17.

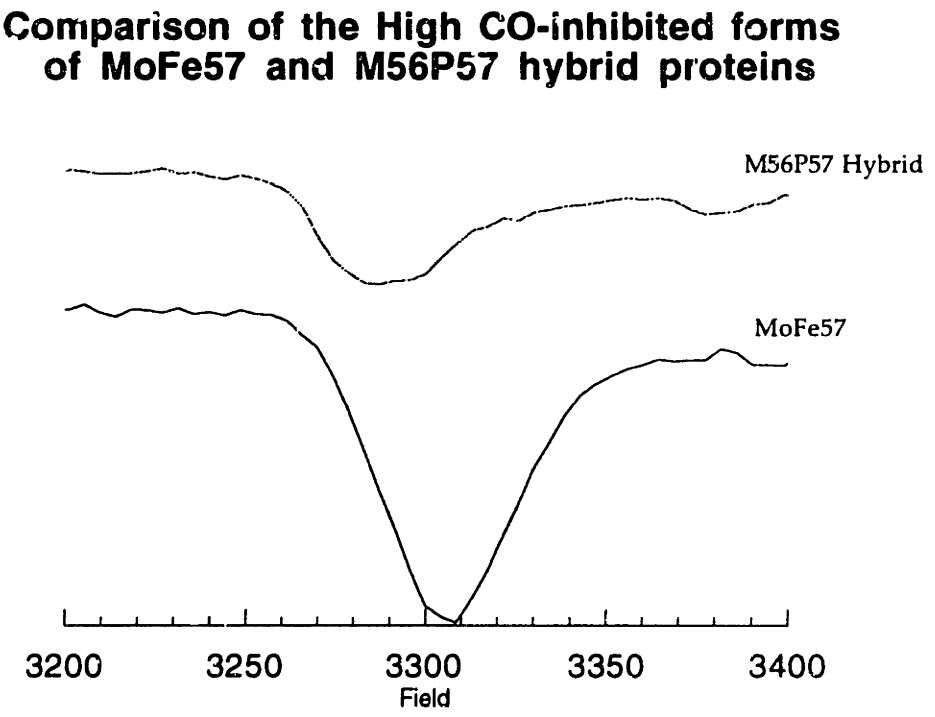


Figure 4.20: Comparison of the g=2 region of MoFe57 and M56P57 hybrid proteins.

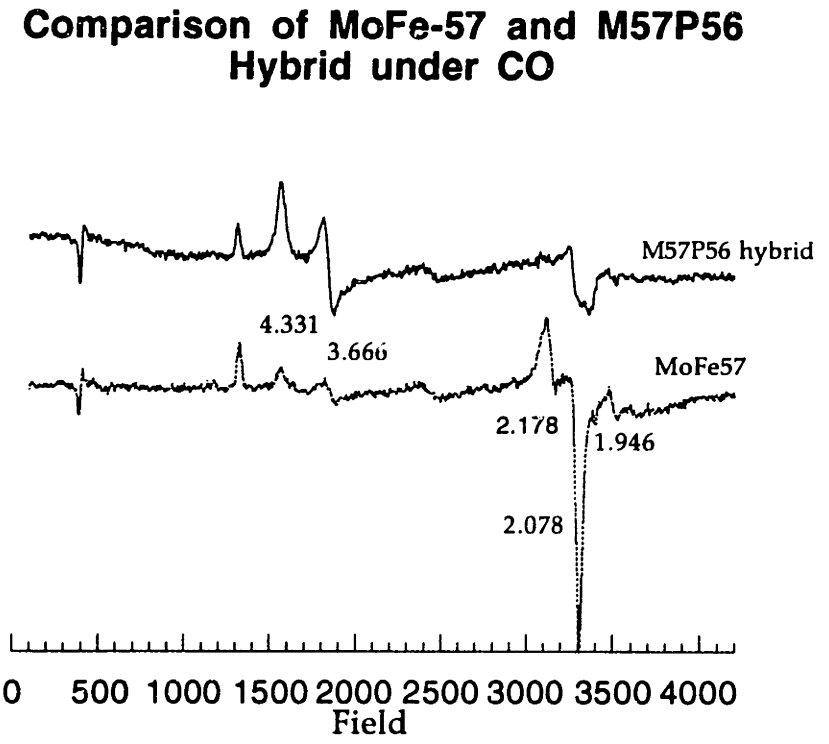


Figure 4.21: Comparison of the high CO-inhibited forms of MoFe57 and M57P56 Hybrid proteins.

EPR conditions have been described in figures 4.15 and figure 4.16.

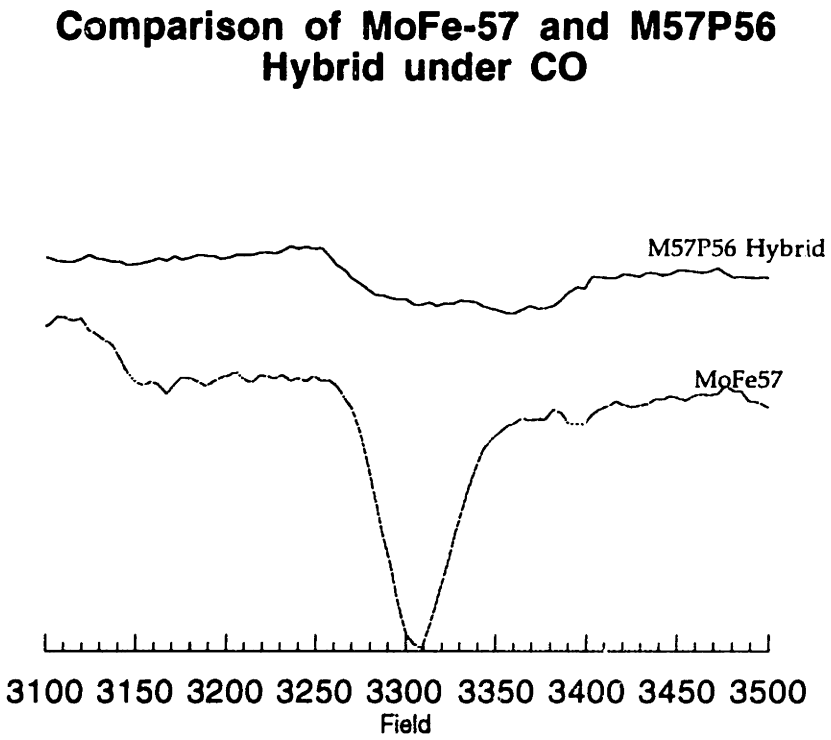


Figure 4.22: Comparison of the *g*=2 region of MoFe57 and M57P56 hybrid proteins.

The CO-inhibited EPR signals of the M56P57 hybrid are sufficiently intense for comparison with the MoFe57 sample. Unfortunately, the M57P56 hybrid sample did not produce a good quality signal due to incomplete turnover. The purpose of these experiments is to determine the site of CO binding. If the site is at the P cluster, then the M56P57 hybrid in its CO-inhibited state should show hyperfine broadening similar to that present in the globally enriched ^{57}Fe MoFe sample. Examination of the g = 2 region of the CO-inhibited form of M56P57 appears to not have any of the broadening that is present in MoFe57 sample (figure 4.20).

It is difficult to compare the CO-inhibited forms of the M57P56 hybrid and the MoFe57 sample (figure 4.21). Comparison of the g=2.0 region of these two proteins does not reveal any obvious similarities. The M57P56 hybrid sample appears to still contain large quantities of reduced Fe protein which is masking the high CO-inhibited EPR signals.

The analysis of the ^{57}Fe ENDOR is ongoing. Preliminary results of the low and high CO-inhibited states of the MoFe57 protein (figures 4.23 and 4.24) indicate that the signal is produced by 3-7 irons. The iron have hyperfine tensors ranging from 10-40 MHz. These ENDOR experiments were done at Q band (~35 GHz) frequency. Repeating these experiments at the X band frequency (~ 9.5 GHz) will determine the individual values of the hyperfine tensors.

The comparison of the ^{57}Fe ENDOR of the high CO-inhibited M56P57 hybrid showed that there was no signal attributed to ^{57}Fe. When this is compared to the MoFe57 protein, it strongly suggests that the EPR signal is due to the CO interacting with FeMoco.

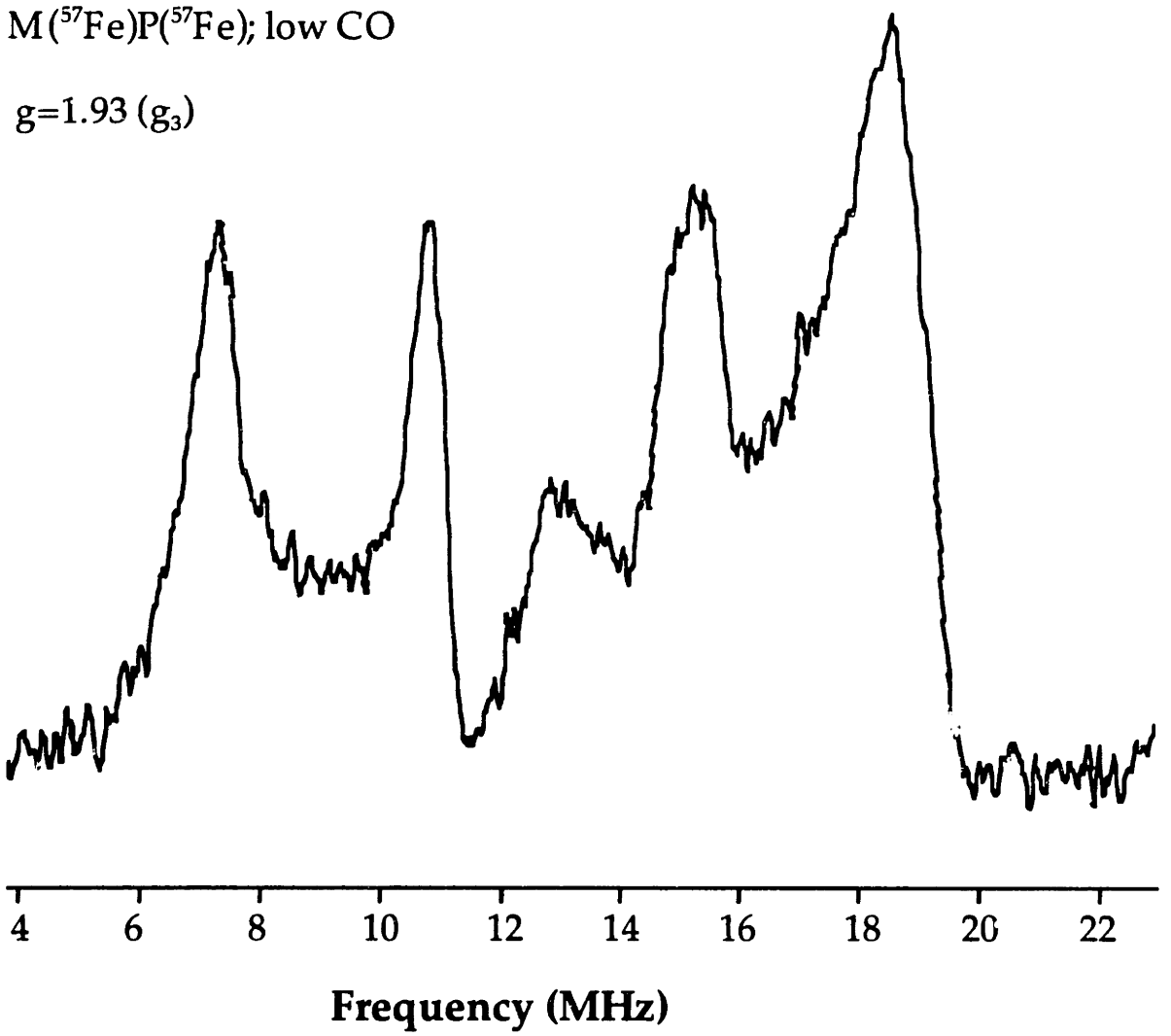
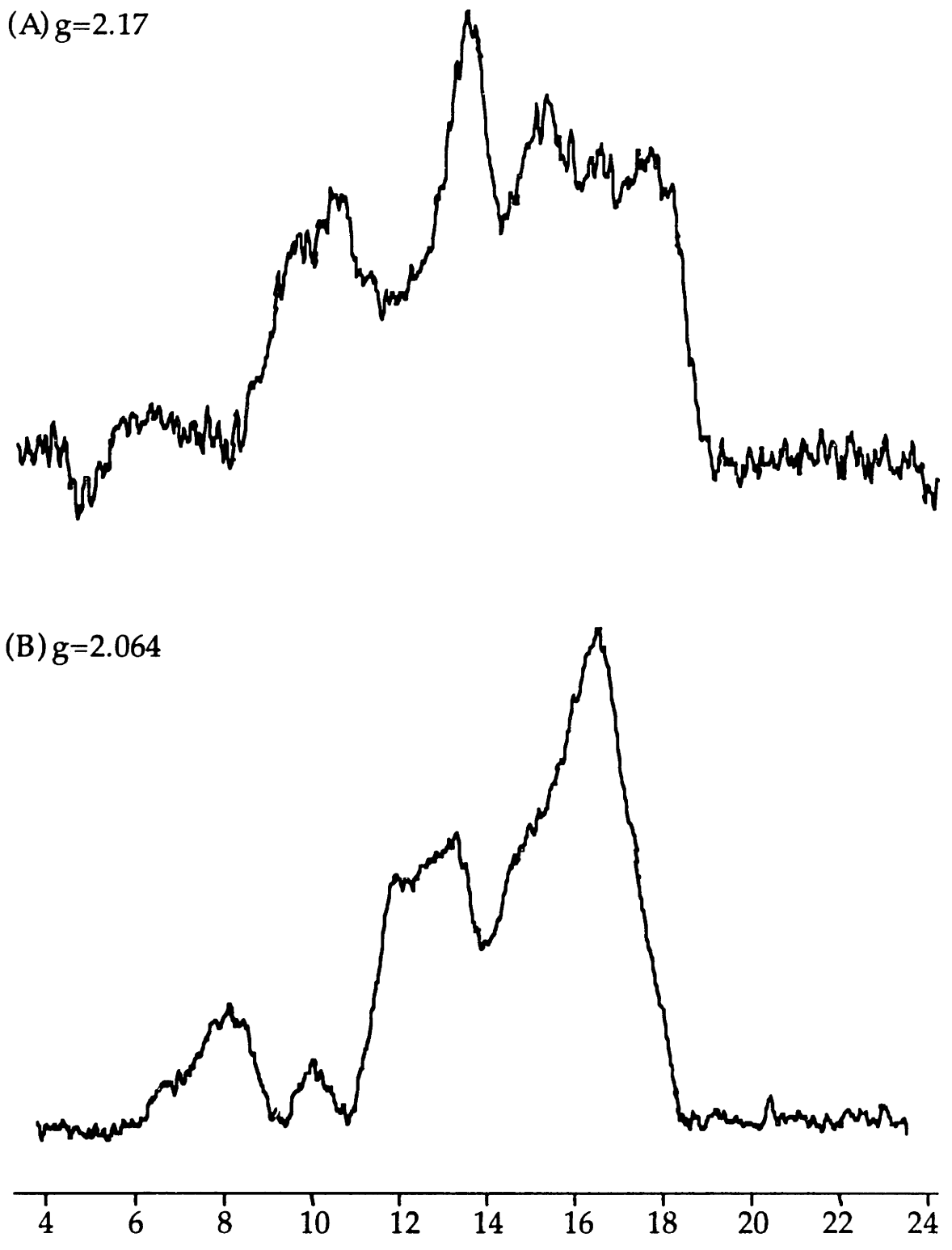


Figure 4.23: ^{57}Fe ENDOR of the low CO-inhibited state of MoFe57 protein. The MoFe57 protein was allowed to turnover under 0.08 atm CO for 1 minute and 10 seconds. Experimental conditions are as follows: microwave frequency, ~35 GHz, modulation amplitude = 67 G, receiver frequency power

= 30 W, scan speed = 1 MHz, TC = 32 msec, temp. = 2 K. (A) was collected at g = 2.17 and (B) was collected at g = 2.064.

Figure 4.24: ^{57}Fe ENDOR of the high CO-inhibited state of MoFe57 protein. The MoFe57 was allowed to turnover for 2 minutes and 50 seconds under 0.05 atm of CO. Experimental conditions are the same as for figure 4.24, except for the different g values at which the spectra were acquired at. For (A) it was g = 2.17 and for (B) it was g = 2.064.

Hi-CO



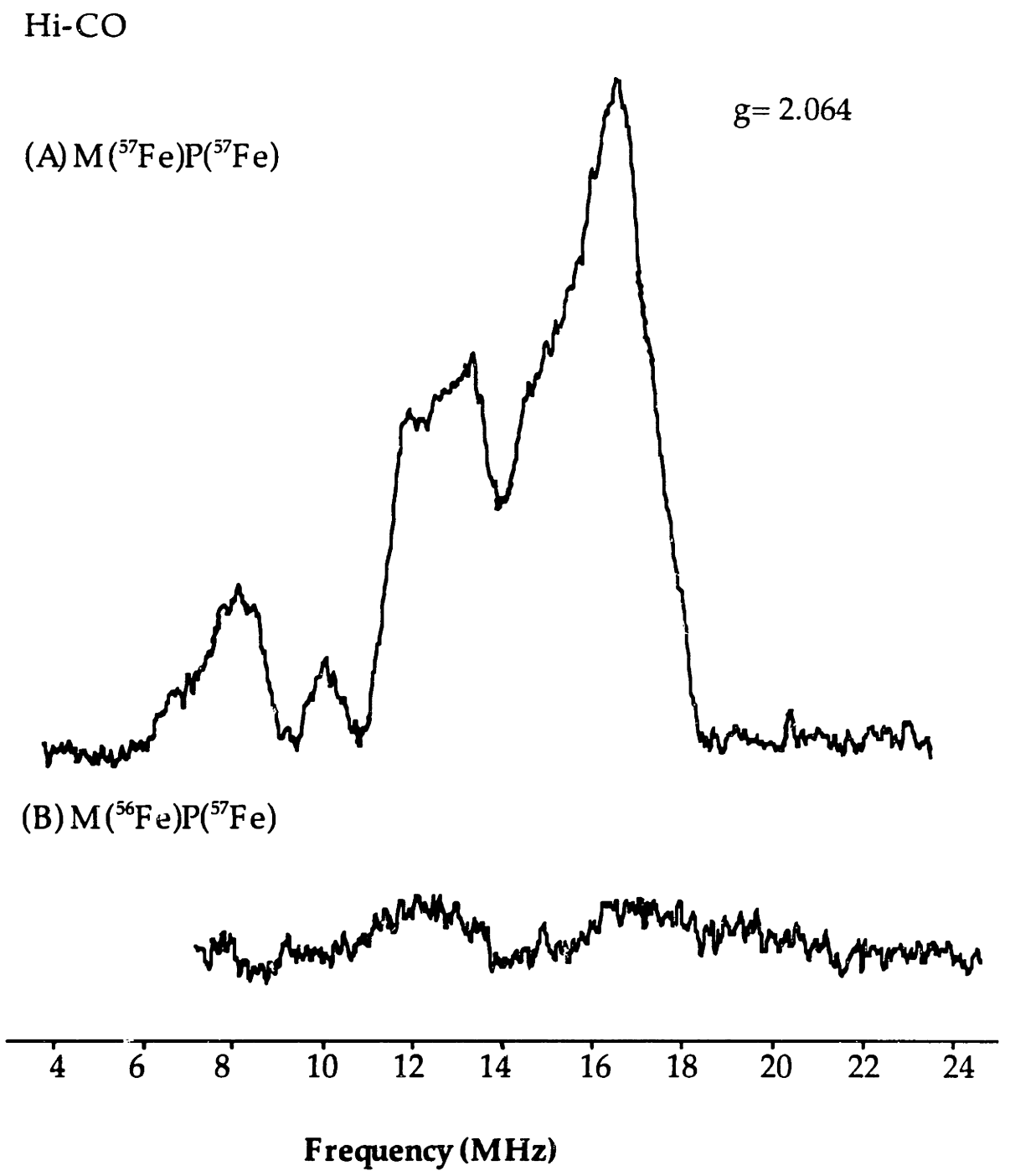


Figure 4.25: Comparison of high CO-inhibited forms of MoFe57 and M56P57 hybrid protein.

The upper spectra (A) is of MoFe57 and the lower spectra (B) is of M56P57 hybrid MoFe protein. The ^{57}Fe ENDOR conditions are the same as for figure 4.23 except that the data was acquired at g = 2.064.

IV. Discussion

There are several suggested functions for the presence of homocitrate in the cofactor. From examining the changes in the substrate specificities when various organic acids are utilized in the *in vitro* FeMoco biosynthesis, one probable role of homocitrate is in the substrate reduction mechanism (Imperial et al., 1989). Kim and Rees (1992) suggest that it might modulate the redox properties of the cofactor by protonating intermediates or by participating in the electron transfer pathway from the P cluster to FeMoco. In the model based on *C. pasteuranium* proposed by Bolin and coworkers (1993a), homocitrate is highly hydrated with at least 12 water molecules. These water molecules appear to form a hydrogen bonded network that includes all carboxylate groups of homocitrate as well as side chain and backbone atoms to the P cluster. Because homocitrate accumulates in high concentrations in the medium of nitrogen-fixing cultures of *K. pneumoniae*, another probable role for homocitrate is in the transport of Fe or Mo into the cell (Allen et al., 1994).

Homocitric acid

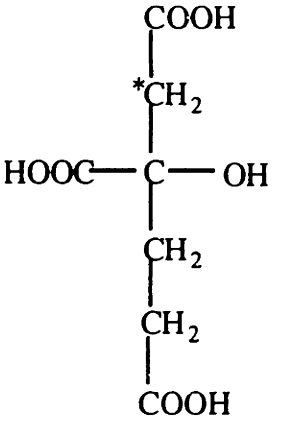


Figure 4.26: Homocitric Acid.
The deuterium label was incorporated into the methylene between the two carboxylic acids and is indicated on the figure by *.

Homocitrate is probably involved at an early step in the biosynthesis of FeMoco because without an organic acid in the *in vitro* FeMoco biosynthesis

system, there is no accumulation of ^{99}Mo in any protein-bound form (Hoover et al., 1988c).

It was thought that by incorporating deuterated homocitric acid (figure 4.26) into the MoFe protein of a *nifV*$^-$ phenotype that the modulation due to the deuterium would be detected on the ^{14}N beat/modulation pattern. From this experiment, the derived nuclear quadrapole parameter was calculated to be 2.4 MHz with an asymmetry parameter of 0.67. Both of these numbers are consistent with previously reported numbers (Thomann et al., 1991; DeRose et al., 1995). It was originally thought that this nitrogen modulation was due to the coordination of Hisα195 to the spin center (Thomann et al., 1991), but now it appears to be due to another nitrogen moiety of a different residue whose proximity to FeMoco is sensitive to certain substitutions at the Hisα195 position. Thomann and coworkers (1991) were able to eliminate the possibility that Hisα442 provided the nitrogen modulation to the cofactor spin state.

There are several conclusions that can be reached from this spectroscopic study. The EPR spectra of the proteins indicate that the samples do not contain any EPR-detectable magnetic impurities. The EPR spectra are typical of MoFe proteins with the g =4.3, 3.9 and 2.0 signals. There are no apparent differences between the ESEEM spectra (figure 4.5) and the Fourier transform of the ESEEM data (figure 4.6) of the wild type and both the deuterated and non-deuterated homocitric acid. This could be due to a number of possibilities. One possibility is that the deuterium in homocitric acid was somehow exchanged off during the incorporation. This probably did not occur because of the restoration of the wild-type growth phenotype. When citric acid is isotopically enriched, the isotopes do not exchange readily with the medium. It can then be suggested that the same scenario could be applied to homocitric acid. When Hoover and coworkers (1989) used cellular extracts to incorporate [^{3}H]homocitrate, the tritium label remained within the homocitrate throughout the purification of the MoFe protein. A more likely

scenario is that the deuterium is too distant from the ^{14}N nucleus to exert an influence. It is already known that imidazole nitrogen of Hisα442 does not interact with the electron spin moment of the cluster (Thomann et al., 1991). This is also observed when isotopes of molybdenum are incorporated into the MoFe protein (Hoffman et al., 1982). This indicates that the electron spin moment of the cluster is predominately towards the iron end. Since the modulation decreases with $1/r^3$, the effective limit of ESEEM is between 3.7 and 4.5 å. The distance from the deuterated arm of homocitrate to the center of the cofactor is 4.2 å. Since the effective electron spin moment is estimated to be towards the iron end of the molecule, it is reasonable to assume that the deuterium is outside of the detectable limit of ESEEM. The magnitude of H/D modulations can often be swamped by N modulations.

The use of the isotopically labeled hybrids of MoFe protein provided some very exciting results. The ability to specifically label either the P cluster or FeMoco is a great leap forward in providing a tool for deciphering the mechanism of nitrogenase. There are still improvements required in the method of production of these hybrids. The purification scheme needs to be refined to improve the purity of the proteins. Because the apoMoFe protein is so unstable, one issue that needs to be addressed is whether the apoMoFe protein becomes inactivated before the addition of FeMoco. When McLean and coworkers (1987) made *K. pneumoniæ/A. vinelandii* isotopic hybrids, they were able to convert between 70-92% of the apoMoFe protein into holoMoFe protein using twice the amount of cell paste. The current level of conversion is 50%. The presence of the apoMoFe protein could have affected the purification of the holoprotein, although it seems unlikely that the apoMoFe protein could have remained active throughout due to its instability. It was probably inactivated apoMoFe protein which led to a lower ultimate purity of the labeled product.

The elucidation of the CO-inhibited form of the hybrid proteins showed some insight into the mechanism of nitrogenase. It has already been

suggested that the EPR signal from the CO-inhibited form of MoFe protein is due to an iron-containing cluster (Davis et al., 1979) and that this signal is due to the same cluster in both the high and low inhibited forms (Pollock et al., 1995). There was ^{57}Fe coupling present in the CO-inhibited MoFe57 sample which was absent in the M56P57 hybrid sample. The lack of ^{57}Fe coupling in the CO-inhibited M56P57 hybrid strongly suggests that the EPR signal is due to FeMoco. It is unfortunate that the CO-inhibited form of the M57P56 hybrid has not yet been produced in large enough quantities for an ENDOR experiment. The production of an EPR signal due to the high CO-inhibited M57P56 hybrid would enable the ^{57}Fe ENDOR experiment to be completed and establish definitively the site of CO binding.

From this certain conclusions concerning the mechanism of nitrogenase can be drawn. It is known that if citrate is present in FeMoco instead of homocitrate, seen in *nifV*- mutants, the enzyme exhibits altered substrate specificities and that CO-inhibits the production of H_2. With the ESEEM experiments, it is proposed the homocitrate is too distant from the electronic spin moment to directly interact. Examination of the isotopic hybrids suggests that the CO binding site lies on the cofactor. The suggested mode of inhibition under low concentrations of CO is by interrupting the electron flow to the site of N_2 reduction. The center continues to become more reduced relative to the resting state of the enzyme (Davis et al., 1979; Lowe et al., 1978). As the pressure of CO increases, the electron flow to the cofactor is being interrupted and as a result it becomes more oxidized (Lowe et al., 1978; Pollock et al., 1995). This suggests that both the H_2 evolution and N_2 reduction are occurring on the cofactor. Pham and Burgess (1993) propose that the evolution of H_2 under CO occurs either via a mechanism different from that of H_2 evolution under Ar, relying on the protonation of a residue with a pKa of ~8.5 or that the binding of CO causes a 0.5 pH unit shift of the pK of a residue from pH 9.0 to pH 8.5. When homocitrate is replaced by citrate, two possibilities could occur. The hydrogen-bonding network required for the efficient transfer of protons from the outside of the protein to

the active site could be disrupted or the homocitrate could be protecting the H_2 evolving site from interaction with CO. Since HD evolution in *nifV-* mutants is absent (see table 4.1), the former possibility seems likely.

V. Summary

The ability to incorporate homocitrate into a *nifV-* mutant to make a wild type MoFe protein was utilized to incorporate a ^{2}H-homocitrate label into the MoFe protein for ESEEM studies. These studies revealed an ESEEM spectrum consistent with a ^{14}N-containing residue within close proximity to FeMoco. The deuteron on the homocitrate was not detected. This result is consistent with the spin moment of the cluster being distant from the Mo end, an observation that tallies with difficulty earlier observed in detecting ^{95}Mo hyperfine coupling on the EPR of the cofactor center in nitrogenase.

The production of isotopically labeled hybrid MoFe proteins enabled the P cluster and FeMoco to have different isotopes of iron incorporated. The production of the CO-inhibited state of MoFe protein using these hybrids and globally enriched ^{57}Fe MoFe protein was investigated using EPR and ^{57}Fe ENDOR. The use of these hybrid proteins for the production of the CO-inhibited state of MoFe protein strongly suggests that the site of CO inhibition is FeMoco.

Appendix A: Guide to Fermentations

Appendix A: Guide to Fermentations

This Guide is meant to provide someone with an easy to follow reference for a successful fermentation run. It is aimed at growing *Azotobacter* and *Klebsiella* cultures, and the time lines and culture recipes reflect that slant.

When attempting to grow bacteria, it is important to utilize sterile techniques. All solutions should be autoclaved for at least 20 minutes on slow exhaust. All glassware should be also sterilized in the autoclave. All plasticware should be presterilized and only opened in the flow hood or should be autoclaved like the glassware. When using the flow hood, all surfaces should be sprayed with a 70 % ethanol solution and then wiped off with paper towels. The flame should be used whenever bacteria are not in the flow hood. If the flame is used while bacteria are in the hood, the chances of heat kill is great. When in the flow hood, one should use latex gloves (which can be sterilized by spraying with the ethanol solution) and a long sleeved shirt or clean labcoat. Sterile transfers of liquids can be accomplished by flame sterilizing the stock container, pipeting out the desired amount of liquid, flame sterilizing the top again and closing the container. The pipette can be moved quickly through the flame and then transferred into the new container after its top has been flame sterilized.

The cultures at any time during a growth can be examined under a microscope. One way to ensure a sufficient sample size is to take a culture aliquot and spin in down in the clinical centrifuge. The supernatant can be decanted off and then the pellet can be resuspended in remaining liquid. The pellet can then be smeared onto a microscope slide, a coverslip applied and then examined under the microscope. It is best to look for culture uniformity when examining the slide. For complete microbiological descriptions of the bacteria, refer to a text, such as Bergey's Manual of Determinate Bacteriology (1974). *Azotobacter vinelandii* are egg-like rods frequently in pairs and look like figure eights and *Klebsiellae pneumonia* look like dome shaped rods.

Modified Burk's Recipe

The stock solutions that are used in constructing the fermentation media should be sterile and only opened in the flow hood. Periodically one of the stock solutions will go "off" and the media will not support growth. It has been reported that the suspect solution is the sodium molybdate (D. Wright, personal communication), but it is often just easier to make up an entirely new set than to determine which of them has degraded. After completing the media, you should check and make sure that the final pH is around 7.4. If it is not, this is an indication the either the recipe was not followed correctly or that possibly one of the stock solutions has degraded. The following is a recipe for 1L of modified Burk's solution
To autoclaved water add the following amounts of the stock solutions:

- 40 mL of a 50% sucrose solution (or 20 g of solid sucrose)
- 3.4 mL of a 1 M sodium chloride solution (58.44 g NaCl in 1 L water)
- 0.1 mL of Iron citrate solution (30 mg/mL of iron citrate). In order for the iron citrate to dissolve into the water, the solution needs to be autoclaved and then stored in a dark bottle in the refrigerator.
- 0.1 mL of sodium molybdate (25 g/L of $Na_2MoO_4x2H_2O$ in 3 mmol/L NaOH)
- 1.0 mL of trace elements solution

  (The trace elements stock solution consists of 0.88 g/L H_3BO_3
  0.44 g/L of $ZnSO_4x7H_2O$
  0.20 g/L $CoCl_2x6H_2O$
  17 mg/L of $MnSO_4xH_2O$
  10 mg/L of $CuSO_4x5H_2O$)
- 5 mL of Burk's phosphates (double this concentration if the culture is not under constant pH control). The Burk's salts stock solution is 160.2 g/L of K_2HPO_4 and 40.8 g/L of KH_2PO_4.
- 2.0 mL of magnesium sulfate (100 g of $MgSO_4x7H_2O$ in 1 L of water)
- 1.0 mL of calcium chloride (40 g of $CaCl_2x2H_2O$ in 1 L of water)

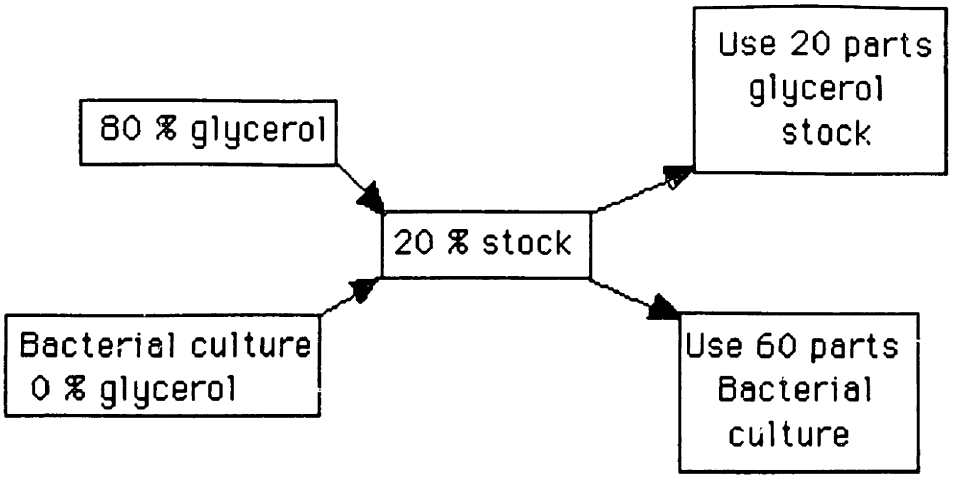
- for solid plates, 20 g of agar
- for derepression media, 0.5 g/L l-arginine chloride for *Av* and 1.0 g/L l-serine for *Kp*.

Making Agar Plates

To make agar plates, first autoclave the sugar and agar together in the appropriate volume of water. Make sure that the solids are dissolved when the container is brought out of the autoclave and put into the flow hood. The solution must be cooled down enough that when the phosphates, magnesium sulfate and calcium chloride are added, they will not co-perceptive upon further cooling, but not so cool that the media hardens before the plates can be poured. A good temperature is one which you can touch the container and not burn your hands. The components of the media are added in a sterile fashion with sufficient mixing. When media is complete, the bottles of the stock solutions are removed from the hood and the flame turned off. The bag of sterile petri dishes are opened in the hood and the media is poured into the bottom of the dish. The depth of the plate should be at least 0.5 cm, usually 1 cm is the norm. The top half of the plate is put halfway over the bottom and the media is allowed to cool. There should not be excessive moisture on the top half of the plate. If there is, it is because too much of the bottom plate was covered. When the media is solid, the plate is wrapped in Parafilm and put media side up in a 30 °C oven overnight. Then the plates can be stored in the refrigerator until used. Depending on the sterile techniques used in making the plates, the plates can be stored for up to six months. After this time, it is better to make fresh plates.

Storage of Culture Stocks

The long term storage of bacterial cultures is usually in a 20% glycerol stock in the -80°C freezer. The glycerol stocks are made up from liquid cultures and sterile 80% glycerol using the following formula



So to make up 1.6 mL of bacterial glycerol stock, take 1.2 mL of bacterial culture and 0.4 mL of the sterile glycerol stock.

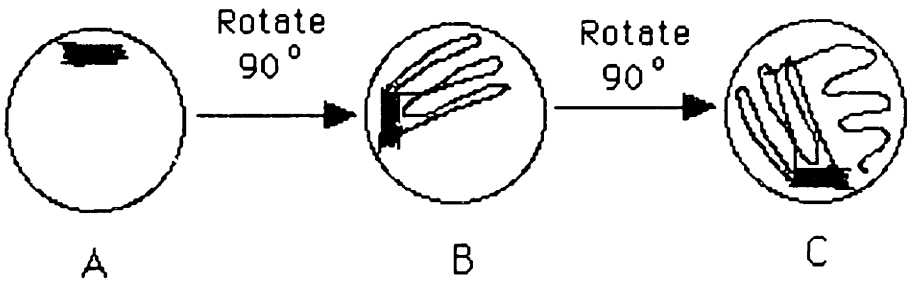
You should plan the fermentations so that you don't have to start a culture directly from a frozen glycerol stock. It is standard protocol in microbiology to start a liquid culture from a single isolate colony on an agar plate. You should also never thaw a glycerol stock and then refreeze it. This induces a lot of bacterial cell death. Once thawed, the glycerol stock should be used and then disposed of in the biohazard waste. A liquid culture can be started from the glycerol stock and then that liquid culture can be plated out onto solid media. Then once the plate has colonies on it, a single isolate colony from that plate can be used to start your fermentation culture.

For short term storage of bacterial cultures, these can be stored on agar plates in the refrigerator. Depending on the type of culture, the plates can have liquid cultures started from them after storage of up to two weeks for UW 45 and up to years for *Av* wt (R. Murray, personal communication).

Plating out cultures

To plate out cultures, take a 5-10 mL liquid culture and pellet the bacterial cells by spinning them in a centrifuge in a sterile falcon tube. The liquid is poured off and the small amount of remaining liquid media remaining is enough to resuspend the cells. Upon resuspension, an aliquot

(usually 50 µL) of the cells is put onto the agar plate using a Pipetman (see diagram below part A).



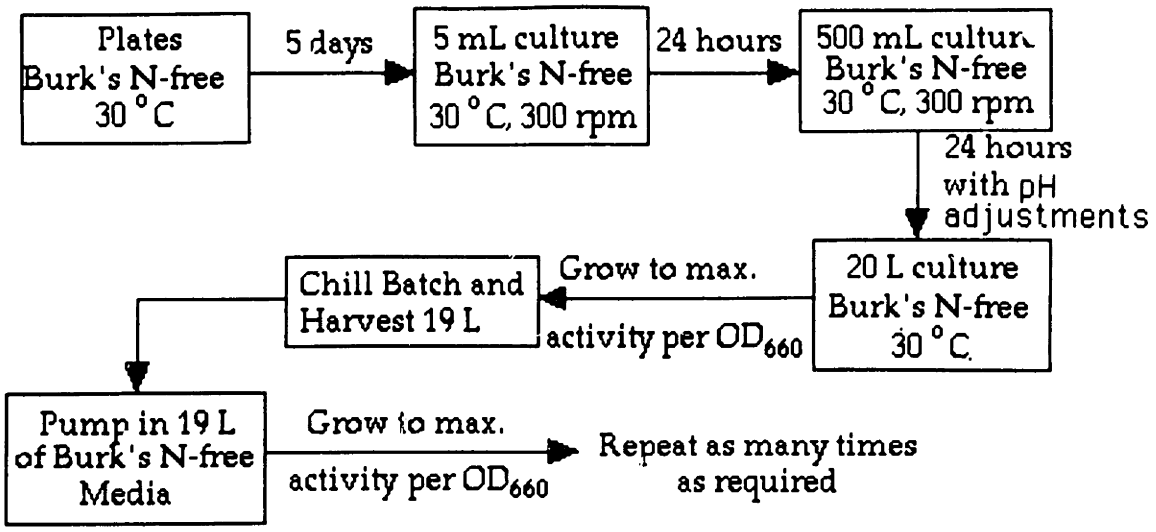
The cells are then serially diluted by smearing out parts of the first smear (B) using a steri-loop. The steri-loop should be dipped in ethanol between dilutions to kill the bacteria present on it from the previous smear. The plate is then rotated again and the cells from a small area of the second dilution are smeared over the rest of the plate (C). It is in this third smear that the single isolate colonies will grow. The plates are then rewrapped in Parafilm and put in the incubator to grow (30°C for *Av* and 37°C for *Kp*) for 3 - 5 days.

Sample Plans for Fermentations:

The following plans start from plating out liquid cultures onto agar plates. Wild type nitrogen fixers are grown without a fixed nitrogen source while mutant strains are grown with a nitrogen source initially (repressed) and then the nitrogen source is removed from the culture and the nitrogenase system is turned on (derepressed).

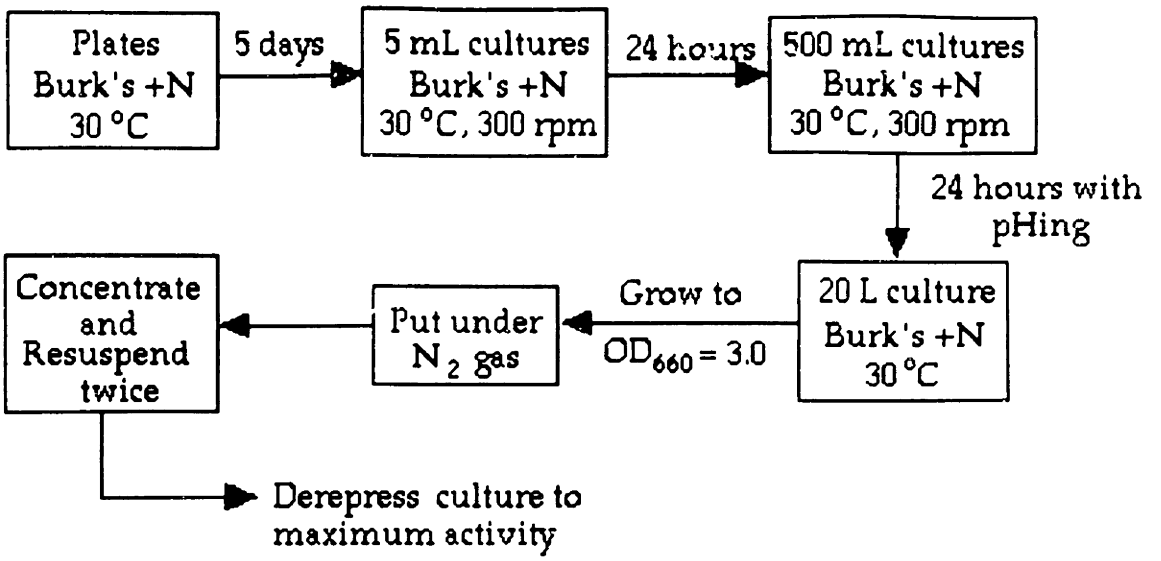
Azotobacter vinelandii wild type fermentation

This fermentation can be run indefinitely using a continuous batch process. A volume of culture can be harvested at a given time and then the remaining bacteria can be used as an innoculum for the next 20 L batch. It is important to check the culture for contaminants at each stage of this multi-day fermentation.



UW 45 Fermentation

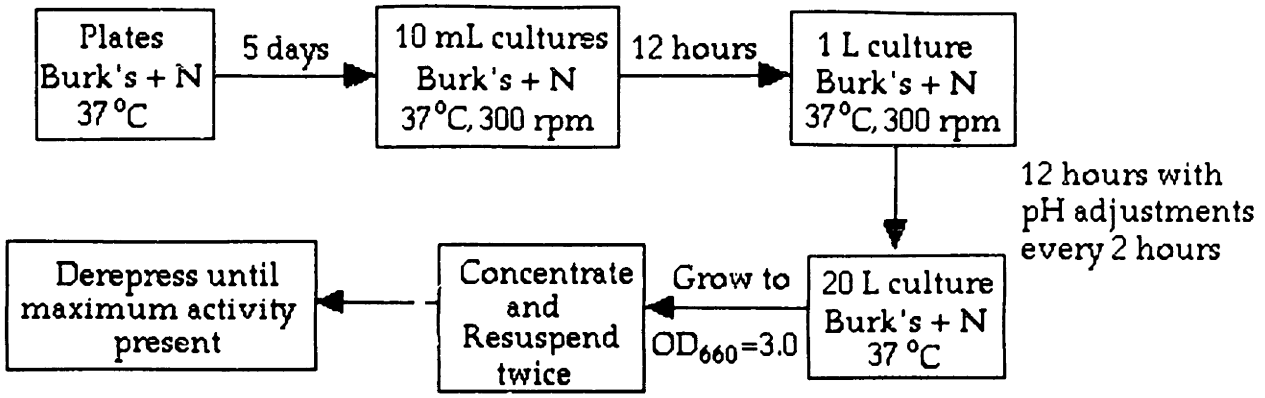
UW 45 will not grow without a fixed nitrogen source. The doubling time for UW 45 is between 3 and 6 hours, compared with 2 - 3 hours for wild type *Av*. Because of the lengthy doubling time, to grow twenty liters of culture up to OD_{660}=3.0 will take around 18 hours. The derepression of UW 45 will then take an additional 2-6 hours. The culture once in the fermenter needs to have the dissolved oxygen content continuously monitored. This results in a 24 hour period from inoculation of fermenter till the end of clean up when the operator needs to be in lab.



The derepression of the culture is under 10 % dissolved oxygen whereas growth under repressed conditions usually is between 10 - 20 % dissolved oxygen.

UN 106 Fermentation

Klebsiella needs to be grown under anaerobic conditions (i.e. under N_2 gas), so there is no need to use a dissolved oxygen probe during the fermentation. UN 106 does produced a large quantity of acidic metabolites, so it is very important to adjust the pH of the 1 L cultures on the shaker table, failure to do so results in a dead innoculum. During the 20 L fermentation, around 600 mL of base will be consumed so make sure that there is at least 1 L. It is a good idea to inoculate the fermenter for the repressed growth late at night and then come back within 8 hours to get ready for the derepression. The derepression times vary from 8-12 hours.



Fermentation Time Lines

It is important to be always thinking ahead during a fermentation nd write what needs to be done, so items will not be forgotten! The following is an example of an outline for a fermentation including the items that need to be prepared before hand. The sections which follow will discuss the operation of the equipment required for fermentations.

A v wt double fermentation

	Bacterial work	Preparation work
Day 1	-Start fresh bacterial cultures growing on agar plates	
Day 5		-Make sure there are sterile culture tubes ready -Autoclave small amount of water to make the Burk's for 5 mL liquid cultures
Day 6	-Start 8 X 5 mL Burk's liquid cultures from fresh plates in am -Just before going home, complete the 2 L of Burk's N-free media and put 500 mL into each culture flask. Store it in the flow hood overnight	-Autoclave 2L culture flasks and 500 mL graduated cylinder plus enough water to make up 2 L of Burk's media
Day 7	-am -inoculate the 500 mL	-Sterilize the fermenter

	cultures with either 5 or 10 mL (depending on the cell density) -pH cultures throughout day	-Dry autoclave the gas inlet, acid, batch and base containers, any glassware for sterile transfer -Autoclave the acid and base stock solutions -Autoclave the fresh media (water, sugar and antifoam only)
Day 8	-am- start 20 L culture in fermenter, grow to maximum activity per OD_{660}, harvest 19 L, pump in fresh media and repeat fermentation as long as required	-Complete the fresh media

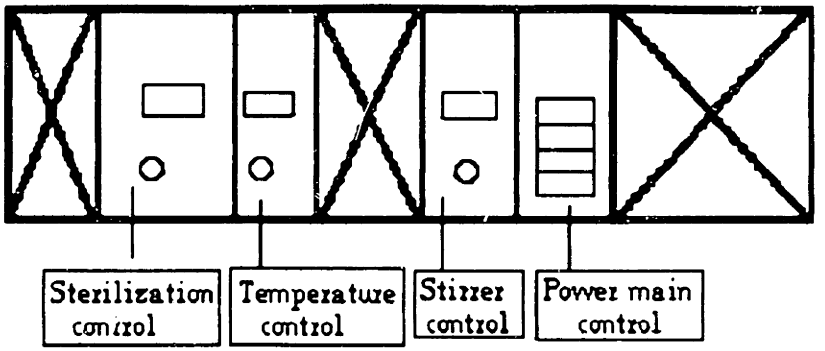
The next part of this guide deals with the fermentation process from the 20 L Chemap fermenter onward.

20 L Chemap Fermenter Operation

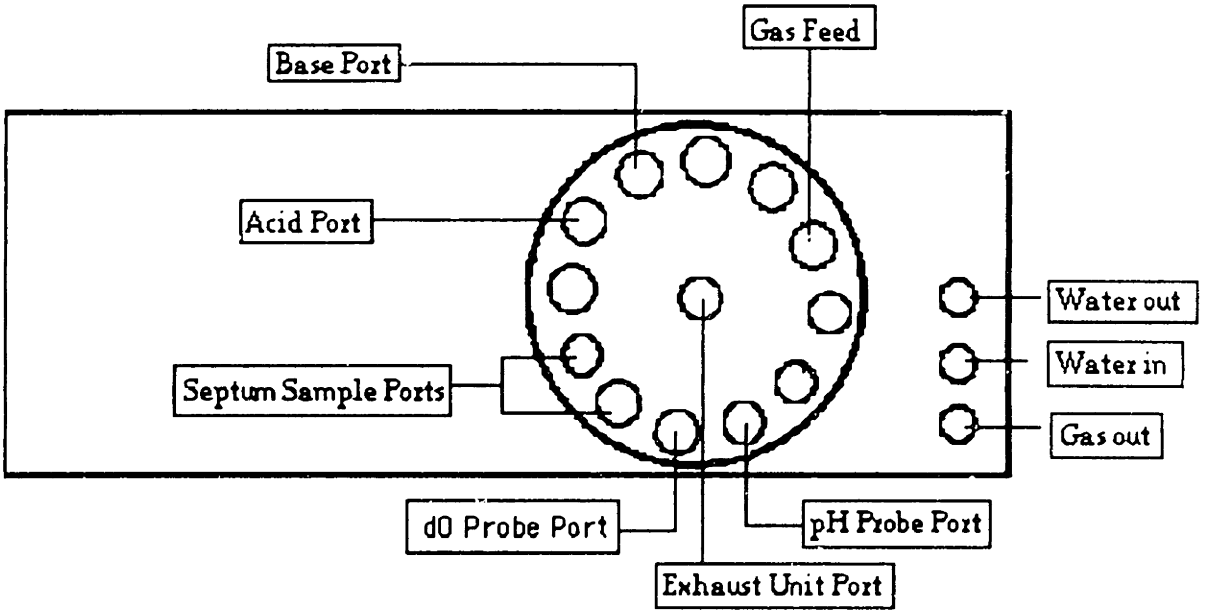
This has been complied by Patti Christie over her six years of fermentation operation and maintenance of the 20L Chemap fermenter. For more in depth fermentation schematics and specifications refer to the Chemap manual. The fermenter has been used successfully in the past to grow *Azotobacter, Klebsiella* and also *Methanogens*. Patti has the most experience growing the first two types of bacteria. The fermenter has been equipped with an external automatic pH controller (New Brunswick) and an external dissolved oxygen controller (New Brunswick) equipped with a galvanic probe. The dissolved oxygen controller is not interfaced with the fermenter, so the oxygen content must be manually controlled by the operator. The fermenter has been modified to enable a rapid chilling to occur. This is accomplished through the nitrogen gas feed being chilled with liquid nitrogen and the water being chilled with a salt bath. While this does not chill as fast or efficiently as a chilled ethylene glycol bath, the net result is that the bacteria metabolism is slowed down sufficiently to enable one to harvest maximum active biomass.

The diagrams of the facility and fermenter are accurate as of 1995.

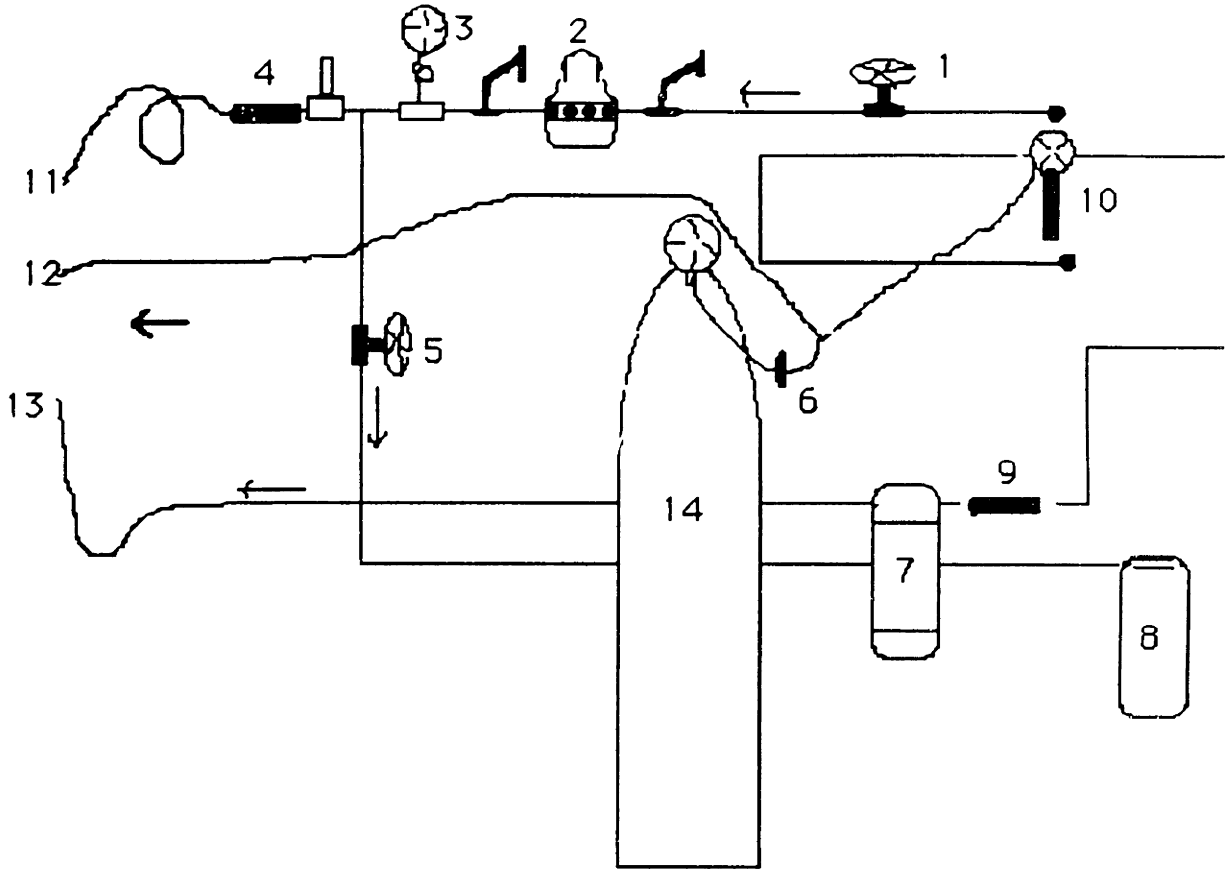
Fermenter Front



Fermenter Top View



Steam, Gas and Water Lines



Explanation of Figure;

1. Main steam valve. This line comes directly from the main high pressure steam line in the sub-basement. MIT adds a preservative to the steam to inhibit the growth of algae and bacteria. If the steam line is completely shut off, these additives precipitate out as the steam cools. It is then of up most importance to continuously bleed the line.
2. Steam trap/regulator. This will increase the pressure in the line to 40 psi.
3. Pressure gauge. This should be at 40 psi.
4. Steam valve to fermenter. This is always shut off when the fermenter is not in use.
5. Steam line bleed valve. This is used to bleed out the water out of the steam line when it is initially turned on completely. This line goes into the drain (8). The bleed valve is turned sufficiently to bleed the steam line continuously.
6. Gas valve.
7. Water filter. This filters the city water before it enters the fermenter. It starts out being white and should be changed when it becomes dark in color. To save on filters, turn on the valve (9) to the filter only when using the fermenter
8. Drain. see 5
9. Water valve. This provides the city water that the fermenter uses to heat and cool the temperature of the culture.
10. House compressed air valve. This is the MIT house compressed air. Would recommend using compressed air tanks for growths, this enables you to control the pressure and quality of the gas entering the culture.
11. Steam to fermenter. This steam line feeds the steam into the fermenter.
12. Gas to fermenter. This gas line feeds the gas into the fermenter
13. Water to fermenter. This water line brings water into the fermenter.

Fermenter Operation

The first item is to open one of the sample ports on the headpiece of the fermenter and drain the water from the fermenter. The fermenter then needs to be filled and drained at least twice with house distilled water to remove any remaining soap from the cleaning. Once you are satisfied that the fermenter is rinsed, add 18 L of house distilled water. The numbering system of the valves refers to the previous diagram.

- Turn on:

  (a) Steam. Turn on valve 1 completely and then drain the water build up by turning on valve 5. Once steam is flowing freely, turn on valve 4 and shut off valve 5. If pressure (look at 3) does not build up, then wait a bit to see if the steam can redissolve the precipitant in the line. If this does not work, call FIXIT.

  (b) Water. Check filter integrity and turn on valve 9.

  (c) all power mains on the fermenter.

- Set temperature on Temperature Control Panel

  toggle up = actual temperature

  toggle down = set point (sp)

  (a) toggle down

  (b) rotate temperature dial until appropriate temperature is observed on LCD. This is 30°C for *Av* and 37°C for *Kp*.

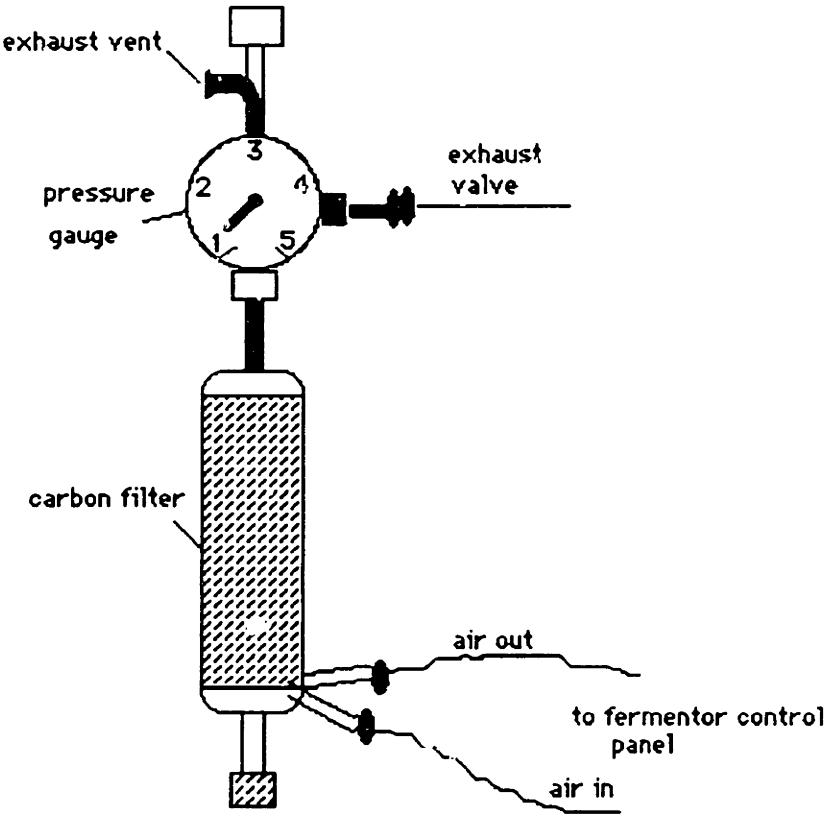
  (c) lock by moving lock bar to right

- Attach combined/exhaust unit to center point in head plate

  (a) open exhaust valve (knob on the top right of unit)

  (b) do not attach condenser water for this will preclude proper sterilization.

  (c) keep the exhaust valve open during sterilization cycle until temperature reaches 95-100 °C, then close exhaust valve. Keeping the valve open speeds up the heating.



- Place pH probe into fermenter

  (a) Take the probe out of the potassium chloride storage solution.

  (b) Rinse off the probe with distilled water. Be very careful about the glass tip of the probe.

  (c) Put the probe into the designated port. Make sure the cap is on.

- Place the dissolved oxygen (dO) probe into the fermenter

  (a) The probe should be stored in blank. When you remove the probe from the solution, check for membrane integrity. There is about 7 mL of 0.1 M NaOH solution inside the probe. Look to see if there are a large number of precipitants in the solution. If there are, change the solution.

  (b) Rinse the probe with distilled water.

(c) Once the probe is in the port in the fermenter, make sure the stainless steel cap is on the top.

- Increase rpm to 300 and lock.
- Add Burk's phosphates and NaCl, and antifoam (0.1%, 2 mL for 20L)
  For modified Burk's buffer, 16.02 g K_2HPO_4, 4.08 g KH_2PO_4 and 3.97 g NaCl.
- Begin Sterilization Loop

(a) At the sterilization control panel, check to see if the switch set at sterilization by steam, heat by water

NB. If setting is different which it should not be, set dial to fill the system for two minutes before proceeding to desired setting.

(b) Toggle to start

(1) yellow light will come on

(c) Set sterilization temperature

(1) on temperature control panel, toggle down to set point

(2) set desired temperature (112 °C) from the sterilization control panel with the set point sterilization knob (sp ster.)

(3) lock sterilization set point

(d) Put safety shield on fermenter

(e) Increase rpm to 500 and lock

(f) wait and wait and wait and wait (heating takes a very long time due to the Teflon coating on the inside of the fermenter)

(g) at 95-100 °C, close exhaust valve.

(h) When temperature reaches sterilization temperature (i.e. 112 °C) crack exhaust valve to vent steam and steam sterilize. The red light on the sterilization control panel will come on when it has reached the sterilization temperature. At this point, it is also convenient to sterilize the sample port at the bottom of the fermenter. Make sure there is tubing around the sample port attached with a worm clamp and that the end of the tubing is in a carboy

(i) After completion of sterilization loop and the temperature is below 90 ^{o}C , open exhaust vent completely.

-watch pressure gauge so as not to create a vacuum (approx. 0.1 bar is an acceptable limit). Creation of a vacuum indicates a failure of the back pressure valve. In event of such a failure immediately equilibrate by pricking a septum on headplate with a needle. If needle is fixed with a filter, sterility should be maintained.

(j) Allow temperature to fall to desired fermentation temperature. Check and make sure the temperature is correct.

(k) Remove the safety shield.

- Attaching the gas inlet filter.

(a) Make a sterile insertion of the end of the gas inlet needle into the fermenter.

(1) squirt 100% EtOH onto the septum

(2) light the ethanol with a match

(3) flame sterilize the needle of the transfer line

(4) pierce the septum with needle of the transfer line. The flame should go out when you screw the needle into the sample port. If you don't feel comfortable doing it this way, blow out the flame just before you pierce the septum. Leave the ethanol on the septum.

(b) Connect the tubing into the gas outlet port on the top of the base unit of the fermenter.

(c) Blow gas through the line to remove any particulate matter. Turn down the airflow and attach the end of the tubing to the gas inlet filter. The fermenter is now left on positive gas pressure (usually nitrogen) until the commencement of the fermentation. Typical settings are 2-5 psi on the second stage regulator and 5% on the base unit.

- Assemble acid and base transfer lines

(1) pour approximately 800 mL of base (either 10 N NaOH or 50% w/v NaOH) and approximately the same amount of 1 N phosphoric acid into their respective containers.

(2) remove autoclaved dip tube/transfer lines from tinfoil and place the dip tube in the flask (making sure that rubber tubing has not been too misshapen by the heat)

(3) put the tubing into the peristaltic pump head

i.e. take key and rotate the pump head as to push the tubing into the groove, making sure that the tubing stays aligned in the opening

(4) flame sterilize the appropriate septum on fermenter

(5) the lines must now be filled with the liquid.

(a) Attach the pH probe to the controller

(b) Select the mode of operation from standby to read

(c) Put the feeds on manual and continue holding them down until liquid comes out of the needle into the fermenter. The base pump works at 6 rpm and the base pump takes approximately 6 minutes to prime itself and the acid pump works at 2 rpm so takes 3 times as long.

(d) Once the lines are primed, put the controller on automatic control, making sure the addition time is short (i.e. 2) and the mixing time is long (i.e. 8).

- Connect the condenser water through the appropriate ports on the base unit, first attaching the water out quick connect
- Turn down the speed of the rotor to 300 rpm.

The fermenter is now ready for you to leave it overnight. The next morning when you get in, the first thing you need to do is attach the dO probe to the controller and then turn on the controller to warm up for at least 30 minutes before you calibrate the probe.

While the dO controller is warming up, there are several operations you can do.

- Complete the medium for the fermenter

  (a) For modified Burk's, add the following to the batch flask;

  800 mL of 50% sucrose solution

  2.0 mL of iron citrate stock solution

  2.0 mL of Na_2MoO_4 stock solution
  20 mL of trace elements stock solution
  40 mL of $MgSO_4$ stock solution

  20 mL of $CaCl_2$ solution

  if required, fixed nitrogen source (for *Azotobacter* use 12.18 g of urea and for *Klebsiella* use 24.36 g of ammonium sulfate). First dissolve the solid in a minimum amount of water and sterifilter the solution.

  (b) Put the dip tube into the batch flask

  (c) Thread the tubing into the Masterflex pump head using the high flow rate Amicon pump.

  (d) Put the needle through the septum in a sterile manner and then pump in the medium. The pH meter will automatically adjust the pH of the completed media.

- The liquid cultures on the shaker table need to have their OD_{660}'s determined.
- The galvanic dO probe must now be calibrated (for complete explanations see the manual.

  (a) Zero: There are four different ways to zero the probe after the instrument has been warmed up for at least 30 minutes. The easiest way to accomplish this is to zero it electronically.

  (i) set the selector switch to galvanic probe-zero position

  (ii) Rotate the span control 5 turns from either end

(iii) Adjust the zero control until the dissolved oxygen meter indicates "0".

There has been nitrogen gas sparging the medium since the day before, so it should also be zero oxygen content.

(I) Turn the galvanic probe to operate and see if it still reads zero. There might be a slight adjustment required, but if there is a vast difference be concerned, there might be something wrong with the probe or the instrumentation.

(b) Setting the 100% oxygen.

(i) Aerate the fermenter with compressed air at the maximum pressure and rotor rotation for at least 3 minutes, making sure that there is not a build up of pressure inside the fermenter. If there is a build up, try opening the exhaust valve a small amount. You will hear gas coming out of the combined/exhaust unit.

(ii) With the selector switch on operate, the span control should be adjusted so that the meter reads 100%. There will be some noise in the instrument due to the vibrations of the fermenter, but the error should be $\pm$ 5 %.

(iii) The dissolved oxygen must now be brought down. First turn off all compressed air gas flow into the fermenter and then sparge it with nitrogen down to zero and then turn down the rotor speed to 300 rpm.

- The fermenter is now ready for you to inoculate

(a) The dissolved oxygen content must be brought up to about 10% with a slow bubbling of compressed air.

(b) The final innoculum is combined in the flow hood (i.e. two 500 mL cultures are poured into one flask.)

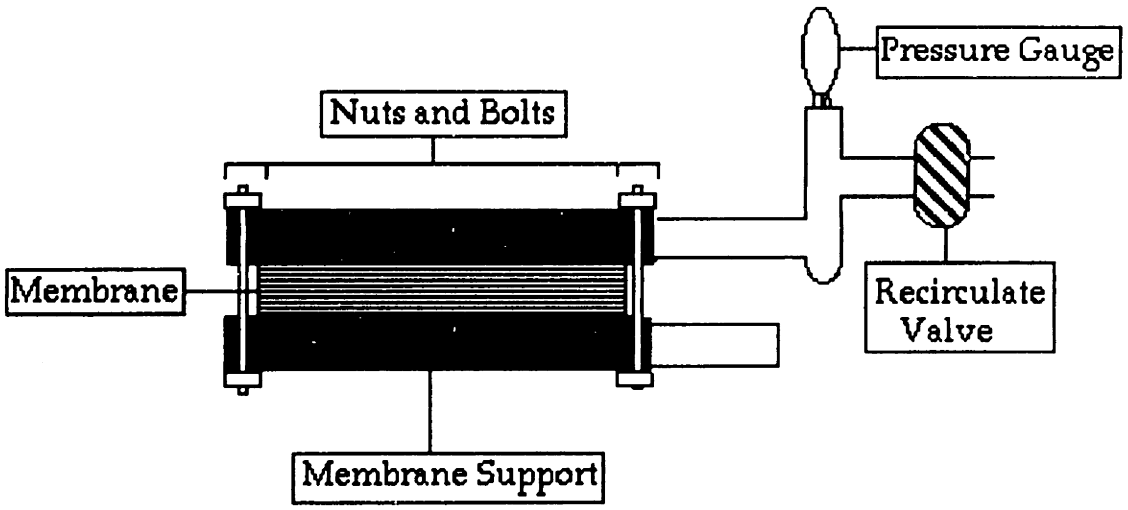
(c) The dip tube is put into the innoculum flask and the tubing thread through the pump. The needle is sterile attached to the fermenter and the bacteria are pumped into the vessel.

- Be aware that the initial 30 minutes of the fermentation requires close attention on the operators part. The dissolved oxygen must be stabilized at between 10-15% by adjustments of the rotor speed, the second stage of the compressed air regulator and the percentage flow on the base unit of the fermenter. It will be very erratic at low cell densities, but once the culture starts to grow, the oxygen content will stabilize.

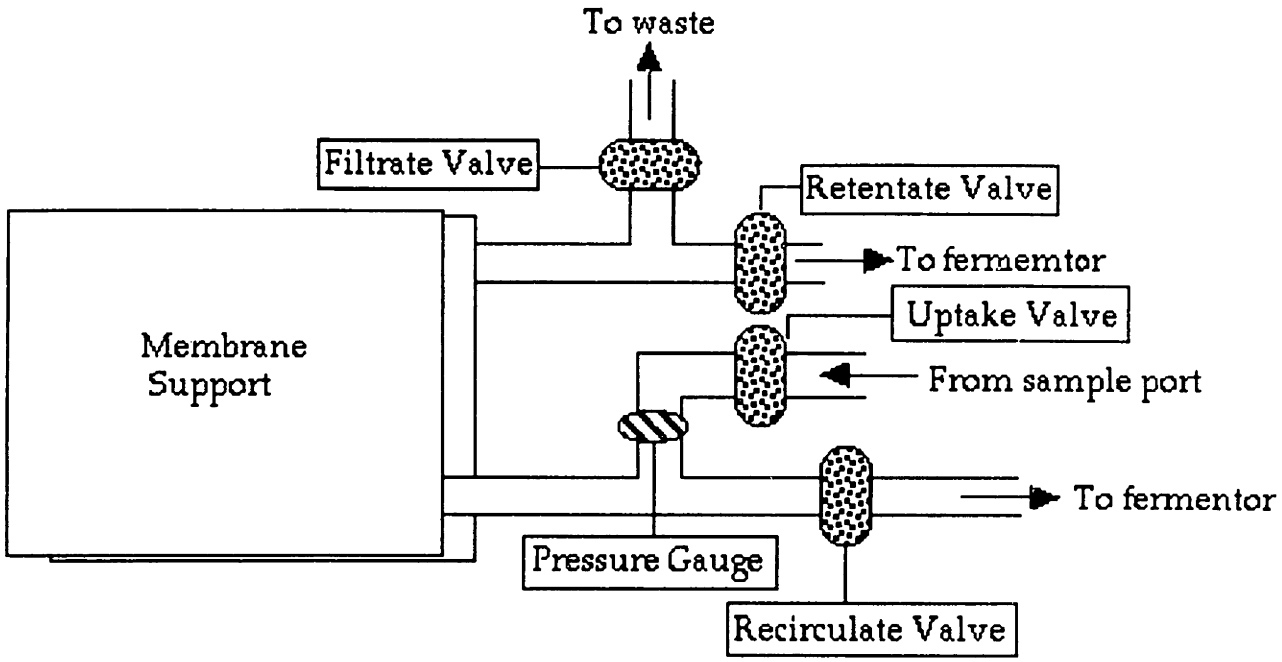
While the 20 L fermentation is occurring, there should be a log kept. Every fifteen minutes, the time, run time, rpm, gas pressure and dO should be written down. The OD_{660} and *in vivo* for nitrogen-fixing cultures should be done every two hours initially and then as the biomass nears its maximum, every hour. When the maximum biomass/activity ratio is achieved, harvesting needs to be done. There are two different means of harvesting, using the Pellicon Concentrator (usually for mutant runs when an initial repression fermentation occurs before the induction of nitrogenase) or the Sharples centrifuge.

Pellicon Concentrator

Pellicon Concentrator Side View



Pellicon Concentrator Top View



The Pellicon Concentrator provides a means of being able to concentrate down the cell broth to volumes of 1-2 L. It is necessary to be able to do this procedure when you have to wash the bacteria of undesirable metabolites. For instance, when the cells need to be derepressed after an initial repressed nitrogenase growth, the remaining fixed nitrogen in the media must be removed before the cells will be induced to produce nitrogenase. The Pellicon Concentrator enables this to be done quickly.

The membrane in the Pellicon must be taken care of. If it is not cleaned and stored properly, then the concentration of the fermentation becomes very difficult. You must realize that after using the Pellicon, cleanup will take about 90 minutes. During the initial fermentation, the wash solutions need to made up. The first wash is a NaCl wash, use about 750 g

NaCl in 18 L of distilled water. The second wash is a 0.1% Triton X-100 (18 mL Triton into 18 L of distilled water) heated up to 45 - 60° C. How each of these cleaning solutions are used will be discussed later on. The Pellicon Concentrator should be stored under 0.1% Roccal (an antibacterial/antialgae solution). You should never store the Pellicon under a bleach solution, it dissolves the graphite fingers in the Procon pump. When it is time to use the Pellicon, it must first have the Roccal solution rinsed off the membrane and out of the tubing system. By circulating about 60 L of house distilled water through, the membrane can be considered free of the preservative.

The operation of the Pellicon concentrator is easy. First, open all of the valves (if the handles are parallel to the plumbing, then the valve is open, perpendicular, it is closed). Start the Procon pump by flipping the switch to on. Then by adjusting the retentate and recirculate valves, the filtration rate can be adjusted. When concentrating the cell broth, it is recommended that a 1:3 ratio of retentate and recirculate flow through be obtained. One should be careful not to pump air through the Procon Pump as the pump head has graphite vanes in it and if they heat up, they will break. Remember to remove all stir bars from solutions before using the Pellicon in them, as this will also break the graphite vanes.

Before concentrating cells, 4 L of 100 mM Tris should be circulated, followed by 3 L of degassed Tris. After the Tris washed, the tubing attached to the uptake valve is connected to the sample port at the bottom of the fermenter. The tubing attached to the retentate and the recirculate valves must have the bored out screws attached to them and then they are attached to the back ports of the fermenter. As long as there is overpressure in the fermenter, the hope is that the culture will not become contaminated with a foreign organism. The tubing attached to the filtrate tube is then put into a carboy with Lysol. The sample port is then opened all the way and the Procon pump started. The concentration is continued until the volume is reduced to 1-2 L being careful not to pump air through the pump head for any length of

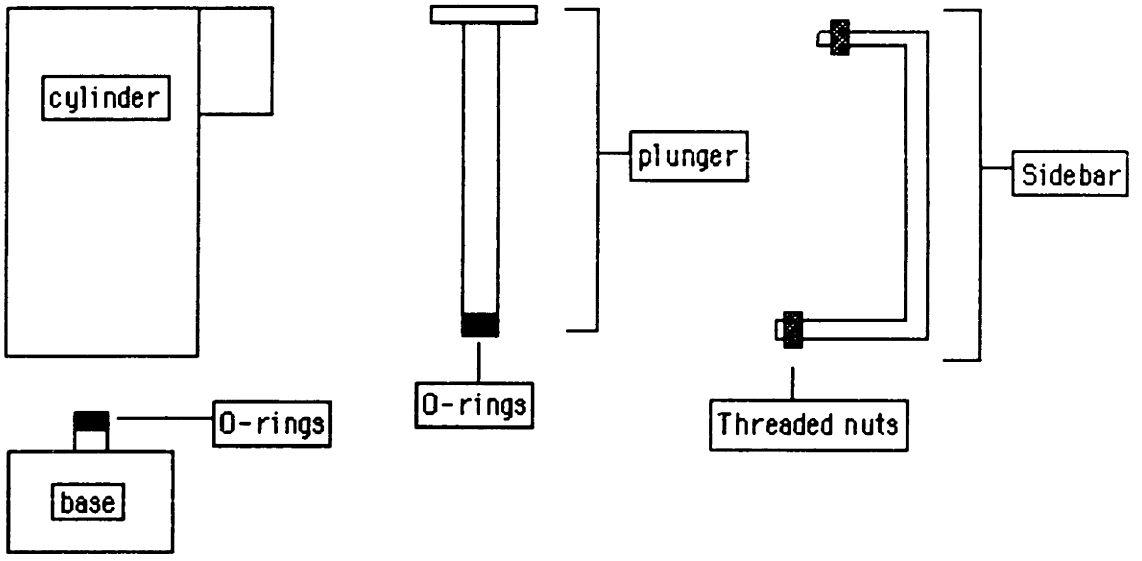
time. The pump is turned off and all of the valves closed. To remove the cell culture from the lines, the uptake and recirculate valves are opened. The sample port on the fermenter is closed and the tubing is removed while simultaneously starting the pump. After the line to the uptake valve is clear, the uptake tubing can be put into the resuspension media. The pump is started and once the recirculate plumbing appears to be washed out, the membrane can now be rinsed off. The retentate valve is opened and then the recirculate valve closed so that all of the media is flowing over the membrane. This reduces the cellular debris on the membrane. After the desired volume of media is pumped in, then the concentration is done once more, more media pumped in and then the derepression is started.

Now the Pellicon membrane must be cleaned. The tubing is first detached from the fermenter and the ends put into a bucket. The NaCl wash is first run through collecting the initial 4-5 L in the bucket. Once an initial wash is completed, then all the ends of the tubing are placed in the salt wash and the Pellicon put into an recirculating mode with most of the wash being forced through the membrane system (i.e. the recirculate valve is shut off completely). Once it appears that most of the cellular debris is removed from the membrane, the detergent (Triton X-100) wash is started. The membrane should be washed for 30-60 minutes with the heat remaining on the detergent solution. Once the membrane has been cleaned, it must be thoroughly rinsed with distilled water. After the water rinse is done, then the Roccal solution is run through the system, and cleaning is complete.

Monitoring Derepression of the Bacteria

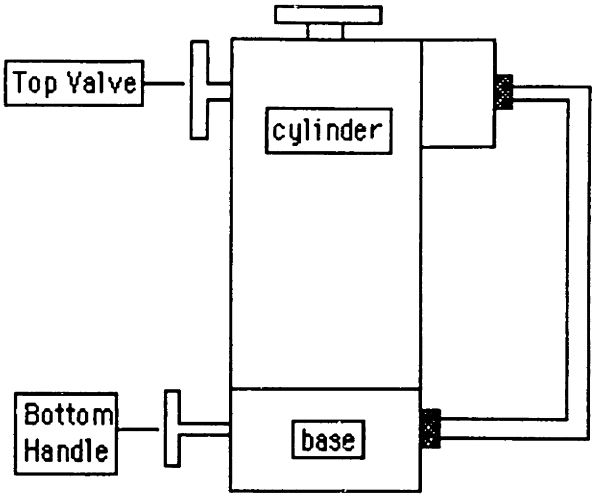
If you are growing a mutant, then *in vitro* assays must done during the derepression to determine the appropriate harvest time. Usually the amount of Fe protein that the bacteria has produced is used to determine the amount of derepression. It is easy to saturate the Fe protein using MoFe protein stocks once the cells have been cracked.

During derepression, the time it takes from sampling till injection into the gas chromatograph (GC) is about 1-1.5 hours. If you have two people doing the *in vitro* assays, the sampling times can be spaced every hour. It will help you doing the assays if you get organized before you start derepressing the bacteria. The cracking and rinsing buffers can be degassed (use 0.1 M Tris pH = 8.0 with 10 mM $Na_2S_2O_4$) and the French Pressure Cell can be put together and checked.



The French Press cell is kept in the cold room in a bucket. Make sure all of the pieces are clean. Grease the o-rings with silicon grease. Put the cylinder on the base and then put the plunger into the cylinder. Then thread the nuts into the base and cylinder and tighten with a wrench. Attach the long cannula to the back of the cylinder, and tighten with a wrench. If your preparation does not have to be anaerobic, then a piece of tubing can be attached to the threaded part of the back of the French Cell. Onto the base, screw into the output needle, and hand tighten. Then the handle needs to have a nylon ball put in and then attached to the base.

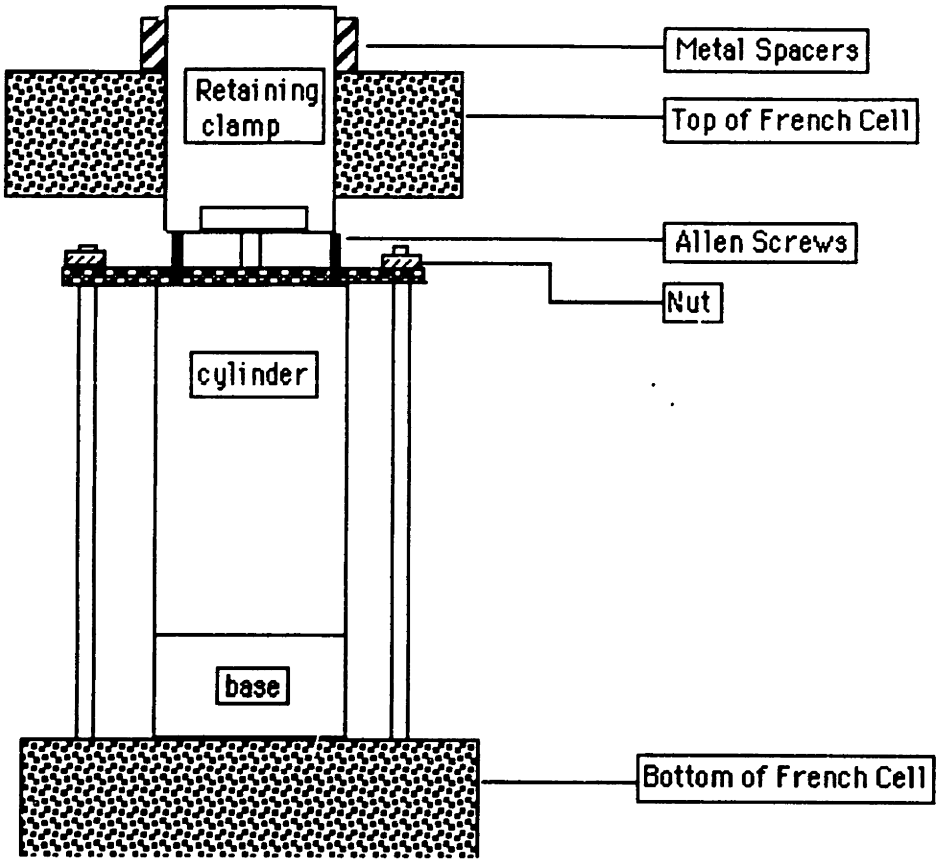
Assembled French Press Cell



The assembled cell needs to be placed into the French Press. The base is placed into position by the three metal spikes on the bottom of the moving press. The cell is held in place by the metal bar. The two nuts are tightened with a wrench and then the cell is held in place with the two Allen screws. The metal retainer clamp is slid onto the top of the French Press and the plunger is lifted so that the clamp is controlling its movement. The metal spacers are then put in to adjust the height of the clamp.

Once assembled, the French cell should be tested for leaks. The liquid is drawn up into the cell by first opening up the top valve, making sure the bottom handle is shut, and putting the end of the cannula into the liquid. The French cell is then selected for down and then the instrument is turned on. The instrument will automatically shut itself off once the bottom of the French cell is at the farthest depressed level. At this point, turn off the instrument and close the top valve. To achieve sufficient shearing forces, the pressure on the upward movement of the cell should reach 1500 psi before opening the bottom handle. To enable the plunger to move upward, the "high" is selected and the power turned on. When the desired pressure is reached, the bottom handle can be opened. The pressure can be maintained by opening up the bottom handle only slightly. If the pressure should

suddenly drop, then close the handle and allow the pressure to build up again. At this point, you should look for any signs of leakage at any joint in the cell. The leaks can be stopped by either tightening the metal pieces or Teflon tape can be put over the threads.



Once the cell has had buffer run through it and tested for leaks, the resuspended cell paste can then be cracked, using the same operation as for buffer. If you have to crack using anaerobic conditions, run reducing buffer through the French cell making sure it is still reducing to methyl viologen paper at the bottom needle. The cells are resuspended in reducing Tris, and sucked up into the cell after a reducing buffer rinse through a cannula. The cracked cells are then collected into a degassed receiving flask which has a bubbler attached.

Checking the Depression of the Bacteria

At every time point, 250 mL of bacterial culture is sampled from the bottom of the fermenter into a centrifuge bottle which is being sparged with nitrogen gas. The sample is then spun for 5 minutes to pellet the cells. The supernatant is then poured off while sparging the bottle and the pelleted cell is frozen in liquid nitrogen. The pellet is then placed in a preweighed vial, the weight of the pellet determined and after the frozen pellet has been degassed, 8 volume of Tris (pH 8.0, 100 mM) is added. Upon resuspension of the pellet, it is cracked in the French Press Cell and the cellular extract is collected. Typically 50 *ul* of extract is tested with stock MoFe protein using the standard acetylene/gas chromatograph assay. Upon maximum derepression activity, the fermentation culture is readied for harvest.

Harvest of Fermentation Culture

To help prevent the degradation of nitrogenase while the harvesting is taking place, the culture is chilled and put under a cold nitrogen gas. The gas feed is switched from compressed air to nitrogen which is run through a dewar of liquid nitrogen. To facilitate the chilling of the bacteria, the cooling water is run through a salt bath (a mixture of NaCl mixed with wet ice) and the set point of the heater is set at 10°C. The culture is cooled for 30 minutes before harvest with the Sharples Centrifuge.

Using the Sharples centrifuge

The Sharples centrifuge will produce a pellet of cells that can be immediately frozen into liquid nitrogen. It is a good idea to put the instrument together before you need it and test it to make sure it is working properly. The Sharples is a air driven turbine system that uses the MIT house compressed air to drive the bowl. The fermentation broth is pumped into the bottom of the centrifuge and the cells are forced to the outside of the bowl and

the supernatant flows out the top. For complete diagrams and description of operation, see the manual (blue binder).

- To assemble the bowl, a liner must be inserted into the bowl. You can buy them from Sharples, but an overhead projector sheet can be cut down to fit inside as well. The bowl bottom is then put on and the bowl bottom wrench is used to tighten. The tool is banged on the floor three times to ensure that the bottom is on tightly.
- The assembled bowl is then inserted into the centrifuge frame.
- The bowl cover is then slipped over the top of the bowl. Make sure the spout of the cover is attached to the discharge tube.
- The square nut is then inserted into the bottom of the centrifuge. This will hold up the bowl to the spindle.
- Attach the drag assembly into the frame pointing it in the desired direction. The tubing that will be used to pump the cells into the centrifuge can now be attached.
- The coupling nut is then tightened using the wrenches, making sure that the wrenches are parallel to prevent any unnecessary torque put on the spindle.
- The cover latch is then placed in the down position
- The front of the frame is then attached using the six wing nuts.
- Turn on the rotation counter
- Attach the cooling water lines to the sink and turn on the water.

The centrifuge should now be checked for maintenance of speed. The main air valve is turned on and then the air flow is gradually turned on using the secondary valve. You should listen for any unusual grinding noises. There will be some "bumpy" noises until the speed is up to about 5,000 rpm. At this point, there should be a constant whine. If is seems very loud, make sure the muffler is on. Once the speed of the centrifuge is at 40,000 rpm, begin to pump in water. There will be some initial slow down in the speed of

rotation, but at no time should the speed drop below 39,000 rpm. If you are unable to maintain the speed, make sure that there is sufficient air flow into the centrifuge (usually around 20-25 psi). If that is not the problem, then something has not been tightened properly. Shut off the air flow, take it apart and retighten. Then try it again. Once the speed is maintained, watch the flow of the liquid out of the centrifuge. It should come out of the tube attached to the discharge tube, not out of the frame cup drain cock. The outflow should be in pulses, and should be full of air, but upon sitting for a few minutes, becomes clear.

Once you are sure the centrifuge is operation, for an anaerobic harvest, one liter of degassed Tris is run through with a continuous argon purge on the top. The fermentation broth can be directly pumped into the Sharples centrifuge from the fermenter. The outflow should be checked for clarity. As a precautionary measure, the outflow should be collected and be killed with a bactericide (i.e. Lysol).

Now the fermentation broth can be spun down. Once the entire culture has been spun down, the air flow is shut off and the bowl is allowed to slow down. While you are waiting for this, the water cooling lines can be detached from the sink and emptied of their water. Once the bowl speed is at zero, the centrifuge can be disassemble in the exact reverse order of assembly. When the bowl is removed from the frame, it can be brought into a glove bag and the bottom removed. The cells will be against the side of the bowl up against the liner. The liner can be removed using tongs and immediately immersed into liquid nitrogen. The frozen cell paste can be broken up into smaller pieces and put into a pre-weighed plastic bottle.

All of the parts of the Sharples centrifuge must be disinfected, washed in soapy water and laid out to dry after a rinsing in distilled water.

Cleaning the Fermenter

Once your successful experiment is completed and the cells frozen away, the fermenter must be cleaned .

- Remove the dissolved oxygen probe and rinse with distilled water. You should inspect the membrane for integrity and then place it into storage solution.
- Remove the pH probe and remove any scum build up with paper towels. Place it into the cleaning solution (0.1 N HCl) and allow the solution to be stirred to clean the probe (usually 15-30 minutes). The probe is then rinsed with distilled water and placed into a saturated KCl solution.
- Rinse out the fermenter with water to remove any loose particulate matter
- Begin the cleaning cycle
  (a) fill up the fermenter with house distilled water
  (b) while it is filling, begin to heat up the fermenter by putting it through a sterilization cycle set at a final temperature of 70°C
  (c) once all of the rotors are covered, turn on the rpm to 300.
  (d) add one scoop of foamless detergent.
  (e) once the fermenter is full, close all ports except the center one and turn up rpm to 600.
  (f) allow fermenter to continue stirring and heating until all of the debris is removed from the inside of the fermenter including the underside of the headplate.
  (g) once the fermenter is clean, turn rpm down to zero and drain the detergent solution
- Rinse out the fermenter at least twice to remove any soap residue
- Fill up the fermenter with 2-4 liters of water so that the stainless steel ball bearings are covered and will not freeze up.
- Go home, have a shower and go to sleep.

References:

Bergey's Manual of Determinate Bacteriology, 8th edition (1974) R.E. Buchanan and N. E. Gibbons (eds. The Williams and Wilkins Company, Baltimore. p.p. 253-255, 321-324.

Appendix B: Team FeMoco's Guide to Protein Purification

Appendix B: Team FeMoco's Guide to Protein Purification

The purpose of this guide is to provide a summary of the optimized purification procedure that has been optimized by David Wright, Trish Humiston and Patti Christie. The raw data from which this guide was written is contained in the notebooks labeled Team FeMoco's "Protein Purification" (1991-1993) and the notebooks of Patti Christie. The organization of the sections corresponds to the order in which items must be undertaken to ensure an efficient process.

The first section is an time outline with brief description of a sample 1.5 kg cell paste purification preparation. It is designed to give you an idea of how an efficient preparation should go. It does not give detailed descriptions of each procedure nor does it tell you what items you should be preparing to start next. To have a successful purification, you must always be thinking about what needs to be degassed next, what dishes need to be washed, and above all use good glove box management. If you do not use excellent glove box management, the box will become too crowded and accidents will happen.

Following the time chart are the detailed instructions for how to do all of the techniques required for a successful purification.

Assay vials

In order to determine the amount of nitrogenase components you have, you need to be able to assay them. This lab uses an *in vitro* ATP-regenerating assay system. The required stock solutions are:

- 200 mM HEPES, pH 7.4 (4.77 g/100 mL). The pH of the buffer should be adjusted with NaOH. This solution is stable and is usually stored in the refrigerator.
- 100 mM $MgCl_2$(2.03 g/100 mL). This solution is stable and is usually stored in the refrigerator.

- 80 mM ATP(disodium salt), pH 7.4 (1.2 g/25 mL). ATP hydrolyzes in water, so this solution is made up only when required. It should always be kept on ice and the pH can be adjusted using cold NaOH.

There are two components required to prepare assay vials. These components are know as RM (reaction mixture) and CK (creatine phosphokinase). Since many of the components in these solutions are not stable at room temperature and because the need for assay vials is great, the RM and CK components are prepared and stored in the -20°C freezer in convenient aliquots which are able to be thawed and used quickly.

RM (Reaction Mixture)

This recipe will provide 100 mL of RM (20 aliquots of 5.0 mL each)

1.374 g creatine phosphate (a.k.a. Phosphocreatine)
50 mL of 200 mM HEPES, pH 7.5
25 mL of 100 mM MgCl2
25 mL of 80 mM ATP

While the ATP solution is being made, the rest of the components can be placed on ice. Then the components can be mixed together by gently swirling. The RM can then be aliquoted into 15 mL Falcon tubes in 5 mL aliquots. The RM tubes should be labeled with RM, date and initials and placed into the -20°C freezer.

CK (Creatine Phosphokinase)

A solution of 25 mM HEPES can be made by diluting 2.5 mL of the stock 200 mM HEPES, pH 7.5 into 17.5 mL Millipore water. Then 40 mg of creatine phosphokinase is dissolved into this solution. This solution is then pipetted into 1.0 mL aliquots into small Eppendorf tubes. These tubes are labeled with CK, date and initials and placed into the -20°C freezer.

Making the Assay Vials

Thaw on ice the frozen aliquots of RM and CK. Each 5.0 mL of RM and 1.0 mL of CK will make about 20 assay vials. While they are thawing, cool 12 mL of Millipore water for each 5.0 mL RM, an beaker to mix together the components, and 20 assay vials. The time required to thaw the ingredients is

2-4 hours, generally dependent on how impatient you are. If you are impatient, the components can be thawed by placing them into your hand. Otherwise, lay the tubes on top of the ice. When everything is thawed, mix them all together with a pipette, in the following ratio:

5.0 mL RM
1.0 mL CK
12.0 mL of Millipore water.

Put 0.9 mL of the mixture into the chilled 13.56 mL assay vials. Place a septum on the filled vial and then place into the -20°C freezer. The assay vials can be stored for up to 6 months, but once they are thawed and chilled on ice, they should be used within four hours.

Assaying for Nitrogenase Proteins

Besides being able to reduce N_2 to NH_3, nitrogenase is able to reduce the triple bond in acetylene (C_2H_2) to a double bond, thus producing ethylene (C_2H_4). This substrate is used in the routine assay of the components. To complete the assay of MoFe or Fe proteins, the following stock solutions are required:

- degassed 25 mM Tris with 10 mM $Na_2S_2O_4$ (to be used to rinse the syringes)
- degassed 0.1 M $Na_2S_2O_4$ in Tris (to provide the reducing equivalents)
- 5 mL of degassed 1 M $Na_2S_2O_4$ in Tris (used to spike the protein solutions
- and to make the degassed Tris reducing)
- 30% trichloroacetic acid (TCA). This is used to stop the assay by precipitating the proteins.

Once the solutions have been degassed and made up, the ensuing steps should be undertaken:

1. Degas and allow to thaw the nitrogenase component you are using as a standard. For instance, if you are assaying MoFe protein you have purified, then a stock Fe protein will be used, and visa versa.

backfills should be argon and the last two backfills will be 10% acetylene in argon.

3. The numbered assay vials are then made reducing by the addition of the 0.1 M dithionite solution. The overpressure is released by a bubbler. The vials are then placed into a 30°C water bath set at 80 rpm for *in vitro* assays.
4. The MoFe protein is usually added first to the assay vial. Upon the addition of the Fe protein, the time is started. The length of the assay is usually 10-30 minutes, whatever time is convenient.
5. The assay is stopped by the addition of 0.1 mL of the TCA solution.
6. 100 µL of headspace gas is injected into the GC. The peak of ethylene produced is reported in GC boxes. The conversion of GC boxes to enzyme activity will be discussed later.

Sample Time Chart for a 1.5 kg Cell Paste Purification:

Day -7 to -1
-regenerate all of the DEAE
-check all of the cracking apparatus, do a dry run of cracking the cells
-stock up on supplies i.e. enzymes, assay vials, gas tanks, Tris buffer, NaCl

Day -3 -degas the DEAE and bring into the box
-begin pouring the first DEAE column

Day -2 -continue pouring the column and pack overnight

Day -1 -spike the DEAE column with dithionite
-make the cracking solutions

Day 1 -7:30 am -make the required cracking solutions reducing
-weight out 1.5 kg of frozen cell paste
-transfer the diluting the cells buffer into the flask with the cells and then put in tepid water bath to thaw
-noon- prepare the MG and chill the cells
-1 p.m. -crack the cells
-2:30 p.m. -start spinning the cells in the centrifuge
-3:30 p.m. -start degassing 0.07 M NaCl in Tris wash and bring into the glove box
-7:45 p.m. -bring the centrifuged cells into the box
-8:30 p.m. -decant of the supernatant from the centrifuged cells and begin to allow it to equilibrate to room temperature
-9:10 p.m. -begin loading the supernatant onto the DEAE column
-start degassing the high and low salt buffers

Day 2 -4 am -begin the Tris wash
-6 am -begin the 0.07 M NaCl wash and bring into the glove box the high and low salt buffers.
-4 p.m. -begin the linear gradient
-8 p.m. -begin collecting fractions

Day 3 -2 am -stopped collecting fractions and started doing assays
-8 am -spiked the fractions containing the Fe protein and brought the fractions containing the MoFe protein into the glove box
-while the protein is concentrating, the 2nd DEAE column is poured, packed and spiked, so that it is ready for the concentrated MoFe protein to be loaded
-8 p.m. -begin loading the protein onto the 2nd DEAE column
-begin concentrating the fractions with the Fe protein

Day 4 -2 am -rinse the lines out with Tris
-3 am -begin the shallow linear gradient
-6 am -start collecting fractions
-the Fe protein should be finished concentrating soon
-11 am -start doing assays
-5 p.m. -bring the fractions with the MoFe protein into the box and begin concentrating them in the Amicon
-begin to pour, pack, and spike the third DEAE column

Day 5 -4 am -begin to load the third column with the protein
-10 am - rinse the lines out with Tris
-11 am -begin the shallow linear gradient
-2 p.m. -begin collecting fractions
-7 p.m. -begin doing assays

Day 6 -2 am- bring the fractions containing the MoFe protein into the box and begin concentrating them in the Amicon
-8 am -pellet the concentrated protein into liquid nitrogen
-Do the MoFe activity assays and the modified Lowry assays
-**Clean up**

Preparation of the Column material:

DE-52 must first be regenerated (even if it is brand new) before use. Refer to the instruction package for information. As of June 1993, the instructions were as follows:

- 1. Wash with 0.5 M NaOH. Using the NaOH, add enough base, so that there are equal volumes of DE52 slurry and base. Let sit for 30-45 minutes to equilibrate, stirring frequently with a glass rod or plastic spatula. After each wash, filter off the base from the DEAE using a Buchner funnel until a crack appears in the material. The number of washes required depends on the number of occupied ion exchange sites and pH of material. If the material is new, 2 washes are usually sufficient. For used DE52, one should wash the DEAE until the filtrate is clear.
- 2. Wash the material with water. The initial rinse can be done while the material is still in the Buchner funnel after the final base. The purpose of the water washes is to remove as much of the base washes as possible before you adjust the pH with Tris buffer.
- 3. Wash the DEAE with Tris until it is at pH 7.4. This process can be facilitated by washing it once with 0.5 M Tris, then two washes with 0.25 M Tris and then enough washes with 0.1 M Tris until it is at pH 7.4. Then wash it several times with 0.025 M Tris to equilibrate the material. It is important that the pH is exactly 7.4; a high or low pH will destroy protein.

The DE-52 is now regenerated and can be either put into the cold room for long-term storage or degassed on a gas train. Check the pH before you put it on the line.

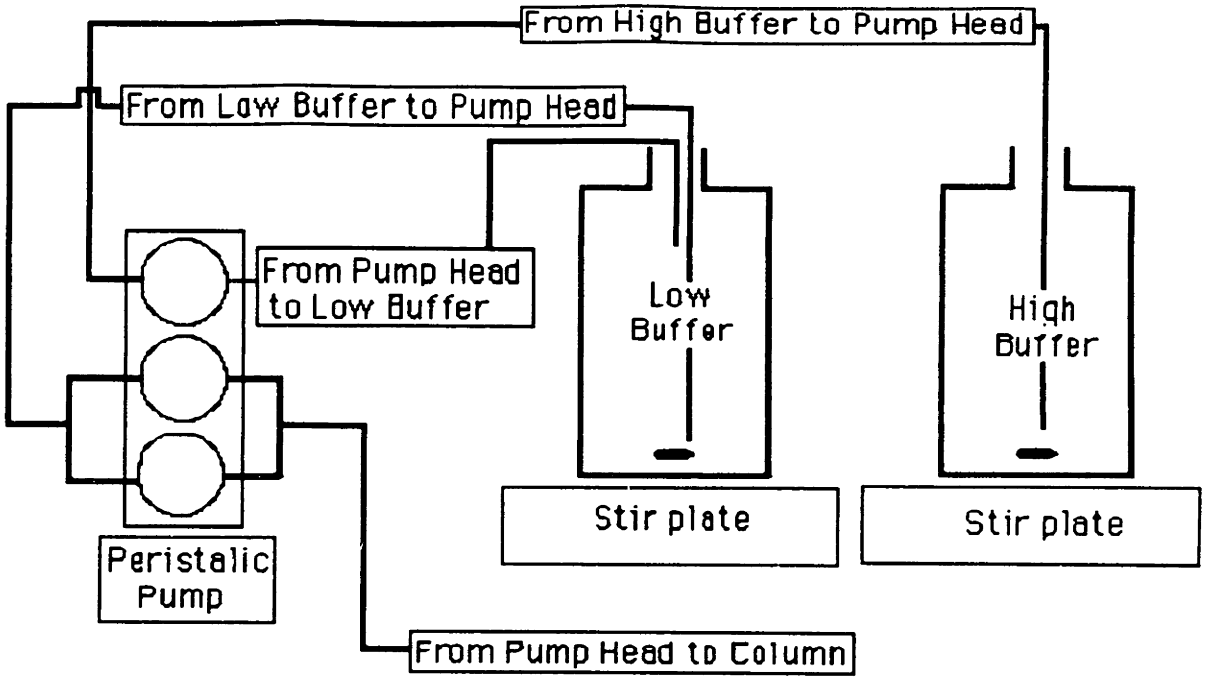
If the material is to be degassed, it has to be slurried and put into Wheaten bottles. The slurry should be thin enough so that when the bottle is swirled on the line, the entire solution becomes mobile. This increases the rate of exchange between oxygen and argon. The DE52 should be degassed on the line for about 6 hours. When it is backfilled, it may be taken off the line, shaken, to allow the gas to be equilibrate throughout the material and placed back on the line. This will help reduce the amount of oxygen introduced into the anaerobic box when the bottle containing the material is opened.

The Pump and Tubing System:

The tubing system consists of three peristaltic pump heads and the appropriate size Masterflex tubing (see figure below). It is very important to ensure that the tubing system is working before you bring it into the box. This saves a lot of trouble and frustration. The usual tubing size is Masterflex size 13 for the pump heads and 14 for the rest of the tubing. Silicon tubing is fine for aqueous systems.

The pumps consist of one pump head for the high buffer and two pump heads for the low buffer. Be sure to label each end of the tubing because all the pump heads have to be threaded in the same direction. Typical labels are as follows:

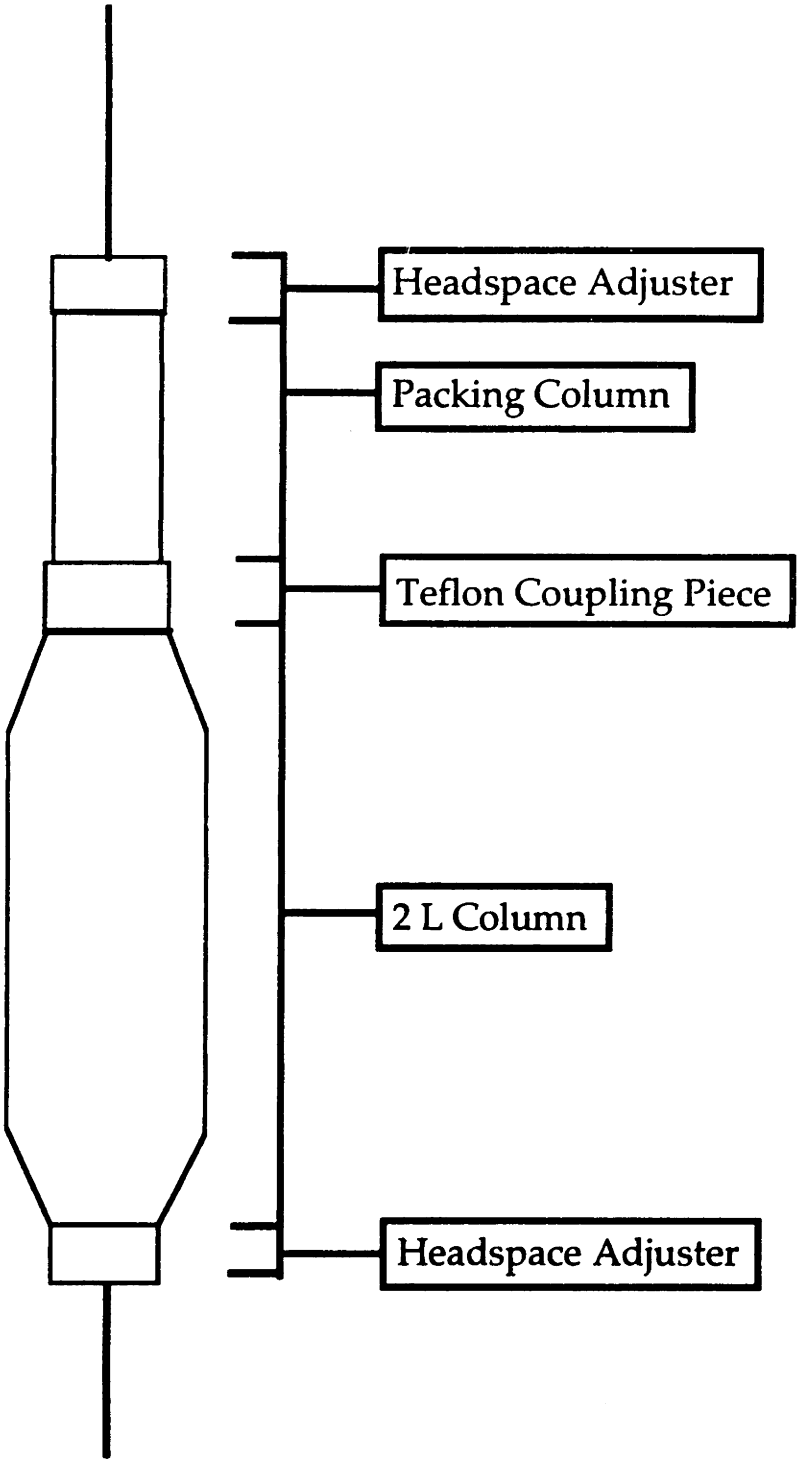
- High tubing: one end: from high buffer to pump head
  other end: from pump head to low buffer
- Low tubing: one end: from low buffer to pump head
  other end: from pump head to column



If the tubing system works outside the box with water, then the pumping system can be brought into the box with the pump heads already threaded. It is good laboratory practice to rinse the lines out with water once you are finished, especially after running a high salt gradient. If you do not rinse out the lines, once the water evaporates the salt remains behind to clog up the tubing.

Pouring and Packing a Column:

For the purpose of this guide, unless otherwise stated, the concentrations of Tris buffer is 25 mM, pH = 7.4 and the $Na_2S_2O_4$ is 2 mM. All solutions should be prepared with Millipore water.



Set up the empty column in the box with the reservoir attached. To ensure even packing, everything needs to be level in all directions (i.e. front to back, sideways). Make sure that the column bottom is turned off. Pour some buffer in the bottom of the column. Shake up the DEAE slurry and pour into the column. Depending on the size of the column, this could take more than one

bottle of slurry, so make sure that all of the necessary bottles are shaken up before you start pouring. Once some of the DEAE has formed a bed on the bottom of the column, then open the bottom of the column and allow for the excess buffer to flow through using gravity to pack the column. Once the level of the buffer is just above the column bed, more column material must be added. First stir the top of the bed using a glass rod, and then add additional slurry of material. Once the column bed has packed by gravity and the level of the material is well into the reservoir, then the top of the column can be put on.

Make sure that there are no air bubbles in the tubing and that the buffer is flowing through the top of the headpiece. If the level of the buffer is at the absolute top of the glass column, open the bottom of the column and put the headpiece onto the top of the column with the controller on the lowest possible setting. Do not worry about the buffer flowing from the top, this limits air bubbles that are trapped. Once the head piece has been screwed on, then the column packing can begin.

It is best to **gradually** start to increase the flow rate of the column. The pumping system can be put on infinite loop (i.e. all the ends come out of the same reservoir, including the column flow through) as long as there is no reducing buffer in the system. Packing usually takes overnight.

Once the column is packed, the excess DEAE must be removed. This is best accomplished by removing the reservoir with the headpiece still attached and placing it into a beaker. This causes a vacuum that is released when the headpiece is removed. This minimizes the spillage of column material. The headpiece can once again be put on and the column flow rate once again brought back up to maximum speed.

Spiking the Column:

Once the column has been packed, it must be spiked with sodium dithionite to scrub the column of its oxidizing equivalents. The volume of 1M $Na_2S_2O_4$ in 25 mM Tris depends on the column size. Usually for the 2L

columns, 30 mL of dithionite is used. The spiking solution is added to the column and the speed of the buffer flow through is reduced to allow the dithionite to equilibrate on the column. The buffer that is run through the column must now contain 2 mM dithionite and can not be recycled through the column due to degradation byproducts of the dithionite. Once the column is reducing to methyl viologen paper, then it is ready to be used.

Cracking the Cells

The following procedure is written on a 1.5 kg cell paste scale and using the APV Mantin Gaulin Cell Homogenizer (MG). The scale of the preparation can be varied according to what is required. When using the French Press to crack cells, usually a 1:2 dilution of cells in 0.1 M Tris, pH 8.0 is used. The large scale cracking uses pH = 7.4 Tris but still at 0.1 M concentration. A detailed description of the large scale preparation can be read in Thanos Salifoglou's notebook #1 pages 49-61. The changes in the method are included in this guide. He gives a good description of the various connection of the tubing, transfer lines and glassware. It is a very good introduction to the procedure.

Days before:

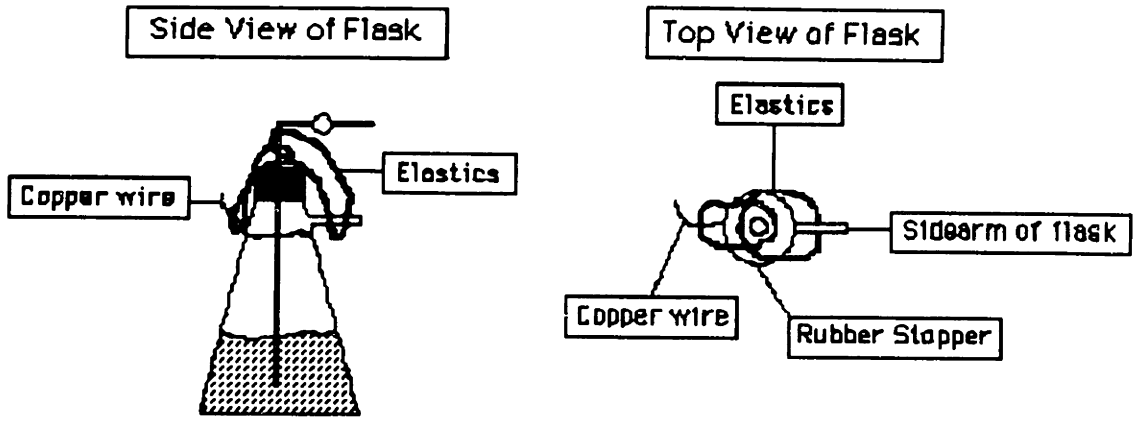
Make sure that all of the cracking apparatus is in working order (i.e. all joints are greased, all connections are tight) and nothing needs to be fixed. Do a dry run of the homogenizer to ensure that it is working and that you can keep all of your solutions reducing.

Day before:

Prepare the following solutions and degas them. When attaching the headpieces, use elastics and copper wire to hold the stoppers in the vacuum flasks.

- Diluting the cells - 1.75 L of 0.1 M Tris, pH 7.4 (Solution #1)

- Make MG reducing - 1.8 L of 0.1M Tris, pH 7.4 (Solution #2)
- Rinsing out the MG - 1.8 L of 0.1 M Tris, pH 7.4 (Solution #3)

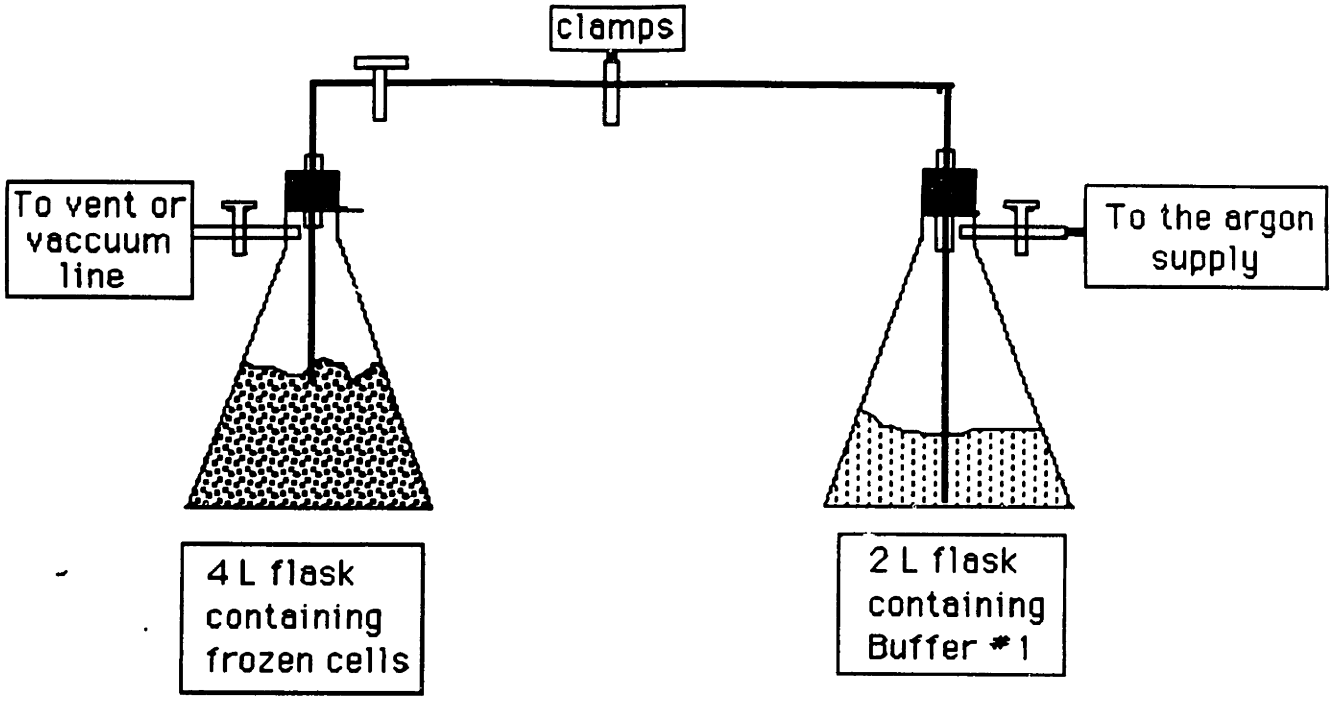


Day of cracking:

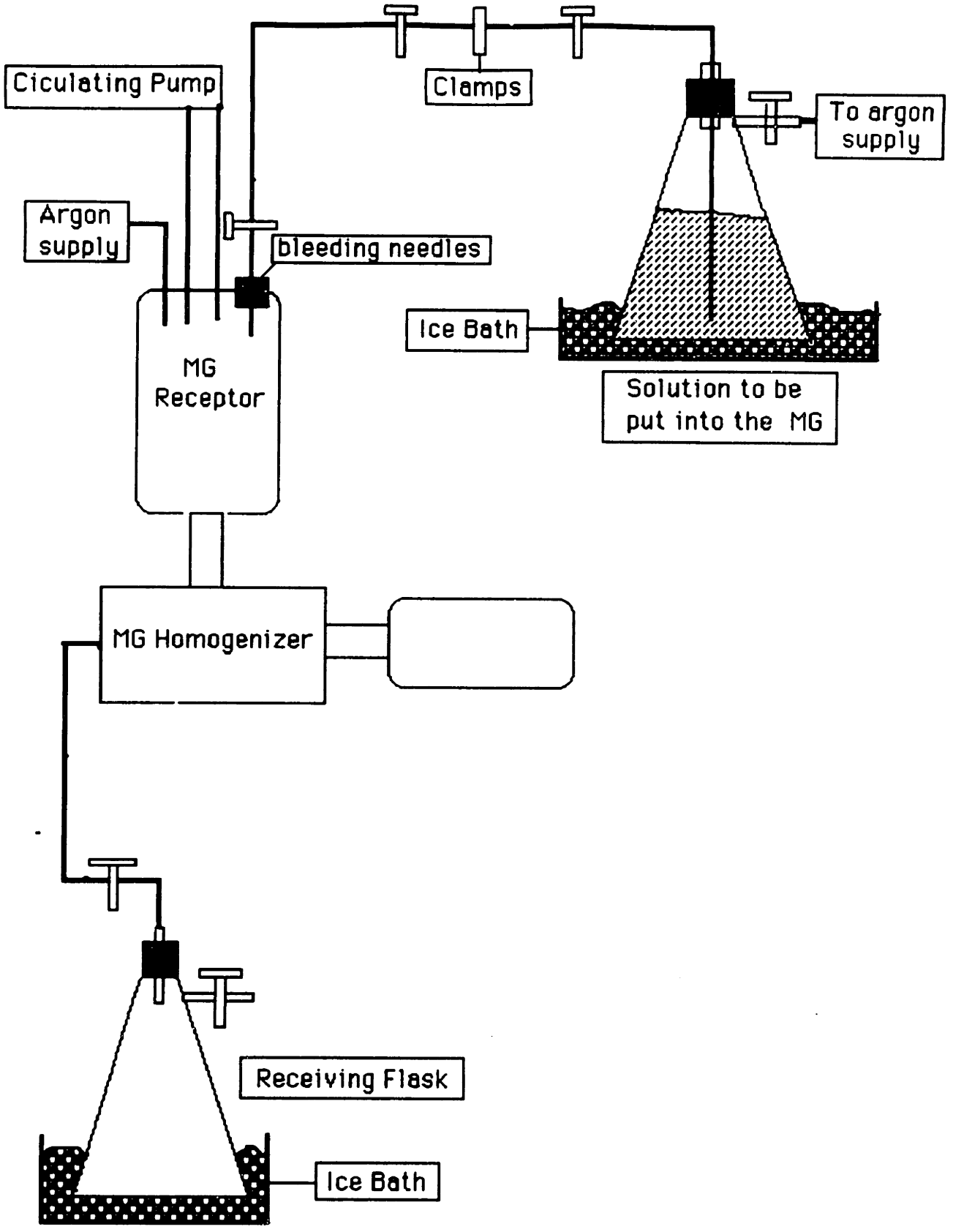
If the cells are stored in the -80°C freezer, equilibrate them in buckets full of liquid nitrogen. This will make the transfer of the cells easier.

Solution #1 (diluting the cells solution) must be made reducing (20 mM) either by (a) cannulating enough 1 M dithionite solution or by (b) adding solid dithionite. Either addition is made when the flask holding the solution is over pressurized with argon. After the addition of the dithionite solution, the flask should be cycled under vacuum a few more times to remove any oxygen that was introduced into the flask.

Approximately 1.5 kg of cells must be put into the Cell paste flask and 50 mg of each RNAse and DNAse are added. The cells are cycled 5-7 times on the gas train. The cells should be under maximum vacuum for 1 minute during each cycle. The vacuum/backfill with argon cycle will take approximately 5 minutes. Once the cells have been degassed, then the diluting cell buffer must be added anaerobically to the frozen cells. The transfer line must first be degassed from the cell paste container by purging the lines with argon.



This is accomplished by over pressurizing the lines with gas and then releasing the ball and cup glass joint. This process should be repeated until the air in the line has been completely replaced with argon. To transfer liquids anaerobically, the vessel from which one is transferring is put under argon and the vessel to which you are transferring is put under vacuum. Once the level of the liquid reaches 3 liters (the maximum volume that can be centrifuged at once), then the cells are put under argon and the transfer line is disconnected (once the appropriate stopcocks have been closed). Check to see if the buffer in the line is still reducing to methyl viologen paper. The cell paste can now be put into a tepid water bath and allowed to thaw. This will take about 3-4 hours. One should swirl the cells periodically to help them resuspend and bleed the over pressure of argon in the vessel by cycling the stopcock on the side arm of the flask. The other cracking solutions should be made reducing as this point. If you are cracking more than one set of cells, the diluting buffer for the second set needs to be degassed at this point.


Ciculating Pump
Clamps
To argon supply
Argon supply
bleeding needles
Ice Bath
MG Receptor
Solution to be put into the MG
MG Homogenizer
Receiving Flask
Ice Bath

Once the cells are thawed and into solution, then the homogenizer must be prepared while the cells are chilling on ice. First ensure that there is enough argon in the tank to purge the homogenizer. Next, start up the circulating pump. This is used to keep the cracked cells chilled. If the pump does not begin circulating the water immediately, it is due to air bubbles trapped in the pump. These can be removed by lightly tapping the pump in the water and ice mixture. Once the pump is circulating, make sure that there is sufficient ice to keep the circulating water cold. You will have to remove the water before you add more ice to keep the top of the pump from being submerged.

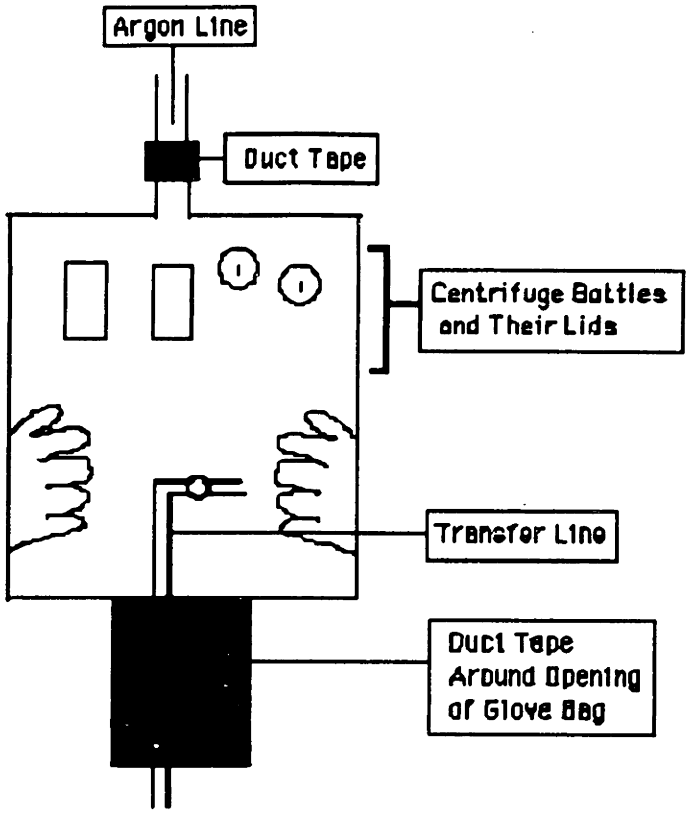
Before operating the homogenizer, you should read the manual. An abridged version of the operation follows. Make sure the bottom valve is in the closed position. The solution that you are going to homogenize is first pushed into the homogenizer by using argon, ensuring that all of the lines are purged before you open the stopcocks to the degassed solutions. Turn on the homogenizer and listen for any unusual sounds, i.e. grinding or the piston not moving back and forth correctly. Then begin the apply the pressure by turning the hand wheel. When the hand wheel is initially turned, the needle on the pressure gauge will fluctuate. The needle will be steadied by turning the dampener. Once the needle is steadied, the appropriate pressure can be applied through the turning of the hand wheel. The solution is then homogenized for the appropriate time. The pressure is released by backing off on the hand wheel and releasing the dampener. The solution is then pumped out by opening up the bottom valve and using the homogenizer to pump out the solution.

During the cracking procedure, there should be a positive pressure of argon throughout the homogenizer. If at any time stoppers pop off **don't panic**; the container is over pressurized and you haven't let oxygen in. To crack the *Av* cells the following order of solutions is used in the homogenizer:

1. The homogenizer is first made reducing by running the "Making MG reducing" (Solution #2) through it. It is best to do this in two stages. The first half of the solution is to ensure that the MG is working and that the solution stays reducing until you pump it out and the second half is done just before you put the resuspended cell solution through the MG as a final oxygen scrub.
2. The chilled resuspended cell solution is then run through. While you are pushing it into the homogenizer, you should swirl the solution to make sure that the unsuspended cells at the bottom of the vessel get into the MG. The cells are cracked at 9000 psi for 8 minutes.
3. As soon as the MG is being used, the cell receiving flask needs to be degassed with 6.5 mg of leupeptin and 5.0 mg of pepstatin in the bottom. This ensures that once the cells are finished being homogenized, then the cell extract can be pumped into this flask. The receiving flask first has to be connected to the bottom of the MG and the appropriate lines purged before the cracked cells can be pumped into the flask.

It is at this time that at least three people are helpful, to make this as efficient as possible.

- **Person A** -will do the heat treatment on the homogenized cell extract. The cell extract is heated at 65°C for 5 minutes on a pre-equilibrated water bath set at 85°C. This entire procedure takes approximately 28 minutes for 3 L of cell extract to heat up to the desired temperature with the gentle swirling of the flask. The cell extract is then cooled by swirling it in a bucket of ice for 20 minutes. The homogenate is then ready to be transferred into centrifuge bottles.
- **Person B** - Will start thawing the second set of 1.5 kg of cells. Following the same procedure as the first set.
- **Person C** -will start preparing for the transfer of the cell extract into centrifuge bottles. This is done by using a glove bag.



The bag is duct taped to the argon line on the bench in the prep room and the centrifuge bottles and lids are put into the bag. Make sure you have 12 bottles and 12 matching lids with O-rings intact before you put everything into the glove bag. If you put some methyl viologen paper in the glove bag, you can check to see if the homogenate is reducing after the transfer. The glove bag is purged and then closed up with duct tape with the stopcock end of the transfer line in it.

- **Person D** (if possible) -acts as a rover, cleaning out the MG, helping with the transfer of the diluting buffer to the second set of cells, putting the rotor into the centrifuges.

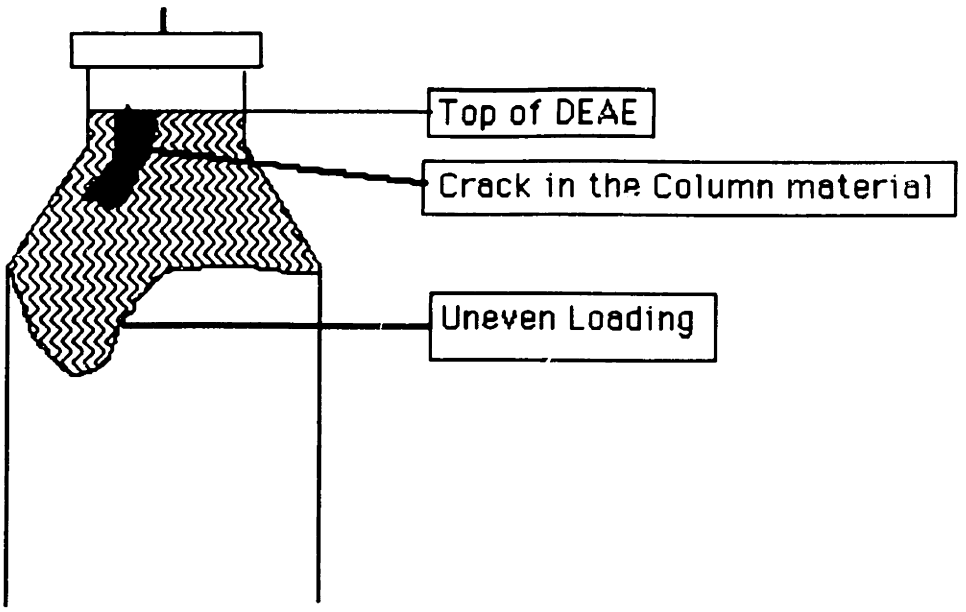
Once the heat treatment is completed, the glove bag should be under argon and the cell extract is pushed into the centrifuge bottles from the receiving flask through the transfer line. You have to fill up the bottles to just below the lip of the bottle in order to have all of the 3 L of cell extract go into the centrifuges. Make sure all of the lids of the bottles are tightly closed.

The homogenate can be checked to see if it is reducing before opening up the bag and removing the bottles. The cell extract is spun at 10 - 13 K for 4-6 hours at 4 ^{o}C. The ultimate goal is to get a firm pellet that will not go soft in the time it takes to get into the box.

Running Column #1 - DEAE

The centrifuge bottles are brought into the box and the supernatant is decanted into a 3L round bottom through a long-necked funnel. Try to not get any of the cellular debris from the pellet into the supernatant. This will only plug up the tubing and it is not part of the cell that you want. You should check and see if the solution is reducing. One liter of reducing Tris is added on top of the supernatant. The supernatant is then allowed to equilibrate to room temperature for about an hour. The warming up of the supernatant prevents the top of the column from cracking due to the temperature differences in the supernatant and the column buffer. At this point you should indicate the volume of the supernatant (i.e. draw a line on the round bottom indicating the volume, then measure the volume once the flask is out of the glove box) and save a sample (i.e. 0.5 mL) of the supernatant for assays. This will give you a starting point of protein activity, concentration and purity.

The protein solution is then pumped onto the column using the low lines (the high lines can be kept in the Tris buffer on infinite loop) Watch and make sure that the ends of the lines are kept in the solution. The protein solution is really dark so this can be difficult. Watch the top of the column for uneven loading.



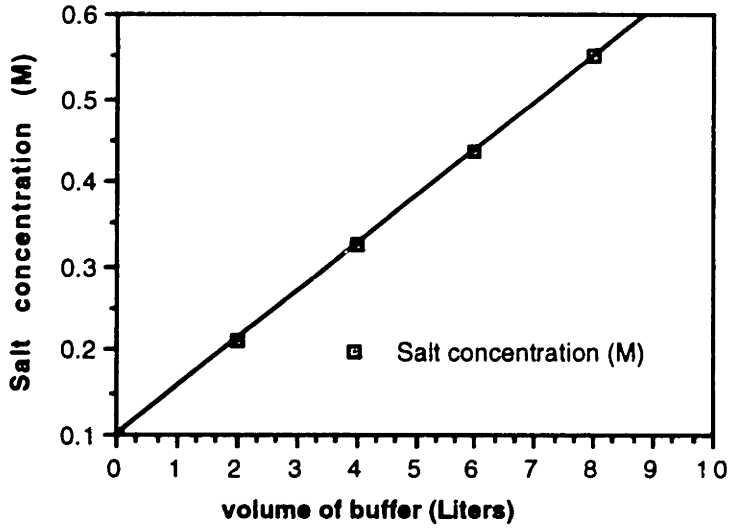
This can be due to a number of things including; (1) the glass frit popping out of the head space adjuster, (2) cracks in the bed of the column or (3) that the column is not level. As you are loading the column, there might be some darkly colored flow through. Don't panic, this is not your protein. If uncertain, a simple activity assay will confirm this. If you have to take off the top of the column at any point during the loading process, rinse out the lines and the head space with Tris before you take off the top in order to clear protein from the tubing.

Once all of the protein is loaded, rinse out the lines with Tris. This enables all of the protein to be loaded onto the column before you start the gradient and also allows you to see the top of the column to check for cracks. After the Tris wash, then the low binding proteins are eluted with a wash of 0.07 M NaCl in reducing Tris. Four liters of this wash is usually used for the 2 L column. There will be a lot of light coffee or yellow colored proteins eluded off the column but the MoFe and Fe proteins, which are black/brown/dark in color will stay on the column.

The linear gradient is then started. The high buffer contains 0.55 M NaCl in reducing Tris and the low contains 0.1 M NaCl in reducing Tris. As the gradient is run through the column, you can see the bands of proteins

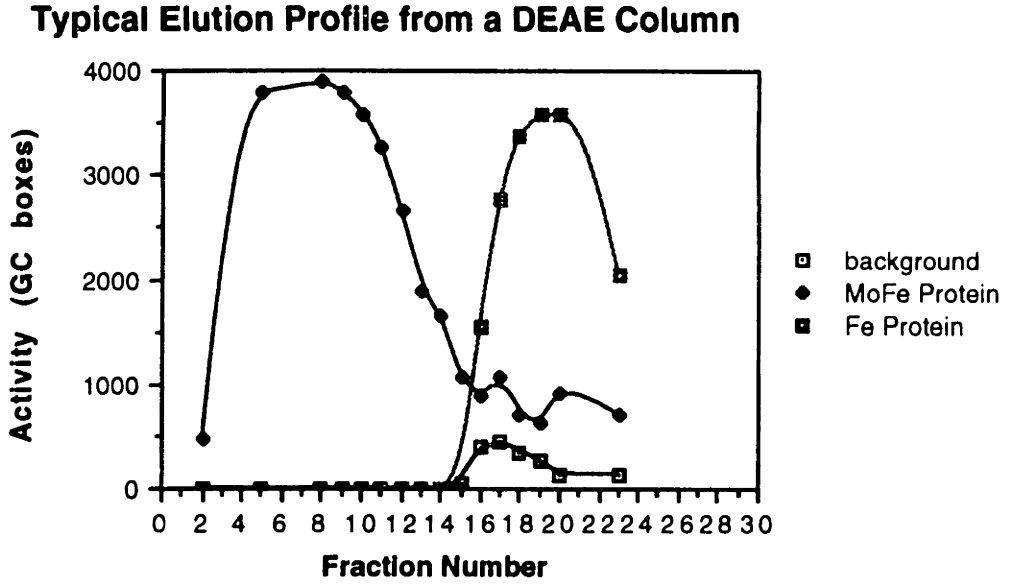
begin to develop. It is a good idea to draw pictures of the developing bands. You do not need to start collecting fractions until the dark brown/black band begins to elute off the column. The initial flow through will be coffee colored which is oxidized flavoprotein. When you do start collecting fractions, a good volume to collect is 150 mL. Collect fractions until the Fe protein has come off. This usually occurs when either: (1) the flow through does not have any color anymore, or (2) when there is less than 1 L left in both the buffer reservoirs. A column gradient profile will indicate when the buffer is the necessary strength to elute the MoFe and Fe proteins (see figure below). The MoFe protein elutes at 0.18-0.25 M NaCl and the Fe protein at 0.25-0.35 M NaCl.

Determination of Salt Concentration on a Column



The activity assays must now be done. If is not necessary to do every fraction. A good strategy is to do every second or third one and then at the critical points do every fraction. You should check and make sure that the fractions are still able to reduce methyl viologen paper. If there are not any residual reducing equivalents, spike the solution with a 1 M $Na_2S_2O_4$ in Tris stock. The best way to visualize where the MoFe and Fe proteins are is to draw a column profile, with the axis labeled fraction number and activity (see figure below). First do a background to find out where the Fe and MoFe bands

overlap. Depending on the concentration of the protein in the fractions, anywhere from 25 - 50 µl is a sufficient volume to assay. Then, using the most concentrated Fe protein and MoFe protein fractions, do the cross reactions. You will have to go back and assay the fractions at the cross over point of the bands. By looking at the elution profile, you can determine which fractions contain the MoFe protein which ones contain the Fe protein.



From the graph above, the MoFe protein is contained in fractions 2-15 and the Fe protein is contained in fractions 16-23. You will always be able to separate the MoFe protein from the Fe protein, but it is difficult at this salt concentration to remove the contaminating MoFe protein from the Fe protein. The MoFe protein in the Fe protein can be separated using a high salt gradient on a sizing column (see further purification of the Fe protein).

Calculation of Yields of Protein:

To calculate the yield of protein from the column, you must convert the GC boxes of ethylene produced to nmoles acetylene reduced/min/mL. This requires you to know the amount of ethylene per GC box using an standard. To make up the standard sample, degas the calibrated round bottom (i.e. a 500 mL round bottle that has had its volume measured) and a

180 mL Wheaten bottle. Remove the round bottom from the gas train backfilled with argon. Remove the Wheaten bottle under vacuum and backfill it with ethylene. The excess pressure from both of the containers is removed by attaching a bubbler (a disposable plastic syringe with water in it). Then 50 μL of ethylene is removed from the Wheaten bottle with a gastight syringe and put into the round bottom flask filled with argon. Then 100 μL of that mixture is injected into the GC. To calculate the nmole of ethylene in the round bottom flask, use the following formula:

$$n = \frac{PV}{RT} = \frac{1\text{atm}\left(\frac{0.1\text{mL}}{\text{Vol. of RB}} \times 0.1\text{mL}\right) \times \frac{1\text{L}}{1000\text{mL}}}{0.08026\frac{\text{L.atm}}{\text{K.mol}} \times 295\text{K}}$$

n = nmoles ethylene

Then, you must calculate the nmoles of ethylene per box (the Magic Number). From the injection of 100 μL of the standard you injected into the GC, measure the number of boxes. Take the number of nmoles of ethylene you injected (calculated from the above equation) and divide by the number of GC boxes. This will give you the "Magic Number" (M# for short).
To calculate the activity of the sample, use the following formula:

$$\text{Activity} = \text{M\#} \times \frac{\frac{\text{Sample (GC Boxes)}}{\text{Standard (GC Boxes)}} \times \frac{\text{vol. of Headspace}}{0.1\text{ mL}}}{\text{Amt. of sample added} \times \text{min. of assay}}$$

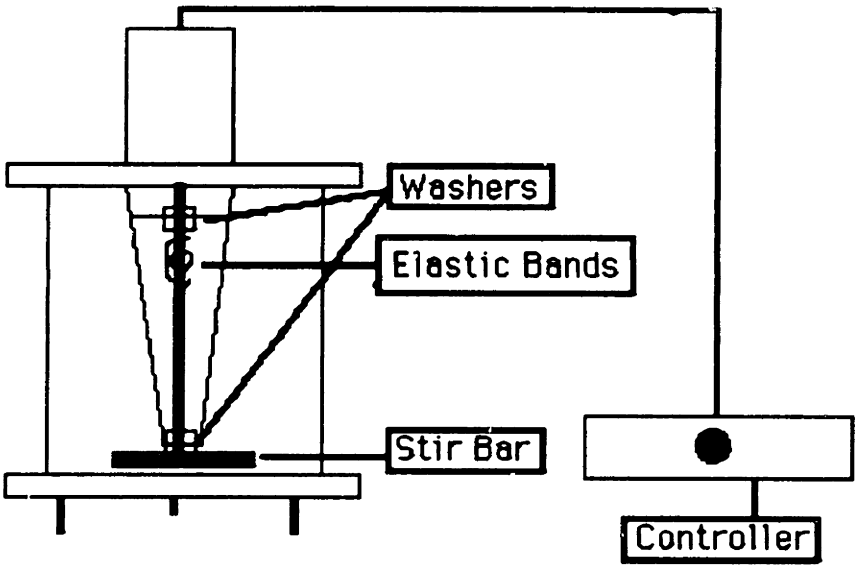
This will give you the activity of the protein sample in C_2H_2 produced/min/mL of sample.

To calculate the number of grams of protein in the sample, you must first multiply the activity by the dilution factor then by the total volume of the sample. This will give you the number of units present in the sample. To convert units of activity to grams of activity, you must divide by the

specific activity for the given organism. For *Av*, the number is 2400 units/mg of protein and for *Kp*, the conversion factor is 1800 units/mg.

It is also very important to calculate the specific activity of the sample. The specific activity of a sample refers to the nitrogenase activity per total gram of protein present. Modified Lowries (after TCA precipitation) will determine this number. For a complete description of this procedure, see the detailed instructions at the end of this guidebook. It is not necessary to do Lowries on all of the fractions collected from the column. It is sufficient to do this assay on the concentrated fraction from the first, second and third DEAE columns to see an improvement in the purity of the protein.

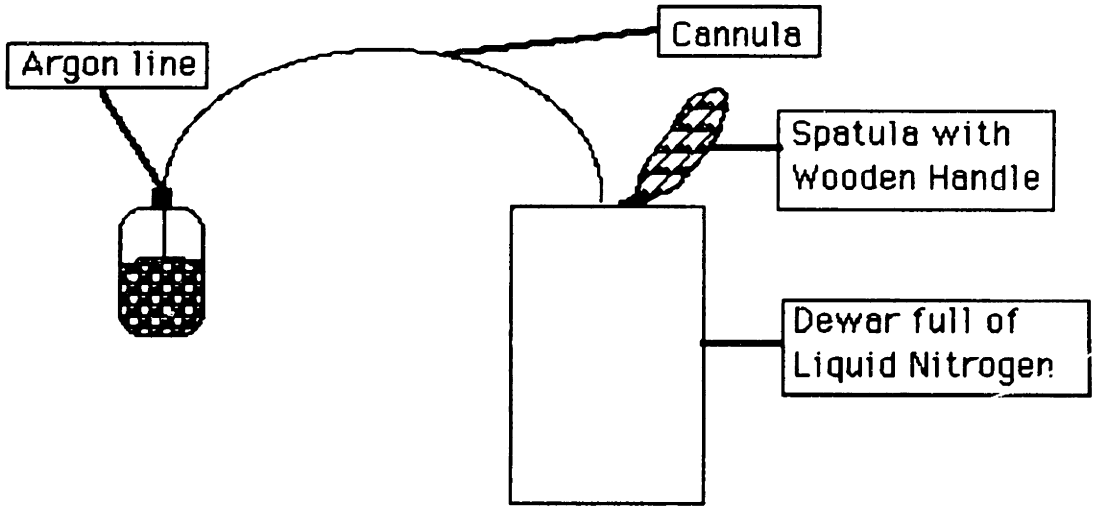
Concentrating Protein in the Amicon:



The fractions containing the MoFe and Fe proteins are then concentrated separately in the 2.5L Amicon. You should concentrate at least 10 fold. Use a YM 100 membrane for the MoFe protein and a PM 50 (or PM 30) for the Fe protein. The Amicon should have reducing Tris buffer run through it before you put the protein in it because the membranes are preserved with sodium azide and this needs to be washed out before your protein gets put on. This Amicon is able to have up to 125 psi of pressure run

through it. If the Amicon stops stirring, then the elastic on the shaft needs to be changed. Due to the high usage of this Amicon, the plastic washers do not stay in place. The elastic band keeps the top washer held tightly around the shaft of the stir bar. The MoFe protein should be concentrated before the Fe protein. When the desired concentration volume has been reached, the protein is pumped out of the Amicon. First, the Amicon is stopped and the pressure is released from inside the vessel. The pressure of the gas running the Amicon is reduced to no more than 5 psi. Application of this pressure into the Amicon will enable the protein to be pumped out with the minimum amount of protein denaturation.

Once the protein has been pumped out, then it must be pelleted. You should first put electrical tape around the septum; this will prevent them from popping off during the cannulation.



The protein is pelleted by pushing it out of the bottle, through a cannula and into a dewar full of liquid nitrogen. The bottle with the protein is put under argon on a gas train with Schlenk lines with needles on the ends. The cannula is then purged with argon by placing one end in the headspace of the bottle with the protein. The cannula is then put into the protein solution and the protein is pushed out of the bottle and into the liquid nitrogen. The stream of protein is protected from the oxygen by the dithionite. As the

protein is frozen in the liquid nitrogen, it should be broken up into small pieces by a spatula cutting through the protein stream.

The Fe protein can be spiked with dithionite and put in the cold room. It is stable for about 24 hours. If you are not going to be able to concentrate it within that time, pellet it into liquid nitrogen through a cannula. The MoFe protein will be put down another DEAE column and the Fe protein will be eventually run down a S-200 sizing column in 5 mL aliquots or through a MonoQ column in 3 mL aliquots.

Second DEAE column

While the MoFe protein is concentrating, the second DEAE column should be poured, packed and spiked, so that you are ready to load the protein when it is finished concentrating. Because the protein is in about 0.25 M NaCl, the protein solution needs to be diluted with reducing Tris at least four fold to a salt concentration less than 0.07 M NaCl, so that the protein will bind to the column. Take a sample of the protein before you start loading it onto the column. Once the protein is loaded onto the column, the lines are rinsed out with reducing Tris and then the linear gradient is started. A low salt wash is not necessary because those proteins have been removed during the first DEAE column. The linear gradient for this column is shallower than the first. It runs from 0.1 M NaCl in reducing Tris to 0.35 M NaCl in reducing Tris. The gradient is run, fractions are collected and activity assays are completed. When doing the assays, an Fe protein sample must be degassed and thawed for the cross-reaction assay. Once again, the elution profile should be drawn and the fractions chosen by observing the graph. The chosen fractions are then concentrated in the 2.5 L Amicon ten fold.

Third DEAE column

A carefully run third DEAE column can increase the percentage of MoFe protein in solution. This third column is done the same as the second DEAE column. The tighter the band of MoFe protein is, the better the

resolution; so load the protein solution carefully. This third column is required to separate the MoFe protein from proteins which elute at the lower salt concentrations. Watch the movement of the MoFe protein down the column and make careful cuts in fraction collection. When you concentrate the protein band, do so in two parts; the middle fractions and the two side fractions. This will increase the purity of the protein solutions.

In addition to MoFe activity assays, TCA precipitate Lowry protein determination should be done at this point. This procedure has been modified from one obtained from Normand Cloutier. This will determine if the MoFe protein makes up at least 50% of the total protein in solution. This is required to crystallize the MoFe to homogeneity.

Modified Lowries (TCA precipitation)

It is important to determine the purity of the MoFe protein before you attempt to crystallize it. If it is not 50% of the total protein in solution, the crystallization procedure will not be successful. The modified Lowries are used because of interfering substances (i.e. Tris) in the protein solution.

The following stock solutions need to be on hand:

- 24% (w/v) trichlorocacetic Acid in water
- 2% (w/v) Sodium deoxycholic acid in water
- Lowry Reagent A - 100 g Anhydrous Sodium carbonate, 20 g sodium hydroxide in 1 L of water
- Lowry Reagent B - 1% (w/v) Cupric sulfate (1 g $CuSO_4.5H_2O$ in 100 mL water)
- Lowry Reagent C - 2% (w/v) Sodium Tartarate (2 g $Na_2C_4H_4O_6.2H_2O$ in 100 mL water)
- Folin's and Ciocalteu's Phenol reagent (2.0 Normal)
- Bovine Serum Albulum (BSA) stock solution of 0.3 mg/mL. The one that is easiest to use is the preweighed vials (Sigma) which can be diluted up to the desired concentration. It is best to use the buffer system to dilute up the lyophilized protein.

The unknown protein concentration samples must be diluted so that the resulting concentrations falls in the linear range of the assay. The linear

range is from 0-0.3 mg/mL total protein. Once the protein samples have been initially diluted then the sample test tubes are made up. If you use 13 mm x 100 mm test tubes, they will fit directly into the HP UV/Vis spectrophotometer. This will make the reading of the samples quicker. The following is an example of typical dilutions used for this assay:

Test tube #	Am't protein added	Am't water added	Total vol.
1	0 mL	1.0 mL	1.0 mL
2	0.2 mL BSA	0.8 mL	1.0 mL
3	0.4 mL BSA	0.6 mL	1.0 mL
4	0.6 mL BSA	0.4 mL	1.0 mL
5	0.8 mL BSA	0.2 mL	1.0 mL
6	1.0 mL BSA	0 mL	1.0 mL
7	0.1 mL dil prot. #1	0.9 mL	1.0 mL
8	0.25 mL dil prot #1	0.75 mL	1.0 mL
9	0.50 mL dil prot #1	0.5 mL	1.0 mL
10	1.0 mL dil prot #1	0 mL	1.0 mL
etc.			

All the samples should be measured using the same Pipetman. Once the samples have all be made up, the procedure is as follows:

- Add 8.3 µL of sodium deoxycholic acid solution to each tube and vortex
- Add 0.3 mL of TCA solution to each tube and vortex
- Centrifuge to produce a well spun down pellet. Depending on the speed of centrifugation and the type of centrifuge being used, the times vary. For instance, using a clinical centrifuge, the time is between 30-60 minutes, but for the GSA adapted rotor, the speed is 5.5 K and it takes at least 60 minutes. The longer the tubes are spun, the better the pellet.
- Decant off the supernatant using an aspirator
- Add 1 mL of water. The pellet will probably not resuspend, but don't worry the Lowry solutions will do that.
- Make up the Lowry solutions with the following ratios: 30 mL of reagent A, 1.5 mL of reagent B and 1.5 mL of reagent C. Mix together well and add 1 mL to each sample. Vortex the samples to ensure that the pellet goes into solution. Let sit 15 minutes at room temperature.

- Dilute the stock Folin's reagent in a 1 to 10 ratio with water. For instance, I mL of reagent is diluted by 10 mL of water. After the first 15 minute incubation, add 3 mL of diluted Folin's to each sample and vortex. Let sit additional 45 minutes at room temperature.
- Read absorbances at 660 nm.

The standard curve should give a correlation of at least 0.990. The unknown protein samples can then have their concentrations calculated based on their absorbances.

If the MoFe protein solution is at least 50% to the total protein, then the next step in purification, crystallization can be undertaken. If the MoFe protein is not the desired purity, then additional DEAE columns should be undertaken, making careful cuts in the fraction collection.

MoFe Protein Crystallization

The purpose of the crystallization procedure is to produce homogenous solutions of MoFe protein. The procedures has been adapted from Burgess et al., (1980) and has been optimized using *Av.* There are also other crystallization procedures in the literature (see: Shah and Brill, 1973; Burns et al., 1970; see also Burns and Hardy, 1984). Paul McLean in this lab also worked on crystallization of MoFe protein, and his notebooks are also a good source, especially notebook #7, starting on page 52.

This procedure produces homogenous material, **if you follow the following rules and follow the directions outlined in this guide.** The starting protein solutions should be at least 30% MoFe protein. The best yields occur when the starting percentage of MoFe is around 50% of the total protein. Based on a 50% MoFe protein solution that is concentrated to **at least 50 mg/mL MoFe,** you should be able to precipitate out 50% of the total MoFe protein. This is the maximum amount of MoFe you can expect to produce. You can use that as a guideline for determining the volumes of buffer

solutions you will have to add. This procedure has been use to crystallize 3 grams of MoFe out of a solution of 6 grams of MoFe protein, and also has been used on a solution of 0.5 grams of MoFe. You should always be trying to get half of the MoFe crystallized out of solution each time you do a preparation.

Supplies required:

-100 mL graduated cylinder (used to make the buffer solutions 2 mM $S_2O_4^{2-}$)
-funnel (solids funnel with a wide top to pour out from centrifuge bottles.)
-Wheaten bottles -5 X 180 mL
-2-3 X 60 mL
-12 X 9.56 mL (to make up samples)
-some 25 mL
-syringes - 1 mL and 100 µL
-Pipetmen -1000 µL and 200 µL
-tips for both
-plastic pipettes - 5 mL, 10 mL, 25 mL
-pipette helper
-conductivity meter and probe
-septum
-centrifuge bottle(s)
-glass rod
-scintillation vials and caps (8 plus enough for samples)
-2 X 3.48 g $Na_2S_2O_4$ (for stock 1 M solutions)
-400 mL Amicon (or the appropriately sized Amicon) plus membranes (YM 100)
-stir plate
-glass Pasteur pipettes and bulbs

Stocks required:

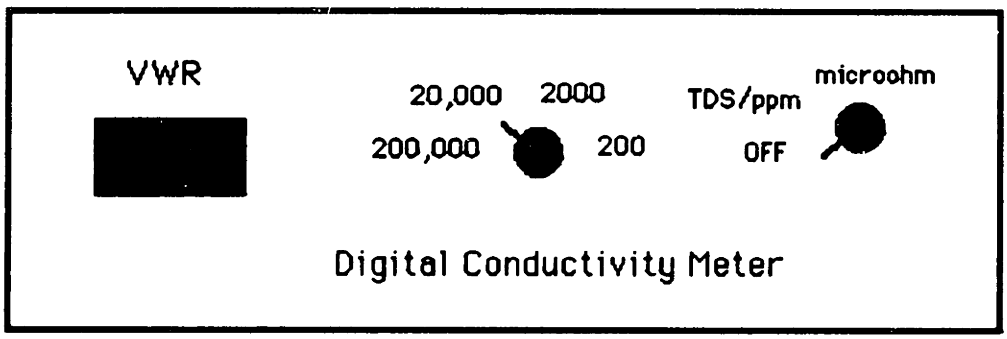
-2 M NaCl in 25 mM Tris, pH = 7.4 (use for standard curve for conductivity)
-5 M NaCl in 25 mM Tris, pH = 7.4 (use for making all solutions 0.25 M NaCl)
-25 mM Tris, pH = 7.4
-0.028 M NaCl in 25 mM Tris, pH = 7.4
-0.25 M NaCl in 25 mM Tris, pH = 7.4

Day 1 - Getting Started with the MoFe Protein:

Day one of the procedure will take 12 hours. It will take longer if you are not organized, **so get organized** and do parts of the experiment in parallel! The majority of the time is spent concentration the protein solution, so you should have it concentrating in the Amicon as soon as possible. First, degas the frozen protein solution on the gas train and begin it thawing in tepid water on the lab bench. Once the solution is thawed, bring it into the glove box. The next step is to prepare the Amicon for use in the glove box. Rinse out the Amicon with reducing Tris (25 mM Tris plus 2 mM $Na_2S_2O_4$). All of the solutions should be made reducing with **2 mM $Na_2S_2O_4$**. If you use higher concentrations of dithionite, if will affect the solubility of the MoFe protein. Before you start concentrating, take a sample of the thawed protein (MoFe start). Then pour the protein solution into the Amicon and begin concentrating. Meanwhile, turn on the water bath or shaker table. Make sure it is set at 38°C. If you are using the shaker table, make sure that the setting is 150 rpm.

Operating the Conductivity Meter:

For this procedure to be effective, you must know the concentration of NaCl in the protein solutions. **All of the solutions contain 25 mM Tris, pH = 7.4 and 2 mM $Na_2S_2O_4$**. The conductivity meter is easy to operate.



There is a manual for this instrument. The following is an abridged version of the instructions. To measure conductivity, set the meter for microohms. The multiplication factors for the scales that you will use in this procedure are 10^3 for 200,000, 20,000, and 2,000. The probe should have a plastic cover over it. The probe does not have to be stored in liquid. It should be rinsed in 25 mM Tris between measurements, and should also have the excess buffer removed by gently shaking it before you place it in the next solution.

In order to measure the conductivity of the protein solutions, you have to make up a standard curve using known concentrations of NaCl to calibrate the instrument. An opportune time to do this is while the protein is concentrating. Before you start making up the various solutions, be sure to label the tops and vials of the solutions. The following chart gives directions on how to make up the required solutions for the standard curve. The conductivity readings are approximate. The numbers can vary plus or minus 10%, but should always result in a standard curve with a linear regression of at least 0.98. While you are making up the standard curve, you should be collecting a sample of the filtrate from the Amicon. You will use this to measure the starting conductivity of the protein solution.

mM NaCl	25 mM Tris req'd	2M NaCl req'd	1M $Na_2S_2O_4$	conduct
0	15 mL	0 mL	3 µL	2.0×10^3
14	14 mL + 0.89 mL	0.105 mL	3 µL	3.6×10^3
28	14 mL + 0.79 mL	0.210 mL	3 µL	5.0×10^3
56	14 mL + 0.58 mL	0.420 mL	3 µL	9.0×10^3
112	14 mL + 0.16 mL	0.820 mL	3 µL	17.0×10^3
224	13 mL + 0.32 mL	0.82+ 0.82 mL	3 µL	24.0×10^3
336	12 mL + 0.48 mL	0.82 + 0.82 + 0.82 mL	3 µL	36.8×10^3

To measure the conductivity of 0 mM NaCl, use the 2000 scale. The rest of the concentrations can be read on the 20,000 scale except for the last two concentrations, which use the 200,000 scale. Using a linear regression program, determine the equation for your standard curve. Use the standard

curve to determine the concentration of NaCl in the Amicon. The concentration of NaCl **must be** 250 mM in order for this procedure to be successful, so adjustment of the salt concentration will probably be necessary.

Crystallizing the MoFe Protein:

Concentrate the protein solution until it is **at least** 50 mg per mL. It is important to have it as concentrated as possible. Patience is required! Once the required concentration is reached, turn off the pressure to the Amicon, put the Amicon back on the stir plate with the top off, and turn on the stirrer.

You must calculate how much reducing (2 mM $Na_2S_2O_4$) 25 mM Tris you must add. The following is a sample calculation:

(a) you have 50 mL of 250 mM NaCl solution

(b) you want to have the final concentration to be 28 mM NaCl

$$M_1V_1 = M_2V_2$$

$$(250\ mM)(50\ mL) = (28\ mM)(V2)$$

$$V_2 = 446.43\ mL$$

(c) since you already have 50 mL, the volume of reducing Tris you have to add is 446.43 - 50 = 396.43 mL

You want to add the Tris slowly with a Pasteur pipette while the solution is stirring. The protein will begin to precipitate out as you get closer to the required salt concentration, but you must add all of the Tris . These initial crystals will act as seeds for the rest of the crystals. Once the buffer is completely added, stir the solution to insure even mixing. The diluted protein solution must be carefully poured out of the Amicon and into the centrifuge bottle(s), (use the funnel). The bottle(s) are then brought out of the box, and put into a 38°C water bath or shaker table for 60 minutes. Heat up the centrifuge and rotor to 38°C by spinning it for an hour at 38°C (the centrifuge is usually set at 4°C).

After the aging of the solution is complete, centrifuge the solution at

38°C, 10,000 - 13,000 rpm for 30-60 minutes (times vary depending on how big your pellet is, you want to have a very dense pellet). Reset temperature on the centrifuge to 4°C.

Bring the centrifuge bottle back into the glove box, and decant the supernatant into the Amicon. Take a sample of this solution and label it S_1. Adjust the salt concentration with the 5M NaCl solution so that the concentration is 0.25 M NaCl. Use the following formula:

$$M_1V_1 + M_2V_2 = M_f(V_1 + V_2)$$

For the example preparation, the numbers would be:

$$(0.028)(446.43) + 5(V_2) = (0.250)(446.43 + V_2)$$

$$12.5 + 5V_2 = 111.61 + 0.25V_2$$

$$4.75V2 = 99.11$$

$$V_2 = 20.87 \text{ mL}$$

The supernatant can now be concentrated while you continue working with the pellet.

Resuspension of the Homogenous MoFe Protein:

The MoFe pellet must now be washed with 0.028 M NaCl in 25 mM Tris Add enough buffer to cover the pellet (i.e. 50 -100 mL). To ensure that the pellet gets thoroughly washed, it should be broken up with a glass stirring rod. Put the centrifuge bottle back into the centrifuge (which is now at 4°C) and spin at 10,000 -13,000 rpm for 30 minutes.

Bring the bottle back into the glove box and sample the supernatant (S_2). Decant it into the Amicon and add enough 5 M NaCl solution to make the solution 0.25 M using the above equation and continue concentrating. Any MoFe protein that is in the S_2 is due to residual protein that was left in the bottle from S_1. There should be little or no activity in this sample.

You now want to suspend the pellet by adding an amount of 0.25 M NaCl in 25 mM Tris (i.e. 100 mL). The resuspended MoFe will be between 5-10 mg/mL, so use that as a guideline for the volume of buffer you add. Use a

glass rod to stir up the pellet and try to dissolve it. It won't all go into solution. There will be some white material and possibly some black rubbery precipitant. The resuspended protein is spun at 4°C, for 10 minutes, at 10,000 - 13,000 rpm.

Bring the solution back into the glove box. This is your crystallized MoFe solution. You should continue to concentrate the pooled supernatants to a reasonable volume (i.e. 150 mL). Be sure to take samples of both the MoFe crystallized and MoFe pooled (typical dilution factors, 1:20). Be sure to measure the volumes of these solutions to get an accurate estimate of the amount of protein present. Then pellet the protein samples separately into liquid nitrogen.

MoFe Sample summary:

At the end of the day, you should have the following samples:

	typical dilution factors
-MoFe start	1:15, 1:20
-MoFe S_1 (supernatant 1)	1:20
-MoFe S_2 (supernatant 2)	1:5
-MoFe crystallized	1:20
-MoFe pooled	1:20

There are internal checks for the recovery of the MoFe protein. MoFe pooled units of activity should equal MoFe S_1, and MoFe S_2. MoFe start units of activity should equal MoFe pooled and MoFe crystallized.

Day 2 - Assays "R" us:

This day you must do MoFe activity assays and modified Lowries (i.e. TCA precipitated Lowries). Earlier in this guide are the necessary procedures and calculations. You could also run SDS Page gels (i.e. Phastgels) to see the purity of the prep.

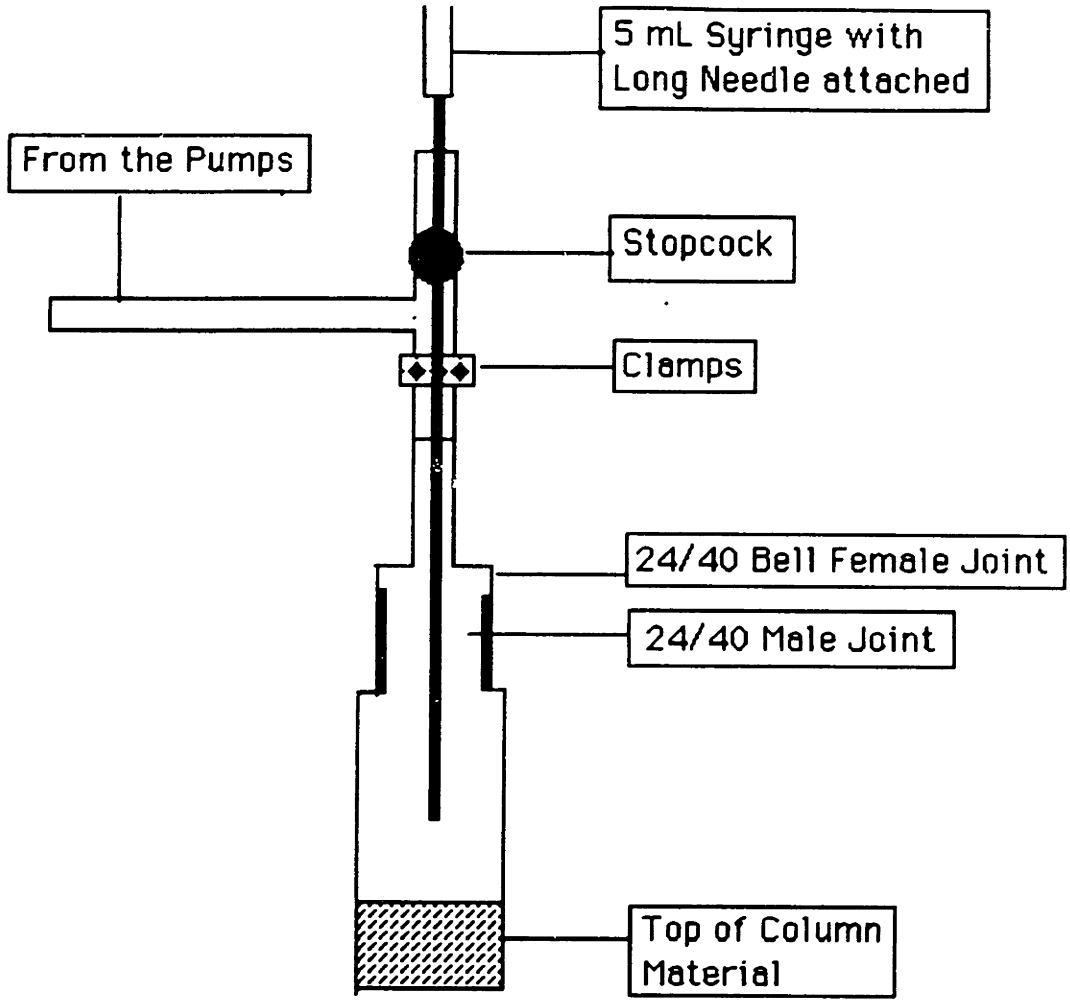
Further purification of the Fe protein

Once the Fe protein has been eluted off the first DEAE column and has been concentrated, it must be further purified. There are two different methods utilized in this lab. One way is to pass it down a Schlenk modified sizing column on the bench. The second way is to use a Source 15Q column inside the glove box.

The purification of the Fe protein using the sizing column will be discussed first. In order to disrupt the electrostatic interactions between the MoFe and the Fe protein, the column has to be run at high salt (0.5 M NaCl in 25 mM Tris, pH = 7.4, 10 mM $Na_2S_2O_4$).

The column material is either S-200 HR or S-300 HR and is poured aerobically in the running buffer. The packing speed of the column is dependent on the length, and inner diameter of the glass column as well as the column material, so it is best to check with the company's literature for the correct values (i.e. Pharmacia's Gel Filtration theory and practice). When you are pouring the column, remember to have a reservoir (i.e. a funnel) on top of the column to enable you to pour the majority of the column material at one time. This will help make the column as free from imperfections as possible.

Once the column has been packed with degassed buffer , then it must have its oxidizing equivalents removed from it. This is accomplished by scrubbing it with a 1 M $Na_2S_2O_4$ in Tris solution. Because the column is outside the box, the easiest way to load the column is to use a gas tight syringe and manually place the material on top of the column bed. You have to use a syringe with a long needle. It is preferable to use a straight needle which will make it less difficult to gain access to the top of the column through the headspace adjuster.



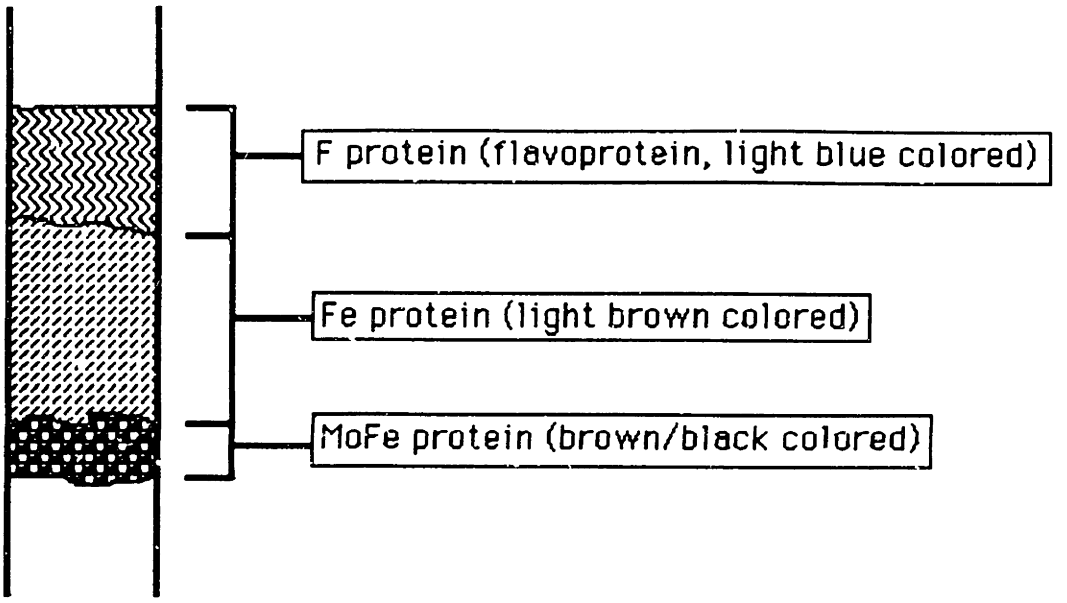
When loading the dithionite spiking solution, it is not necessary to have a tight band. You can just syringe the solution into the headspace above the column bed. Loading protein is a different process.

Once the column has been scrubbed and the running buffer is reducing at the end of the column (this can be tested by using methyl viologen paper), then the column must be checked for imperfections. This is accomplished by passing myoglobin (about 50 mg) down the column. The degassed solid protein is brought into an anaerobic box and dissolved in 4-5 mL of the running buffer with 0.5 mL of degassed glycerol added. The glycerol makes the protein solution denser than the buffer which aids in loading the protein. The protein solution is then brought out of the box and loaded into a gas tight 5 or 10 mL syringe, that had been rinsed out with reducing buffer. The

protein solution then must be carefully pushed out of the syringe drop wise onto the top of the column bed. If you release the protein too far away from the top of the column, then you will get dilution of the protein, which will result in a wide band. If you load the protein too close to the column bed, then you may pit the even surface of the column material which results uneven loading of the protein.

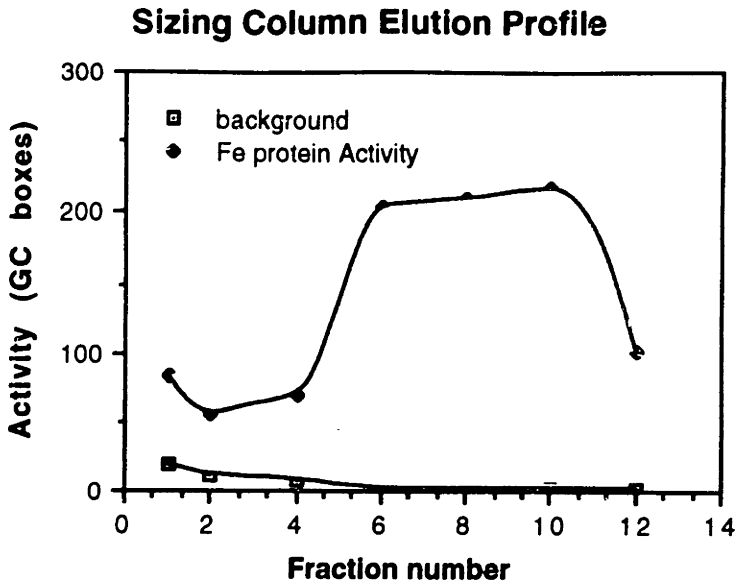
The myoglobin is very close in MW to the Fe protein, so it is used to calibrate the column. The myoglobin will separate into yellow and orange bands. The volume of buffer required to elute the myoglobin is the void volume, and this must be measured using a graduated cylinder. This will give you an idea of the volume of buffer the has to pass through the column until the Fe protein is eluted.

The Fe protein is purified on the sizing column in usually 5 mL aliquots depending on the column and required purity of the Fe protein. The sample is prepared and loaded on to the column the same way as the myoglobin solution. The Fe protein sample that is loaded onto the sizing column is a mixture of MoFe protein, Fe protein and J protein. You will probably be able to see the colors of the bands until halfway down the column, so it is important to know the void volume of the myoglobin. This will give you an idea when to start collecting fractions.



The above picture gives an example of what the bands might like about halfway down the column. The volume of the fractions you should collect is about 5-7 mLs. The F protein can be collected in a single fraction. There will be some overlap between the three bands. The overlap between the MoFe and Fe proteins can be determined by activity assays and the Fe and F proteins overlap by be observed by the blue tinge of the Fe protein.

The fractions in which you think the Fe protein is in have to be assayed. A typical well run column gives the following elution profile:

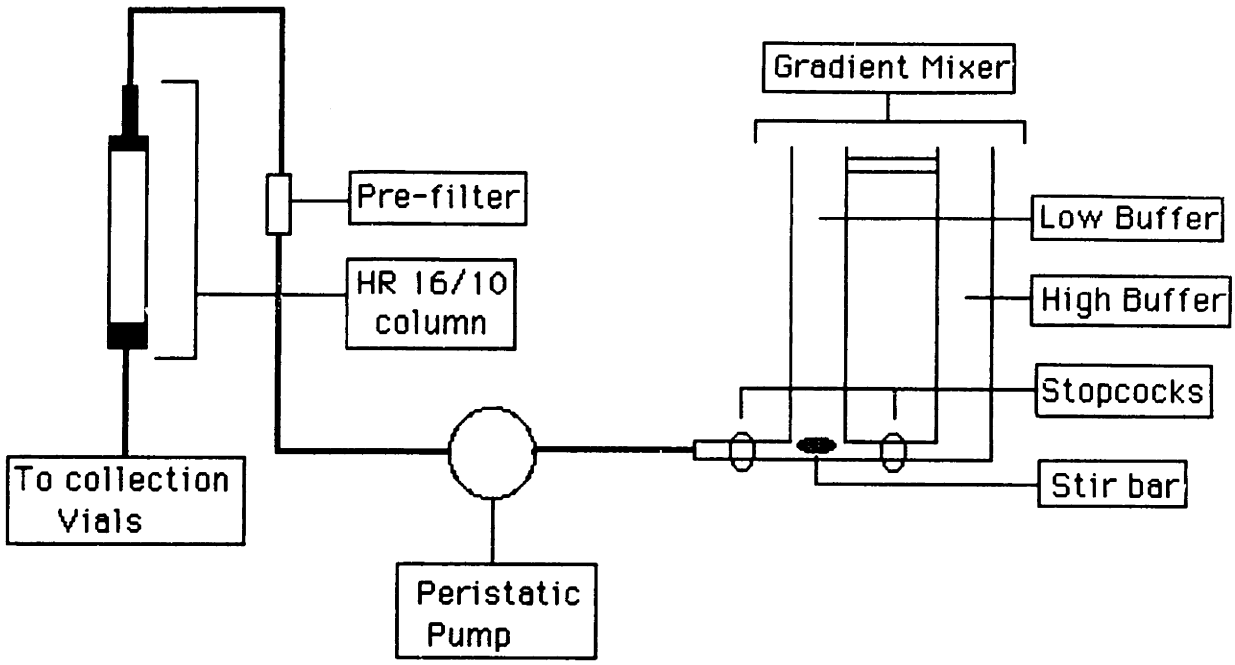


The fractions which you want to keep depends on the final use of the Fe protein. It is better not to use the fractions which contain the F and Fe proteins. If you want the Fe protein to use in assaying FeMoco, then the background should be at most 1.2 GC boxes. For assaying MoFe protein, it is only necessary to know what the background value is and subtract that. From this sample column, if there is not any contaminating F protein in the last few fractions, then all the fractions are good for assaying MoFe protein, and fractions 7-12 are good for cofactor assays. The chosen fractions must be concentrated and frozen away. A good volume to freeze in each vial is about 2 mLs. If the sample has a background and you want to be able to do accurate calculations, then a background assay should be done every time you thaw the sample.

A second way to further purify the Fe protein is to use a Source 15Q column. Source 15Q from Pharmacia is essentially the same material as Mono Q, but Pharmacia doesn't sell Mono Q in bulk. Source 15Q is a high affinity anion exchange material. A 16/10 HR column (from Pharmacia) was packed using Source 15Q. The column can be used in an anaerobic FPLC system or by utilizing Masterflex tubing and peristaltic pumps. Because of the nature of the column packing, all solutions need to be sterifiltered and/or passed through a pre-filter before they are eluted onto the column.

Concentrated Fe protein in 3 mL aliquots can be purified using this column to produce 4-6 mL of protein with little or no background MoFe activity.

HR 16/10 modified FLPC System



The column material needs to be prepped before one can use it. The anion sites on the column must be first occupied with chloride ions. This is usually done the day before one wants to use the column. This can be accomplished by eluted 2 M NaCl in 25 mM Tris (about 20-50 mL) through the column. The column is then spiked with a solution, less than 1 mL of dithionite in Tris and then the excess dithionite is washed off with reducing Tris overnight at a low flow rate.

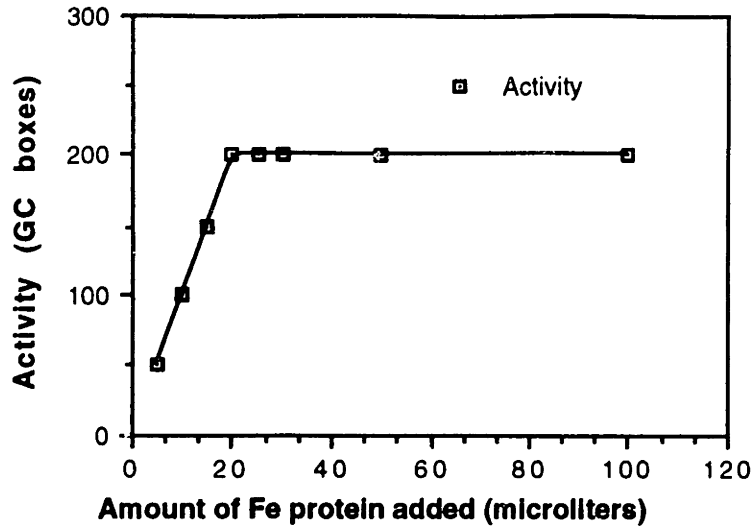
The protein must first be diluted at least 1:5 to enable it to bind to the column. The protein then is ultracentrifuged for an hour to remove any particulate matter. The supernatant is decanted off and the solution is sterifiltered through a low protein binding filter. The protein is pumped on at half the speed at which you will develop the column, without the pre-filter in line. If you use the pre-filter at this time, the Masterflex tubing and pump system can not handle the resulting backpressure. Once the protein is

pumped on, the tubing is rinsed with sterifiltered Tris and the pre-filter is put back in-line. The column is developed using a linear gradient consisting of 230 mL of 0.05 M NaCl in 25 mM Tris and 0.5 M NaCl in 25 mM Tris. The fractions are collected in 13.56 mL vials and the first eight fractions should be clear and be between 10-12 mL in volume. Once colored protein begins to elute (this is the MoFe protein), the fractions should be 5-7 mL in volume. The assays for background and Fe protein activities are done the same way as described for the sizing column.

It is important to regenerate the column material after the preparation is finished. Depending on the nature of the sample, regeneration or cleaning-in-place is required. To regenerate the material, the remaining proteins can be eluted off by running between 15 to 50 mL of 2 M NaCl in Tris through the column. The column is then equilibrated in Tris buffer. If the column material appears to still have proteins bound to it, then with reversed elution flow, wash the column with 1 M NaOH. Pharmacia recommends that the linear flow rate be approximately 40 cm/h with a contact time of 1-2 hours. This is usually sufficient to clean up the Source 15Q. The column is then rinsed with around 100 - 200 mL water and then Tris until the pH is back to 7.4. The material is now ready to be prepped for usage again. If the column is not going to be used for a long period of time, it should be stored under a 20% ethanol solution with the column ends capped.

Once the Fe protein is concentrated, then you must determine the saturating amount of Fe protein that is required for assaying MoFe protein. This is accomplished by doing a titration of a known concentration of MoFe with varying amounts of Fe protein.

Determination of saturating Fe protein concentration



From this graph, it can be determined that 20 µL of this Fe protein sample saturates this known concentration of MoFe protein. Each Fe protein sample will have a slightly different saturation level, so this titration must be done on every Fe protein sample.

References:

Burgess, B.K., D.B. Jacobs, and E.I. Stiefel. 1980. Large-Scale Purification of High Activity *Azotobacter vinelandii* Nitrogenase. *Biochim. Biophys. Acta.* 614: 196-209.

Burns, R.C. and R.W.F. Hardy. 1984. Purification of Nitrogenase and Crystallization of Its Mo-Fe Protein. *Proc. Roy. Soc., B* 172: 319-325.

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McLean, P. 1985. Notebooks from the Laboratory of W.H. Orme Johnson, MIT Chemistry Department. Notebook #7, page 52.

Shah, V.K., and W.J. Brill. 1973. Nitrogenase IV. Simple method of purification to homogeneity of Nitrogenase components from *Azotobacter vinelandii. Biochim. Biophys. Acta.* 305: 445-454.

Appendix C: Guide to FeMoco Extractions

Appendix C: Guide to FeMoco Extractions

The column method of cofactor extraction was developed in the Orme-Johnson lab by A. Hickman, D. Wink, P. McLean and others (see McLean et. al., 1989 and Wink et. al., 1989). The original preparation used MoFe protein immediately off the second DE-52 column at a concentration of 30 mg/mL and with the MoFe protein constituting 30% of the total protein in solution. It was found that when this procedure was attempted to be up-scaled, that there were problems. The entire procedure was then optimized and up-scaled using homogenous MoFe protein solutions and the entire extraction process was examined as to understand the processes involved in each step. This work was undertaken by D. Wright, T. Humiston and P. Christie.

This guide is meant to provide an outline for the entire cofactor process, including all the necessary preparation and insights that have been discovered by the many extractions performed in this lab. One of the important aspects of cofactor extractions is patience and being able to adapt to what happens. Most of the anecdotal remarks have occurred because the extraction did not go according to the plan. As long as the glove box stays below 1 ppm O_2 and you are careful, you should produce active cofactor.

Preparation for the Extraction of FeMoco

There is not order to the task described below. To be efficient, one must multi-task these jobs.

Glassware:

All glassware used in the extraction of FeMoco, or glassware that comes into contact with something used in the extraction of FeMoco, must be new or freshly washed in a KOH/EtOH bath prior to use. The use of a base bath is critical as the KOH etches the surface of glassware exposing a fresh clean surface. To wash glassware in KOH/EtOH (Base bath), remove any previous sharpie or tape remnants by rinsing with methanol followed by house distilled water. The glassware should then be allowed to soak in the base bath

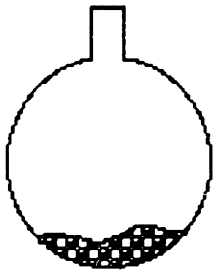
for a minimum of 6 hours. After soaking, the glassware is rinsed with Millipore water and allowed to soak in Millipore water for a minimum of 4 hours. After this soaking cycle, the glassware is exhaustively flushed with Millipore water to remove any vestiges of the base. Incomplete rinsing will leave a KOH residue which will destroy the cofactor. If the need for glassware is not urgent, a convenient timetable for washing is to simply soak glassware in the base bath and in Millipore overnight. While such a procedure may seem devised by paranoids, it should be noted that some research groups have found the issue of clean/safe glassware such a troublesome issue that they only use glassware one time for FeMoco extractions (W. Newton, personal communication).

Molecular Sieves:

Molecular Sieves, 4Å, are purchase from Linde Chemicals. Linde sieves are used because they are the only ones which don't contaminate the solvents with a fine powder. The molecular sieves must be activated by heating at temperatures greater than 125°C for 24 hours prior to usage. To insure a supply of activated sieves, a 600 mL beaker can be filled with the sieves and placed in a drying oven at the desired temperature. Do not allow them to cool under ambient atmospheric conditions as they will become readily saturate with O_2 and H_2O from the environment.

Solvent Preparation:

All solvent used (NMF, DMF, and Acetonitrile) are from Aldrich and are "Sure-Seal" HPLC grade. **Use of any other source for these solvents can result in failed extractions.** Prior to distillation, the solvents are stored over activated Linde molecular sieves, 4 A, under a blanket of inert gas (He or Ar) for at least 24 hours prior to distillation. Fill the bottom of a 1-3 L single-neck round-bottom flask with activated molecular sieves up to a depth so that when sitting on a cork ring the depth of the sieves goes to the top of the cork ring.

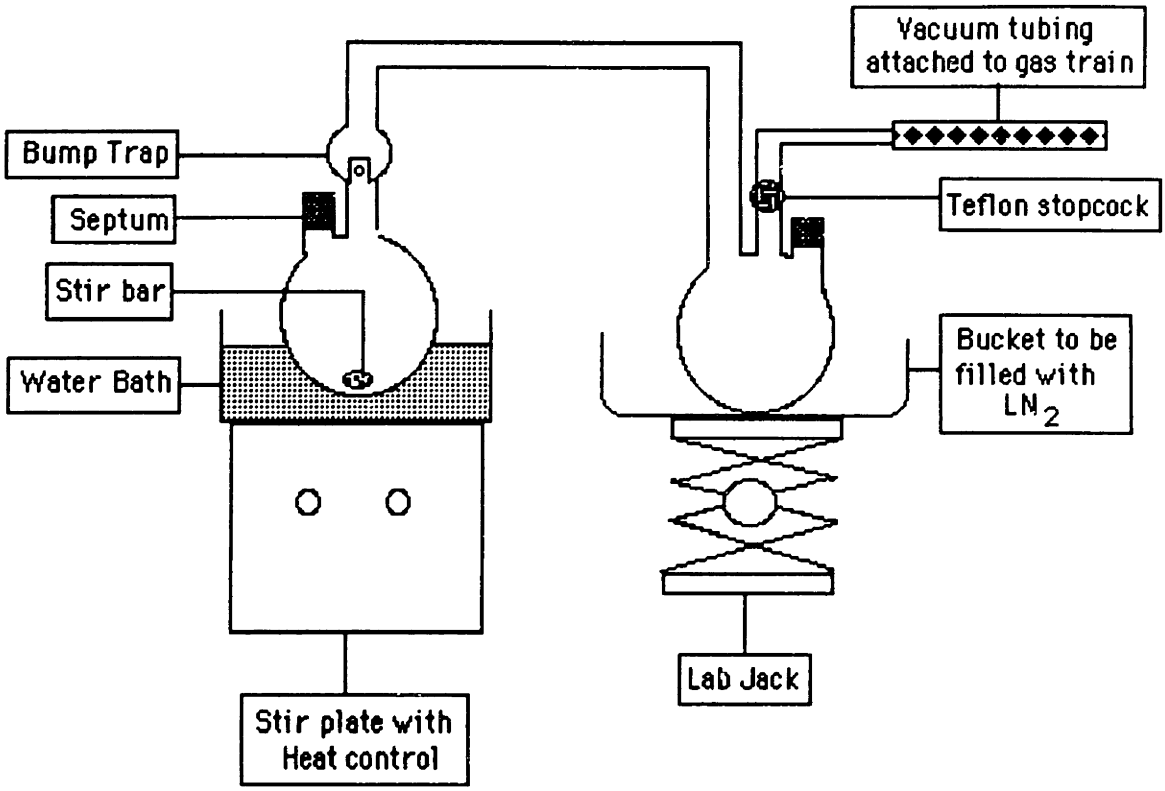


As the sieves are hot they should be allowed to cool before solvent is added to the round bottom. To do this place a septum in the neck and continuously purge the round-bottom with an inert gas. As one of the major sources of cofactor degradation is water, a bubbler **must not be used** for required pressure relief of any solvents used in the extraction process. Simply bleed the vessel with a needle. Once the activated molecular sieves have cooled to near room temperature, the solvent may be added to the round bottom. It is not necessary to transfer the solvent anaerobically, simply pour the solvent into the round bottom. After the solvent has been poured, pass another blanket of inert gas over the remaining head space. Periodically, swirl the round-bottom to insure that all of the solvent interacts with the molecular sieves.

The distillation of the solvent is performed in one of the single-piece distillation appparati in the lab. Distillations are done on a gas train reserved exclusively for organic solvents. If the train has not recently been used, purge it with an inert atmosphere for 5-10 minutes prior to use. On distillation days, the pump should be turned on first thing in the morning and the train should be pumped down to <0.05 mm Hg. This is done by cycling the train 3 times between vacuum and inert gas, pumping down 10-15 minutes for each vacuum cycle. A reading of <0.05 mm Hg, obtained from the attached McLeod gauge, verifies that the pump is pulling the required vacuum for removal of O_2 during the distillation. If a reading of >0.05 mm Hg can not be obtained, first check stopcocks in the train. If the stopcocks are not making a good seal, turn off the pump, shut off the Ar gas flow, remove the stopcocks, and clean and regrease them. If the stopcocks do make a good seal, the pump oil needs to be changed.

Connect the distillation apparatus as shown below. **Do not forget the stir bars or to tape the septa.**

Vacuum Distillation Set-Up



It is helpful to first soak the vacuum tubing in methylene chloride before attaching it to the distillation apparatus. Flush the tubing between the train and the stopcock on the distillation apparatus 3 times verifying a vacuum of <0.05 mm Hg at the end of the cycle. Next evacuate the distillation apparatus in 3 cycles verifying that the vacuum is <0.05 mm Hg before a cycle is repeated. For the 3 L distillation apparatus, this step can take between 45 minutes to an hour per cycle. For the 1 L apparatus, the cycles should take about 20 minutes.

When the final cycle is complete, the solvent may be added to the still by rapid canulation of the solvent into the left half of the apparatus under vacuum. This step must be watched closely to minimize the amount of air that is introduced to the distillation apparatus. Degas the solvent (for 1 L it is

an hour and for 3 L it is about 2 hours), cycling with inert gas at least 3 times. This step is critical in removing any residual O_2 introduced during various manipulations of the solvent.

After degassing, strict anaerobic conditions and techniques must be observed. The distillation of the solvent is begun by placing the right half of the apparatus into liquid nitrogen and the left half into warm water. To ensure efficient distillation of the solvent, the water should be the same depth as the initial level of solvent. The apparatus from the bump trap to the bucket of liquid nitrogen should be wrapped in glass wool. Distillation of 3 L of solvent will take 3-5 hours. The distillation is complete when there is only enough solvent remaining on the left hand side to completely cover the stir bar.

The next step requires two people. Remove the heated water with one person holding the water bath and the second person removing the stir plate. This end of the apparatus is then placed on a lab jack on a glove to prevent star cracks from forming in the apparatus. The liquid nitrogen tub is removed by one person and replaced with a glove and the lab jack by the second person.

Slowly backfill the distillation apparatus with inert gas and allow to thaw overnight. Make sure that the septum have been taped around the ground glass joint as well as over the top. This will prevent the septum from popping off and destroying your day's work. The apparatus should be observed for 30 minutes after being placed under a blanket of inert gas to insure that the septa are securely fashioned to the apparatus and the over pressure is stabilized. Once the solvent has thawed completely (this will be 2-4 hours for 1 L and overnight for 3 L), it can be canulated into a degassed round bottom flasks or 180 mL Wheaten vials for transfer and storage of the solvent into the box. If round bottoms are used, they should be new or washed in base bath (KOH/EtOH) prior to use. Round bottoms not prepared in this manner contain soap and other unknown agents from previous usage which will destroy the cofactor.

Distilled DMF will slowly degrade in the glove box. You should use freshly distilled solvents for every extraction. If that is not possible, solvents that have been distilled in the last two weeks have been used with satisfactory results for extractions.

Preparation of Organic Dithionite:

The organic dithionite is prepared by the method of Lough and McDonald (Lough and McDonald, 1987). A 2.5 x 1.5 cm column (Econocolumns, Bio-Rad, new or base bathed) of Bio-Rex 5 anion exchange resin (Cl^- form) is made anaerobic by elution of 1 L of rigorously degassed H_2O. The column is then converted to the dithionite form by the slow elution of 600 mL of 50 mM $Na_2S_2O_4$. The column is washed with degassed H_2O until the dithionite is not longer detectable with methyl viologen paper. The resin is treated with degassed 0.5 M $[Bu_4N]OH$ in H_2O. The pH and reducing ability of the fractions are monitored with pH paper and methyl viologen paper, respectively. When the pH suddenly increases, the elution is discontinued. The organic dithionite is in the fractions which are reducing to methyl viologen paper and the pH is constant. The eluent is evaporated on a liquid nitrogen chilled rotovap at 30°C to give a pale oily residue. The material is then heated to 80°C under vacuum for an additional 6 hours causing the solidification of a well-formed product and a color change to an intense canary yellow.

***nif B*:**

FeMoco is assayed by a reconstitution assay using a cofactorless mutant, *nif B*. The most common strains are *Klebsiella pneumoniae* UN 106 and *Azotobacter vinelandii* UW 45. Conditions for growth and harvesting of these strains may be found in the appendix A, Guide to Fermentations or in the notebooks of Paul McLean, Alison Hickman, David Wink or Patti Christie. Prior to FeMoco extraction, a sufficient volume of cracked *nifB* extract must be prepared.

Approximately 10 g of *nifB-* pellets are placed in a small 50 mL round bottom contained a small stir bar and cycled five times between vacuum and argon. After the headspace has been thoroughly degassed, the cells are suspended in 20 mL of 0.1 M Tris, pH 8.0, 5 mM $Na_2S_2O_4$ if the culture was harvested by Low speed centrifugation (i.e. Pellicon concentrator then spun in a Beckman GSA rotor) or in 40 mL of the same buffer if harvested using the Sharples centrifuge. The cells are slowly stirred at 4°C until they are thawed and evenly suspended. The cell suspension is cracked in the French Press (see section in Fermentation Guidebook) and aliquoted into degassed 12.56 mL vials. Due to the extreme thermal instability and oxygen lability of Nif B$^-$, the aliquots should never exceed 2 mL. The aliquots should be immediately frozen with liquid nitrogen.

The extract should be checked for activity. One of the vials is degassed and thawed anaerobically and then diluted up an addition four fold (i.e. 1 mL of extract should have 3 mL of buffer added to it). You may use pH 7.4 Tris, 25 mM, 5 mM $Na_2S_2O_4$ at this stage. The total dilution for harvesting using the Sharples centrifuge is 1:16 and for low speed centrifugation, it is 1:8. The activity assay usually consists of cross reacting 50 µL of extract with 2.5 µL of stock MoFe protein. This experiment provides a number against which the quality of the *nifB*$^-$ may be judged. If later repeats of this control experiment demonstrate activity reduction of 25% or more, the vial is not longer suitable for FeMoco reconstitution assays and should be discarded. Nif B$^-$ extracts can not be successfully thawed, refrozen and thawed again without loss of activity.

In planning the amount of *nifB*$^-$ extract required for a specific experiment, it must be remembered that each fraction of eluted FeMoco requires 5 reconstitution assays to demonstrate titration. The 2 mL of *nifB*$^-$ extract, that is diluted and additional fourfold, is good for 20 reconstitution assays. Each reconstitution assay requires 0.4 mL of extract.

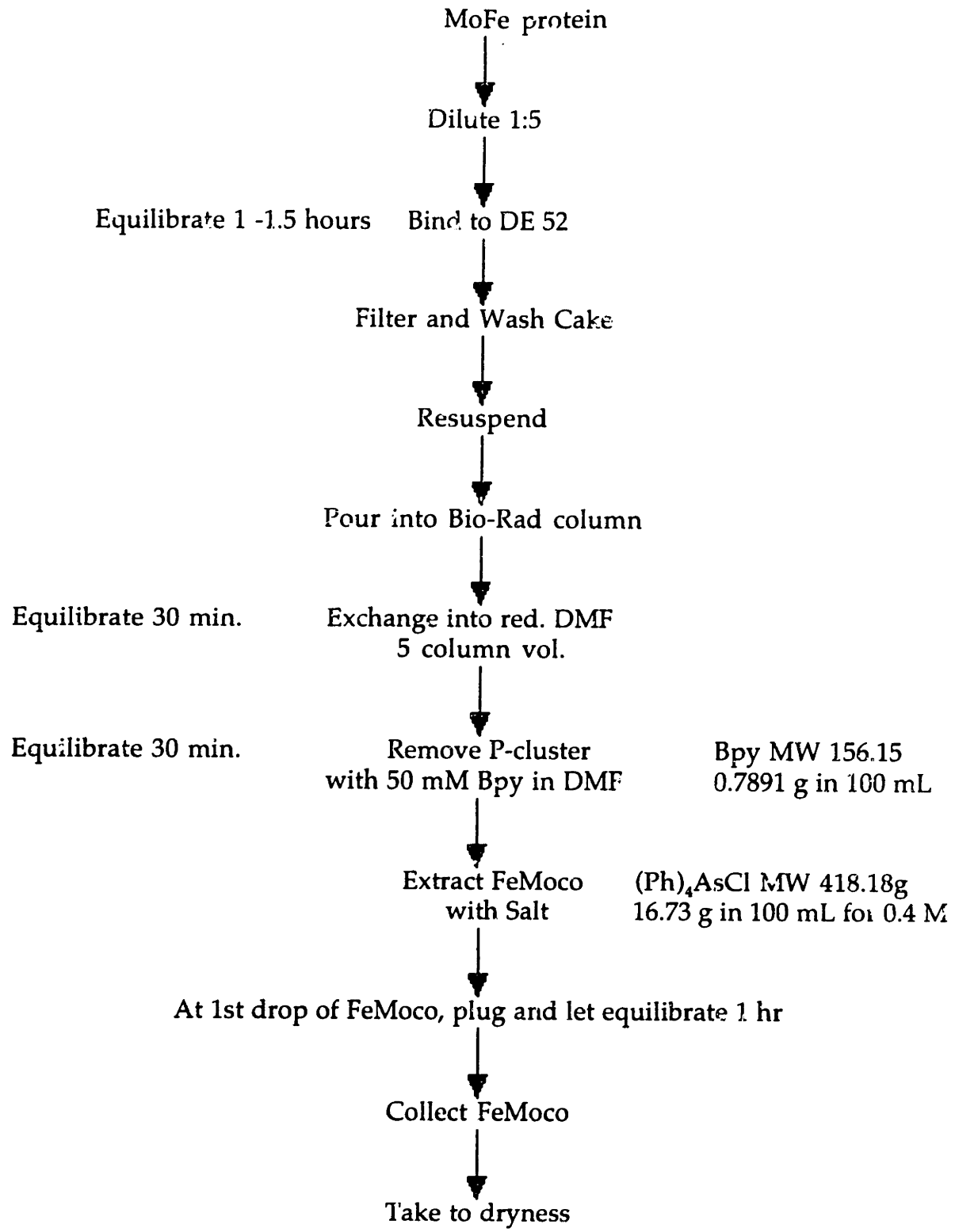
Material Required for Extraction:

Remember that all glassware and accessories are either new or cleaned in a base bath (KOH/EtOH) prior to use.

-extracting salt
-2,2'bipyridine
-25 mM Tris -for diluting up protein sample, pouring column
-DMF -volumes required range from 1.25 L for an 100 mg extraction without desalting to 9 L for a 1 g extraction with desalting on a LH-20 column
-Whatman DE-52 (preswollen DEAE). Only use new material, do not use regenerated material.
-Beakers
-Stir bars
-Filter flasks - 500 mL, 1 L, 125 mL and 50 mL
-Buchner funnel - 9 cm
-fritted glass funnels
-rubber adapters for the Buchner funnel
-Filter paper -9 cm
-Bio-Rad Econocolumns -DMF resistant variety (these can be filled with base bath up to just below the plastic top), minimum of 2, with the dimension chosen for the scale of extraction.
-Glass stir rods -min. 3
-Teflon coated spatulas
-disposable glass pipettes
-goober paper (disposable lab bench covering)
-paper towels
-glass collection vials and polypropylene collection tubes (for after desalting column)
-many extra vials -for waste, aliquoting, sample dilutions, etc.
-glass syringes -for dilutions
-glass graduated cylinders
-tools -slotted screw driver, adjustable crescent wrench, level etc.
-Hose adapters -required for vacuum line in Vac Atm. glove box.
-Worm clamps

The Battle Plan:

The Battle plan should be an outline, with as much or as little detail as one needs of the extraction. It is often useful to include equilibration times, concentrations of required solutions, and amounts of chemicals required to make up these solutions so as to save valuable time during the extraction. A typical Battle Plan is provided below.



This is meant to provide a brief outline of what you will be doing on an extraction day. The next sections will describe in detail what each step in the procedure involves.

Modified Batch Method of FeMoco extraction:

This will describe what happens the day of the cofactor extraction. It will assume that all of the preparatory work has already been done, that everything is in the glove box that is required and that the glove box has been regenerated the night or two nights before and is below 1 ppm O_2. Remember that the extraction will take between 12-24 hours, so be prepared and have patience!

Once the MoFe protein is in the glove box, it should be diluted up 1:5 and a sample (start) of this solution taken (indicate total volume). This sample will be used to determine the yield of the extraction. The DE-52 should have been preweighed (4.5 g of DE-52 binds 100 mg of homogenous MoFe protein) and brought into the glove box in the beaker that the batch adsorption will be occurring. In the beaker, there has to be a stir bar that reaches all sides of the flask. This ensures that the column material will not settle on the bottom when the solution is being stirred. The DE-52 is then suspended in reducing Tris. The volume of Tris used should be 25% of the volume in which the MoFe protein is contained. Check and make sure that the supernatant of the suspended DE-52 is reducing to methyl viologen paper. The diluted MoFe protein solution is then added slowly to the stirring DE-52 slurry. There will be some frothing of the protein, but excessive frothing indicates the stir rate is too high. You should rinse out all of the glassware which came into contact with the protein solution. Keep track of the total volume of Tris added. Let the swirling solution stir for 1-1.5 hours, checking to make sure that all of the DE-52 is stirring and that the protein appears to be binding.

During this time, it is a good idea to get organized for the next parts of the extraction. A 0.1 M organic dithionite solution can be made in DMF. Bottles of DMF can be made reducing by adding a few drops of the stock organic dithionite solution. All the DMF solutions which are used in the extraction must be have the organic dithionite added to them. The 50 mM

2,2'bipyridine (Bpy) solution can be made up in reducing DMF. For 100 mL of Bpy solution, 0.789 g of solid is required. If tetraphenylarsonium chloride mono hydrate ($[Ph]_4AsCl.H_2O$) is going to be the extracting salt, for 100 mL of a 0.4 M solution, 16.73 g of solid need to be weighed out and dissolved in reducing DMF. The vacuum pump for the in box vacuum line also needs to be turned on.

After the equilibration time, stop the stirring, allow the DE-52 to settle and take a sample (supernatant) of the supernatant, making note of the total volume. The supernatant might have particulate matter floating in it. These are fines from the DE-52. The DE-52 is then swirled in the beaker and the supernatant is filtered off using the Buchner funnel and the filter flask. This filtrate should be clear, but if the extraction is with non-homogenous MoFe protein, there might be some frothing. This first filtrate should be saved and a sample taken.

The filter cake is then washed five times with reducing Tris. The cake is then put into a beaker and all traces of the filter cake rinsed off the funnel and into the beaker using reducing Tris. The filter cake is then resuspended and poured into an Econocolumn. The column is packed with 2-3 column volumes of reducing Tris making sure that all of the column material is rinsed off the upper sides of the column.

The column is now ready to be exchanged into DMF. It is very important for all traces of water to be removed from the column. This is best accomplished by having a separate small volume of DMF from which you pipette out of. You should use a fresh pipette every time you need more DMF. The top plastic part of the column needs to be thoroughly rinsed with the DMF to remove all traces of water. Once you are convinced that all of the water is removed from the top of the column, then larger volumes of DMF can be added to the top of the column. To fully exchange the column buffer from Tris to DMF takes at least 5 column volumes. As the movement of DMF occurs through the column, the bound protein will change color from light brown to brown/black upon. You can also see the swirling in the drops

of the flow through from the column and since $Na_2S_2O_4$ is not soluble in DMF, you could see it precipitate in the flow through. Once the exchange is complete. plug the column bottom and let sit 30 minutes. At the end of this equilibration time, the column material should be stirred with a glass rod to remove any channels that have formed. The column is then repacked with 1 column volume of DMF.

When the protein has been exchanged into DMF, it has unfolded and exposed both the P clusters and FeMoco. The P clusters must be first removed by passing a 50 mM Bpy in DMF solution through the column. The column should be eluted with Bpy solution until the flow through turns pink or that there have been 3 column volumes passed through. The column bottom is plugged and allowed to equilibrate for 30-45 minutes. After this equilibration period, one column volume of Bpy solution is eluted through the column and then DMF is eluted through the column until no color remains. The pink color is a $Fe(Bpy)_2$ complex.

Now it is time to extract out the FeMoco. The cofactor is extracted out of the unfolded protein using high salt. One of the more commonly used salts is $(Ph)_4AsCl.H_20$. If this is the salt of choice, then for 100 mL of a 0.4 M solution, 16.73 g of solid needs to be weighed out and dissolved into DMF. The salt solution is then eluted onto the column. At the first drop of FeMoco, which will be black, plug the column and let sit for 1 hour. After the hour, unplug the column and chase off the FeMoco with a salt wash, collecting the flow through into a Wheaten vial. If at this time there is not any flow through, it is because the frit has become clogged with the salt. **Do not panic!** You can batch extract the cofactor from the column material. Add some of the salt solution to resuspend the column material so that you can pour it out. Have a clean, small filter flask ready with a fritted funnel set up. Pour the column material into the fritted funnel and filter off the liquid. This is your cofactor solution. You can rinse the filter cake with a small volume of salt solution. P. Christie has routinely done this type of extraction with yields of at least 70%.

Whether you are able to elute the cofactor off the column or do a batch extraction, the remaining column material should loose its color. This means that you have removed all of the FeMoco. If you still see color remaining, repeat the equilibration step with salt solution.

The majority of the cofactor will be in the darkest colored solutions, but you should save any fractions that have color. You need to take samples of the cofactor solution and determine the volumes in a graduated cylinder. The darkest colored cofactor solution is then put into a pear shaped flask to be taken down to dryness. Depending on the concentration, typical dilutions range from 1:300 to 1:20. At this time, you should also take 25 µL samples for Atomic Absorption (AA) analysis and put them in test tubes. If you are going to determine the iron content, 20 µL samples need to be taken. When the samples are brought out of the glove box, the AA samples need to have 975 µL of 2% nitric acid added to them, wrapped in Parafilm and put in the refrigerator. Because of the high salt concentration, there will be some precipitant in the sample.

At this stage, the cofactor will degrade in a high salt solution. It is important to get it down to dryness as quickly as possible. The quickest way is to use a rotovaporater that uses liquid nitrogen chilled nitrogen gas in the condenser. The flow of the nitrogen gas should be such that the DMF is being condensed up to halfway up the condenser in the rotorvap. Depending on your set up, this could be accomplished by the 2nd stage of the nitrogen tank to be set from 5-20 psi. The cofactor solution on the rotorvap is put in a 30°C mineral oil bath. Pay careful attention to the temperature of the bath and don't turn the heat on unless you can constantly watch it! Heat kills cofactor.

While the cofactor is being taken down to dryness, you can get the reconstitution assays done.

Reconstitution Assays:

The purpose of these assays is to figure out how much cofactor you were able to extract from the MoFe protein. Once you become familiar with the extraction process, you should be able to obtain 70% (for a batch extraction) to 100% yields on a regular basis. These assays involve the reconstitution *in vitro* of the cofactor into *nifB*$^-$ extracts and then the assaying of the produced MoFe protein.

You can start preparing for these assays during the many equilibration steps of the cofactor extraction. For every dilution of the cofactor that will be made, there needs to be 5 of the 8.56 mL Wheaten vials degassed. In addition to the usual 25 mM Tris, 1 M $Na_2S_2O_4$ stock solution, diluting Tris solution and the 0.1 M $Na_2S_2O_4$ in Tris solution, plus all of the necessary pre-made assay vials. You will also need to have aliquots of cracked B$^-$ extract, stock MoFe protein and low background (less than 1.2 GC boxes) Fe protein degassed and thawed. Make sure that the B$^-$ extract is diluted up an additional four fold and then kept on ice.

Once all of the samples are out of the glove box, the first set of assays should be to check the stocks (see chart below):

Vial #	Am't of MoFe added	Am't of Fe added	Purpose of Assay
1	—	40 µL Fe	-check on background of Fe prot
2	5.0 µL of MoFe	40 µL Fe	-make sure Fe prot is active
3	5.0 µL of MoFe	50 µL dil. B$^-$ extract	-make sure B$^-$extract is active
4	2.5 µL of Start	40 µL of Fe	-find out starting units of cofactor
5	5.0 µL of Start	40 µL of Fe	
6	50 µL of supernatant	40 µL of Fe	-checks on unbound MoFe prot
7	50 µL of filtrate	40 µL of Fe	-checks on unbound MoFe prot

It saves a lot of headaches if you make sure the B$^-$extract is active before you do all of the reconstitution assays. Once it is determined that the B$^-$ extract is indeed active, then the reconstitution B$^-$ can be started. The following are examples of reconstituted B$^-$:

Vial #	Am't of cofactor added	Am't of B added
1	----	0.4 mL dil. B$^-$ extract
2	2.0 µL of 1:20 dil. cof.	"
3	4.0 µL of 1:20 dil. cof.	"
4	6.0 µL of 1:20 dil. cof.	"
5	8.0 µL of 1:20 dil. cof.	"
6	10.0 µL of 1:20 dil. cof.	"

Therefore, for each cofactor dilution there will be six reconstituted B$^-$ samples. Each one of these vials can then be treated as holoMoFe protein and is tested against the low background Fe protein for activity. Hopefully the dilutions have been chosen carefully to produce a titration curve for the cofactor activity. For instance, using the reconstituted B$^-$ vials in the above example, the following GC activities could be produced:

Vial #	Am't Reconst B-added	Am't Fe added	GC units	Activity	dil. fact	total units
1	50 µL of vial 1	40 µL	3.90 X 4 (15.6)			
2	" vial 2	"	4.45 X 4 (17.8) 2.2	171.5	1:20	2.02×10^5
3.	" vial 3	"	4.80 X 4 (19.2) 3.6	140.3	"	1.66×10^5
4.	" vial 4	"	5.65 X 4 (22.6) 7.0	181.9	"	2.14×10^5
5	" vial 5	"	6.15 X 4 (24.6) 9.0	175.4	"	2.07×10^5
6	" vial 6	"	6.0 X 4 (24) 8.4	saturating		

When calculating the activity of the cofactor, an additional dilution factor is inserted into the equation. This additional factor is because only 50 µL of the total volume of 0.4 mL of reconstituted B$^-$ extract is assayed. Based on the volume of cofactor required to saturate the B-extract, you can calculate the total number of units of cofactor present in the extraction and by dividing by 2400 units/mg, you can calculate the amount of mg of MoFe protein present.

Back in the Glove Box:

While you have been doing the cofactor assays, you should have been keeping an eye on the cofactor being rotovapped in the glove box. The liquid nitrogen for the gas needs to be filled periodically. Once you are finished with the assays, you can then turn on the heat for the mineral oil bath. Just make sure that the temperature does not go above 30°C.

The cofactor solution needs to be completely dry before it can be stored. The 75% of the liquid should be stripped of quickly but its the last 25% that takes time. The heat will help get the last volume of DMF off. You can check the solid for stickiness and grind up the solid to help it evaporate off the solvent. When the cofactor is completely dry, it will be pale in color, a combination of white salt and tan colored cofactor. If by the end of the day the cofactor is at the sticky stage, a stir bar can be put into the flask and then put on the vacuum line overnight (i.e. 8-10 hours). The next day, you should check it for stickiness and keep it under vacuum until it is completely dry. This two stage drying does not degrade cofactor, but if the cofactor is left in a small amount of solvent, that will kill it quickly. Once the cofactor is in a dry state and closed into the flask, it is stable for long periods of time.

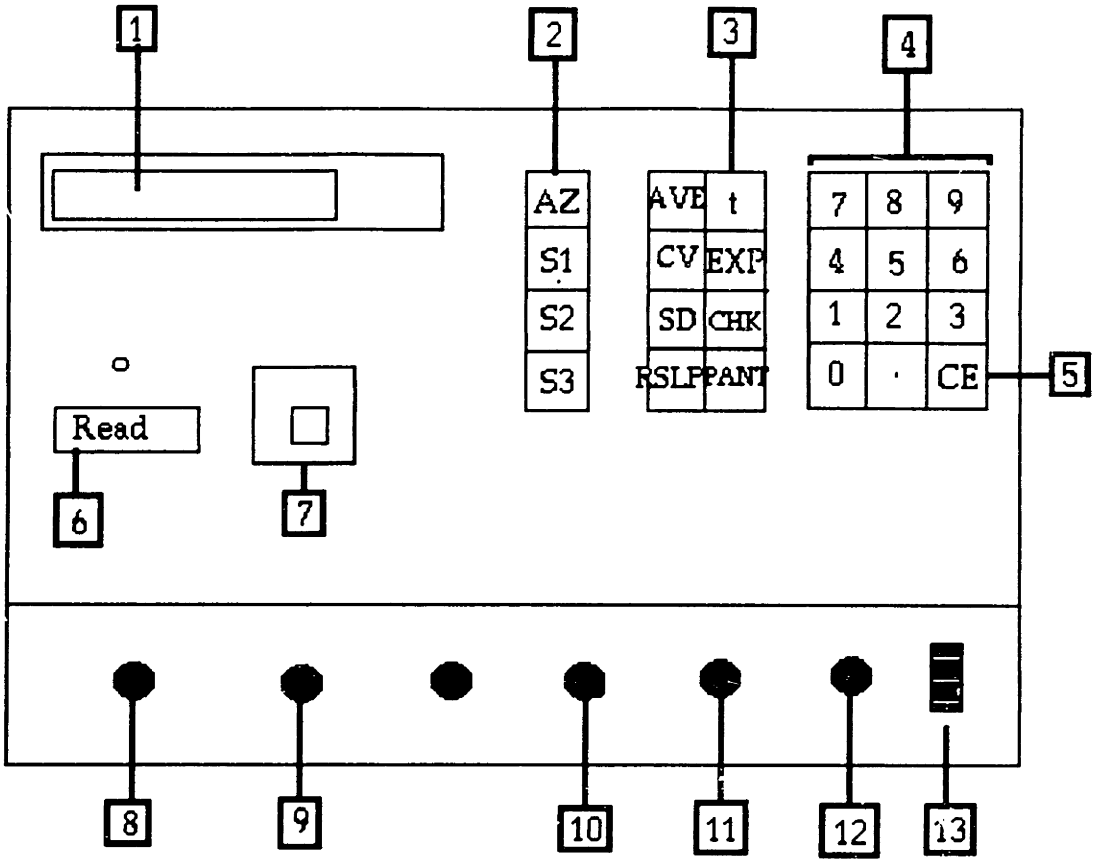
The extraction is now complete. You now have to clean up all the glassware, change pump oil on all of the pumps that came into contact with DMF and finish the analysis.

Atomic Absorption Analysis:

The next stage of analysis involves determination of specific activity of the cofactor by dividing the activity of the nitrogenase activity by the total amount of molybdenum present. Atomic Absorption Spectroscopy is a destructive type of spectroscopy which is able to determine the concentration of metallic elements in a variety of matices. Using a lamp that will generate a characteristic wavelength for the element of choice, the absorption of light is measured. The matrix most commonly used is 2% HNO_3 and depending on the element (i.e. Cr, As, Na) matrix modifiers, most commonly 5 mM $Mg(NO_3)_2$ might have to be used.

The AA spectrometer currently in use in the Orme-Johnson lab is the Perkin-Elmer 2380 equipped with a HGA Graphite Furnace. The instructions that follow are meant to provide information for the user that is covered in more depth in the manual. The manual for the Spectrometer is a very good resource and should be read in addition to this guidebook.

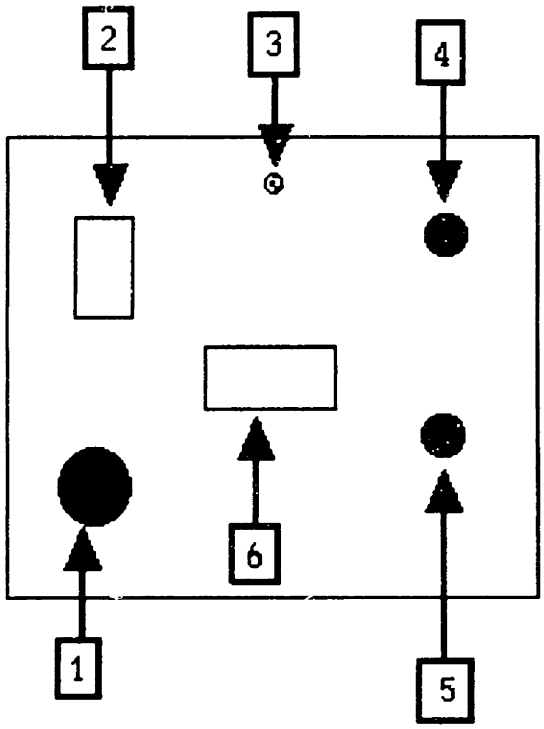
Atomic Absorption Spectrometer Right Front Control Panel



Explanation of the figure:

1. Digital Display and Indicators
2. AZ (Auto Zero) key and Signal dot
3. t (Time) key
4. Numerical keyboard
5. CE (clear) key
6. Read key and signal dot
7. Lamp/Energy display
8. Signal switch. The settings are (left to right): lamp, set up, abs, conc, and em.
9. Mode switch. The settings are (L to R) cont (continuous), hold, pk ht (peak height), and pk area (peak area)
10. Gain control
11. Lamp control
12. BG (background) corrector switch. Should be set on AA EN.
13. Power Switch

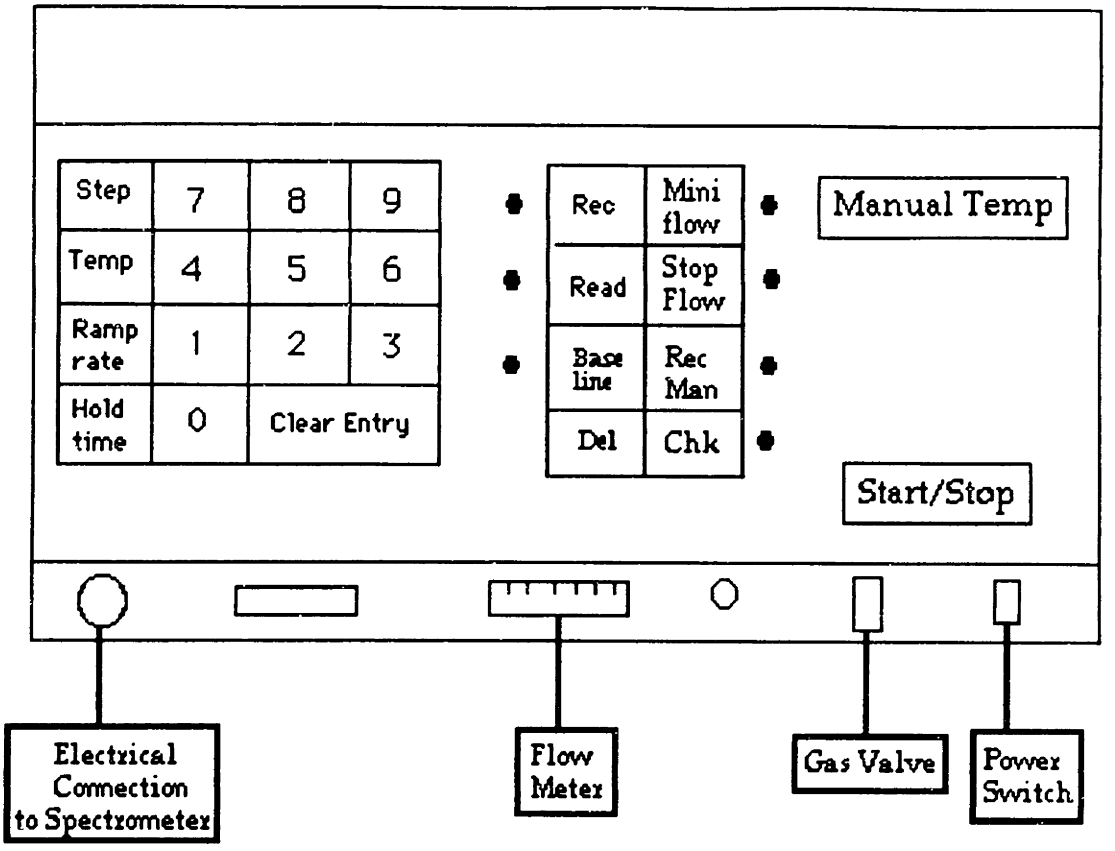
Atomic Absorption Left Front Control Panel



Explanation of Numbering:

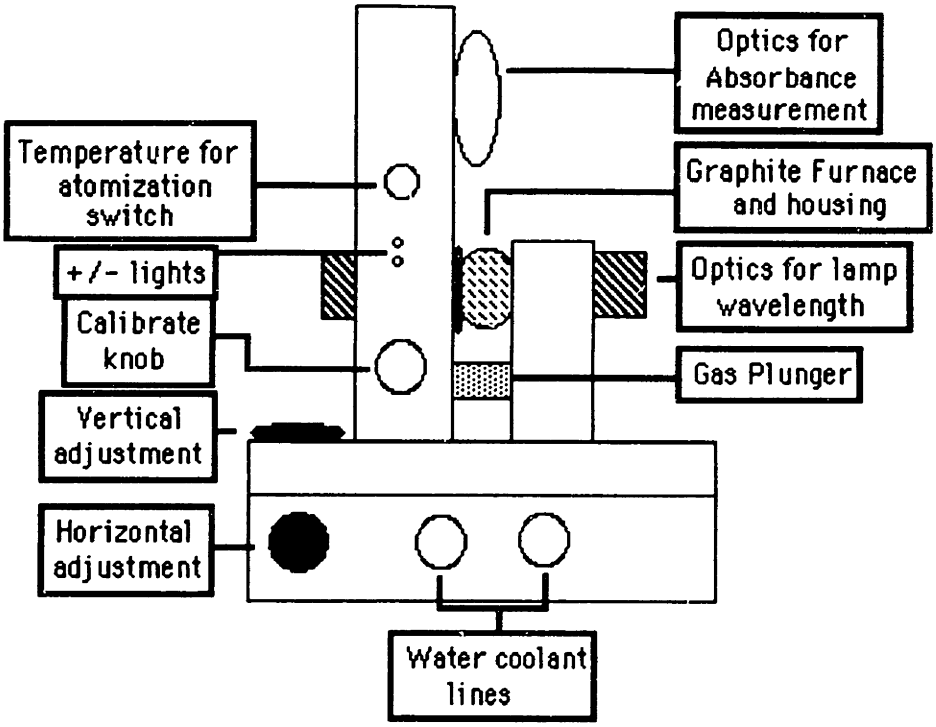
1. Slit width selector. Reading from left to right, under normal bracket: 2.0, 0.7 and 0.2; under alt bracket: 0.2, 0.7 and 2.0.
2. Wavelength drive switch
3. Optics compartment access screw
4. Fine adjust wavelength control
5. Coarse adjust wavelength control
6. Wavelength counter

HGA Programmer for the AA Spectrometer



The is part of the spectrometer into which the programs for the given elements are imputed. Turning the power off eliminates any entered program, so it must be reprogrammed each use. The gas valve has three settings, off, on and open and is used to hold the furnace closed. To program the controller, first press the numerical value, then the function. The entire program can be checked by depressing the chk (check) button, which will cause the red light next to it to flash and then the various functions (i.e. step, temp., ramp time, and hold time).

A.A. Spectrometer Furnace



The furnace unit is the part of the spectrometer in which the sample is heated to its atomization temperature and its absorbance determined. When ever the lamp is on, water should be running through the furnace to keep the unit cooled. This prevents the sample from being prematurely atomized. When the gas, usually argon, is turned on, this will hold the furnace in place as well as purging the graphite furnace to prevent its combustion. The gas switch is on the HGA Programmer (see diagram). Periodically, the optics for the lamp wavelength should be removed from the unit and cleaned with alcohol. The optics for the absorbance measurement should also have the disk directly on top of the graphite furnace cleaned with alcohol. To make sure that the furnace is aligned properly, put the mode (see figure AA spectrometer lower right front panel, #9) on continuous and move the optics for lamp wavelength around, as well as the horizontal and vertical adjustment knobs to minimize the amount of background. Once the background has been minimized, autozero the absorbance and put it back into peak height mode.

There are several items which can be optimized for the particular element you are testing for. The temperature for atomization switch should be orientated for the correct atomization temperature for the element of choice. The three choices are 800-1000°C, 1000-1500°C and 1500-3000°C. At the atomization temperature (usually the second to last step in the program), the + and - lights directly above the calibration knob should flip back and forth. Altering the calibration setting will achieve this required setting.

Periodically the graphite housing has to be changed. It is easiest to refer to the spectrometer manual for this. The housings need to be changed when they are broken or if they are covered in black scorching. The graphite tube also needs to be changed. The graphite tube usually is changed after 3-5 experiments. If you are analyzing high salt samples, the tube might have to be changed every experiment. There are two different types of tubes, ones with platforms and ones without the platforms.

<u>To change the graphite tube:</u>

- Open the furnace by first switching the Gas switch on the Programmer to OPEN and then pulling the right-hand side of the furnace to the right side.
- Remove the old tube.
- Take the new graphite tube and insert it into the right hand contact cylinder, so that the sample introduction hole in the tube lines up with the sample port in the contact cylinder.
- Insert the tube alignment tool into the sample port, while carefully moving and turning the tube so that the top of this tool goes into the sample introduction hole in the tube.
- The furnace needs to be closed with the alignment tool in the graphite tube. The gas switch then needs to be turned to ON.
- Remove the alignment tool

A new furnace needs to be conditioned before use. The program is as follows:

Step	Furnace Temperature	Ramp	Time Hold
1	2650	60	2
2	20	1	20
3	2650	10	10
4	20	1	20
5	2650	10	10
6	20	1	20
7	2650	10	10

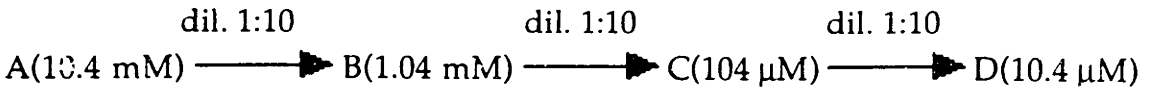
The lamps that are currently in use are usually stored by the spectrometer. If you have to change the lamp, first turn everything off. Then press the button on top of the lamp. Unplug the lamp currently in the instrument and insert the desired lamp. The slit width (seen on the top left half of the control panel) might have to be changed (it is 0.7 for Fe, and 0.2 for Mo). The wavelength needs to be dialed in (see chart on front of instrument). Make sure the Signal knob is on lamp and using the lamp knob set it to 30. The fine adjust and the knobs holding the lamp in place are then optimized to the highest value possible. If this ends of being higher than 30, adjust the lamp energy back to 30.

Example Determination of Molybdenum in a FeMoco Preparation:

The instrument and lamp need to warm up for at least one hour prior to use, so set up the instrument. First make sure the gas is turned on, then turn on the water to cool the furnace, Then turn on the power to both the HGA programmer and the Spectrometer. Plug in the Mo lamp, and turn the amperage up to 30. Now you can go and make up your standard curve and the FeMoco samples.

The procedure for determination of the concentration of molybdenum in the cofactor sample is to compare it with a standard curve with known concentrations. The standard solutions which are currently being used have been purchased from Perkin Elmer. The shipped solution has a concentration of 1000 μg/mL (10.4 mM) Mo and should have the calculations

on the side of the bottle. The first step is to serially dilute out the stock solution using 2 % HNO_3 as follows:



Solution D (10.4 µM $MoSO_4$) is used to generate the standard curve. The following samples are made:

Conc of Mo present	Am't stock added	Am't 2% HNO_3 added
0 µmoles	0 µL	1000 µL
0.1 µmoles	10 µL	990 µL
0.2 µmoles	20 µL	980 µL
0.4 µmoles	40 µL	960 µL
0.6 µmoles	60 µL	940 µL
0.8 µmoles	80 µL	920 µL
1.0 µmoles	100 µL	900 µL

The cofactor samples to which 975 µL of 2% HNO_3 has been added when they were brought out of the glove box, now have precipitant in them. This is just excess salt. The samples need to be spun down and the supernatant pipetted off into another test tube. The cofactor samples now should be further diluted 1:2 (making it a 1:80 dilution) in addition to the initial 1:40 dilution.

After the Mo lamp has been warming up for 1 hour, turn the Signal knob to Set up and the gain to 15. The Signal knob is then put on Abs (Absorbance) and the Mode knob to PK HT (peak height). This should result in the lamp energy reading 75. The HGA Controller must now be programmed for Molybdenum with the following:

Step	Temp	Ramp Time	Hold Time	Additional programming
1	160	1	25	
2	300	1	4	
3	1000	1	4	
4	2650	0	3	Hit **Read** and **Mini Flow** buttons. On the Spectrometer, press **3**, then **t**
5	2700	1	4	

At the end of the programming, press **1, Step** to return to the beginning of the program. You should check the program (by pressing chk and then the various step, temp, time and ramp buttons) to make sure it is correct.

The analysis can now begin. The zero must first be set by injecting the 20 µL of the 0 µmoles solution. Once you have a stable baseline, then the rest of the standard solutions can be injected. Depending on how accurately you inject the samples, the variation between different injections can vary. It usually is a good idea to inject the same sample five times in order to get meaningful numbers. Examples of absorbances for the standard curve are shown below:

Amount of Mo	Sample absorbances	Average
0.1 µmoles	0.020, 0.017, 0.018, 0.020, 0.019	0.019 ± 0.001 (± 7.0 %)
0.2 µmoles	0.047, 0.049, 0.052, 0.050, 0.048	0.049 ± 0.002 (± 3.9 %)
0.4 µmoles	0.112, 0.102, 0.102, 0.107, 0.104	0.105 ± 0.004 (± 4.0 %)
0.6 µmoles	0.167, 0.161, 0.165, 0.163	0.164 ± 0.003 (± 1.6 %)
0.8 µmoles	0.228, 0.220, 0.222, 0.224, 0.229	0.225 ± 0.004 (± 1.7 %)
1.0 µmoles	0.278, 0.291, 0.294, 0.273, 0.284	0.284 ± 0.009 (±3.1 %)

This produces a standard curve with the r value of 0.99. Now the cofactor samples need to be injected. Before you start injecting unknown samples, you should inject the zero again to verify that baseline is still zero. Depending on the concentration of salt in your cofactor sample, there can be a great deal of noise in your absorbances. By having duplicate samples, you should be able to see which absorbance is real. The following are examples absorbances for the above standard curve:

Cofactor sample #1 (1:80) 0.006, 0.020, 0.009 (too low for a significant value)
#1 (1:40) 0.026, 0.028, 0.029, 0.040, 0.018. 0.047
Average absorbance is 0.031± 0.006 (did not use the highest and lowest values for absorbances.

Cofactor sample #2 (1:80) Too close to background
#2 (1:40) 0.027, 0.032. 0.031, 0.038, 0.019, 0.049
Average absorbance is 0.032±0.005 (not using the highest and lowest absorbances.

Using the standard curve, the cofactor sample has 0.138 µmoles of molybdenum. Based on a 1:40 dilution factor, and because for every mole of MoFe protein, there are 2 moles of Mo, the total molybdenum concentration is 11.06 µM. You then divide the value for units/mL by the concentration of

molybdenum to get the specific activity in nmole acetylene produced/min/mL/Mo. For the example that we have been using in this guide, there are units/mL = 3525.3 and a [Mo] = 11.06 μM produces a specific activity of 318.50, which is a very good number. These values for active cofactor usually range from 175 to 280. If the number is low, then there is degraded cofactor present, and it would be best to go back to the preparation to examine what could have happened. The specific activity can also increase as the sample has excess salt removed.

Iron Determination:

Another metal in FeMoco that can be easily analyzed is iron. There are seven times as much iron present in FeMoco than molybdenum. There are two methods currently in use in this lab. One is to do Iron Atomic absorption. The other is a spectrophotometric analysis using ferrozine (Carter, 1971). There is usually a background concentration of iron present in all the samples. This can be reduced by using acid washed test tubes and metal free pipette tips. To eliminate all of the iron from the glassware, it should be soaked in 5 M HNO_3 for 24 hours, then rinsed with Millipore water to remove the acid (usually rinsing at least 10 times), a soak for 24 hours, then a rinse with Millipore water for at least 20 times and then a final drying in the oven. This may seem a little extreme but it has been determined to be the only method that works (personal communications with D. Wright and R. Eisen).

The samples that will be used for iron determination should have been taken before the concentration of cofactor to dryness. Usually 20 μL of cofactor is put into a test tube and then 980 μL of 2% HNO_3 or water depending on the type of analysis is added. If the sample has not been desalted, then there will be a precipitant that can be removed by centrifugation, just prior to analysis. Whether the analysis is going to be done spectrophometrically or using AA, known concentrations of iron need to be made up for the standard curve. If

the spectrophotometric assay is going to be used, the solutions are made up in water, if AA is going to be done, then the solutions are made up in 2% HNO_3. The iron standard from Perkin-Elmer has a concentration of 1.078 mM (see side of container for calculations). This standard needs to be diluted 1:10 and this solution (B) will be used for the analysis.

Standard Conc.	Am't of B added	Am't of H_2O (or 2% HNO_3) added
0	0 µL	1000 µL
1	10 µL	990 µL
2	20 µL	980 µL
4	40 µL	960 µL
6	60 µL	940 µL
8	80 µL	920 µL
10	100 µL	900 µL

For AA analysis, the procedure is the same for the molybdenum analysis except for the following:

- The slit width on the lamp for iron is 0.2.
- The wavelength is 248 nm
- The program is:

Step	Temp	Ramp Time	Hold Time	Additional Programming
1	160	1	25	
2	300	1	4	
3	1000	1	4	
4	2500	0	2	Hit **Read** and **Mini Flow** buttons. On the Spectrometer, press **2**, then **t**
5	2700	1		4

The quicker determination of iron is to use the ferrozine spectrophotmetric assay. The procedure is as follows:

- Make up the standards and sample solutions using Millipore water.
- Make up the Ferrozine assay solution as follows:

  5 mLs of 0.2 N HCl

  5 mLs of 11.3% trichloroacetic acid (TCA)

  4 mL of 10% Ammonium Acetate (NH_4OAc)

  1 mL of ferrozine solution

The ferrozine solution consists of 75 mg of ferrozine and 75 mg of neocuproin in 25 mL of acidic water [25 mL of water plus 1 drop of concentrated HCl]. Once the above are mixed, add 20 mg of Ascorbic acid. Then add 1 mL of this solution to each test tube.

- Incubate the test tubes for 15 minutes at room temperature. If necessary, the samples can be centrifuged to removed precipitants at this stage.
- Read the absorbances at 562 nm.

The following are sample absorbances using the zero as the blank:

Concentration	Absorbances
0	0.00000
1 μM	0.01532
2 μM	0.01443
4 μM	0.06691
6 μM	0.08856
8 μM	0.13414
10 μM	0.16994

Desalting:

The extracted cofactor is now in a dry state in the glove box. Because the cofactor is not particularly stable in high salt concentrations, the excess salt needs to be removed. The first step is trituration, followed by a desalting column, LH-20. To achieve the minimum salt to cofactor ratio, isopropanol precipitation is the final step. Each step will be described separately.

Trituration:

One way to remove half of the excess salt is to triturate the sample. The dry cofactor sample needs to be ground up into a fine powder. You should use a glass rod. Teflon spatulas have a tendency to have the Teflon scraped off which ends up contaminating the cofactor. Once the sample is ground up, a stir bar is added. The sample is then resuspended in half the amount of reducing DMF in which it was eluted off the column. The solution if stirred for about half an hour. The cofactor is soluble in this volume of DMF but not all of the salt is. This means that there will be precipitants remaining in the flask.

Once the solution has been stirred, then the liquid is filtered off using a medium glass frit and filter flask. The filtrate contains the valuable cofactor sample. Samples should be taken remembering that the solution is now twice as concentrated as it was when it eluted off the column.

At this stage, the cofactor solution can have its solvent removed by rotorvapping it down, or it can be further purified by passing it down an LH-20 column.

LH-20:

During the quest for a good desalting column, a vast range of gel exclusion column materials were tested by A. Hickman and D. Wright. These included Alumina, Polyamide 6, G-15, G-25 and LH-20. It was found that the only material that swelled sufficiently in organic solvents and did not reduce the activity of FeMoco were the G-25 and LH-20.

LH-20 can be brought into the glove box dry and allowed to swell overnight in DMF. A column can be poured and packed, and just before use, it should be spiked with organic dithionite to scrub it of its oxidizing equivalents. The triturated cofactor sample can be run through the column and once it elutes off the column, the solution should be collected in polypropylene tubes. The K^+ from the base bath contaminates the desalted cofactor solution if the samples are kept in glass. When the cofactor has eluted off the desalting column, the salt remains. The column should be washed with at least 5 column volumes of solvent to elute off the salt before the column is used to purify additional cofactor.

For large scale desalting of FeMoco, a large DMF resistant column and pump system must be used. A 10 x 30 cm glass column with glass fritted filter paper matrix supports and Kevlar O-rings can have LH-20 poured into it outside of the glove box and then the apparatus can be brought in. The flow of the column can be controlled by a Fluids Metering Pump, model AS41CSC with low flow adapter that can maintain a flow of between 0.5 to 1.0 mL/min. The tubing for this column is polypropylene. The column should be scrubbed

with a 10-20 mL of organic dithionite solution to scrub it of its oxidizing equivalents. After 1 column volume of reducing DMF has been eluted through, a 20-30 mL FeMoco sample can be loaded onto the column. The dark brown band of FeMoco is eluted off in 80 mL and stored in polypropylene tubes.

Desalting of FeMoco can also be done on a smaller scale using a 1.5 x 50 cm DMF resistant Econocolumn. The column material is prepared the same way as for the larger column. Usually 1-2 mL of triturated FeMoco solution is desalted at one time. The column is hand loaded with a pipette and rune using gravity instead of a pump system. Clean-up of the column after use is the same as for the larger column.

Passing the sample down one LH-20 column reduces the salt to Mo ration to 200 to 1. A second LH-20 column further reduces the ratio to 1:20.

Another interesting feature of the LH-20 column is the ability to exchange cofactor solutions into different organic solvents. The extractions are usually done in DMF, but that is not necessarily the solvent of choice. You can pour a column in acetonitrile, add the cofactor sample that is in a small volume of DMF and run the column in acetonitrile, effectively desalting and exchanging the cofactor sample at the same time.

Isopropanol precipitation:

This procedure works because the salt used to extract the cofactor is not very soluble in this solvent, whereas cofactor is. Cofactor can be precipitated rapidly by using a 10 fold excess of isopropanol. Repeated isopropanol precipitations will result in the minimum salt to cofactor ratios. From the work of D. Wink and coworkers, this minimum salt to Mo ratio was determined to be 4:1.

Small volumes (1.0- 1.5 mL) of concentrated cofactor samples, (0.1 - 1.0 mM Mo concentration) can be put into 3-5 mL Wheaton crystallization vials. These vials can then be put into a reservoir containing 20-30 mL of freshly dried and vacuumed distilled isopropanol. This reservoir can be placed into a

container, sealed, and placed in a vibrationaly insulated location in the glove box. After 3 days, black solid can be collected from the crystallization vials. D. Wright using this procedure was able to yield a solid which had the following ratios: Mo:Fe of 1:6-8, Mo:As (the extracting salt) of 1:3-5, and a Mo:Na (contaminating salt) of 1:0-2. Unfortunately these solids were microcrystalline and unsuitable for X-ray diffraction.

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