

Spectroscopic and Mechanistic Studies of Nitrogenase

by

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Submitted to the Department of Chemistry
in Partial Fulfillment of the Requirements for the degree of

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Abstract.

A series of experiments were performed with the intention to obtain some insights into the structure and function of the enzyme nitrogenase. Nitrogenase is a two-component enzyme responsible for the conversion of nitrogen gas from the atmosphere into ammonia which can be assimilated by living organisms. The two components of nitrogenase, separately known as the Fe and the MoFe protein were purified according to standardized procedures, and a number of studies performed using this protein for spectroscopy. Specifically, the techniques used were Electron Nuclear Double Resonance (ENDOR) spectroscopy, and X-ray Absorption Near Edge Structure (XANES).

ENDOR spectroscopy was used to attempt to characterize "resting state" MoFe protein, to probe for the possible presence of hydride ligands. This was accomplished by allowing the enzyme to turn over substrate in D₂O solution. Both the absence of protons or the presence of deuterons, as observed by ENDOR spectroscopy, can be used to probe this question.

Secondly, an attempt was made to more precisely characterize the CO-inhibited state of nitrogenase using both ENDOR and XANES. Carbon monoxide gas completely inhibits the nitrogen and acetylene reduction capabilities of nitrogenase. However the enzyme is still fully capable of reducing H⁺ to H₂ gas under CO, and during this time the MoFe exhibits unusual Electron Paramagnetic Resonance (EPR) spectra. These states of the enzyme were investigated first of all by ¹³C ENDOR, using isotopically enriched ¹³CO gas to detect the presence of CO bound to one of the clusters of the MoFe protein. Secondly, XANES was used to check the relative oxidation state of the enzyme during turnover under CO. Preliminary results show that the form of CO-inhibited nitrogenase generated under low partial pressures of CO appears to be quite markedly more reduced than other states of the enzyme appear to be.

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

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I am also most blessed to have a wonderful group of friends at 216-1/2 Harvard St—thankyou Andrew, Joel, John, Joe, Tim and Jim Derksen and all of the other numerous guests that have made life at “C-flat” very enjoyable indeed.

Thankyou to my church Tree of Life City Church Boston for being an encouragement and a family to me while away from home. God bless you.

Finally I wish to thank Kathryn for being extremely patient through much uncertainty and for maintaining a forbearing spirit whenever I fall short

Thankyou to all of my friends that I haven't had time to mention here. I love you all. Special thanks to Araz and Mary for helping me and supporting me through the “writing” phase of the thesis. Thankyou for being a shelter from the storm..

Psalm 121

I lift up my eyes to the hills—
where does my help come from?
My help comes from the LORD,
the Maker of heaven and earth.

He will not let your foot slip—
he who watches over you will not slumber;
indeed, he who watches over Israel
will neither slumber nor sleep.

The LORD watches over you—
the LORD is your shade at your right hand;
the sun will not harm you by day,
nor the moon by night.

The LORD will keep you from all harm—
he will watch over your life;
the LORD will watch over your coming and going
both now and forevermore.

Spectroscopic and Mechanistic Studies of Nitrogenase.

Robert C. Pollock

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Chapter One: Background and Significance.

1.1 History of Nitrogen Fixation.

It is common knowledge to any high school biology student today that nitrogen is an essential nutrient to the growth of plants. Farmers use ammonium salts as a supplement to the soil in order to ensure optimal growth of crops. It is also common knowledge that these organisms need nitrogen in a useful form for the synthesis of proteins, which are in turn essential for proper cell function and for growth. Since feudal times, it has been known that the planting of legumes seemed to replenish the soil in some way, facilitating the harvest of non-leguminous crops planted in the following season. Most college freshmen in biology are familiar with the process of nitrogen fixation, that is the conversion of nitrogen from the air to ammonia, a form useful to living organisms. It is this process that accounts for the replenishment of soil observed upon cultivating legumes (such as soy bean, peas or string beans).

So with these facts as a foundation, it is difficult for the average twentieth century scientist to imagine that there was in fact a time—fairly recently—when it was *not* known precisely how plants obtained their supply of nitrogen, and the process of nitrogen fixation was vigorously disputed. The field of nitrogen fixation, or at least the study of it, is unique in that in order to address it properly, one must approach the problem from many angles. The attempt to understand nitrogen fixation has become one of the most interdisciplinary efforts of science to date. Thus, it is fascinating to review the history of these studies to observe how the cumulative efforts of many great minds have led in a stepwise process, to the place where an understanding of the molecular details of nitrogen fixation is imminent. The following discussion is by no means comprehensive, but focuses on those results more directly related with the chemistry of nitrogenase, and those results which illustrate the extraordinary science through which the details of nitrogen fixation have been elucidated. Discussion of research on the development of root nodules, and aspects of plant biochemistry has been completely omitted, but is more than adequately discussed in a number of books on this field¹

1.1.1 The Beginning of Nitrogen Fixation Research.

The idea that perhaps plants could take nitrogen from the atmosphere, as well as their carbon, was first espoused by Joseph Priestley and Jan Ingenhousz (the discoverers of photosynthesis). However at that time the means to investigate this hypothesis were not available. Throughout the nineteenth century, as procedures for total nitrogen analysis were developed and refined, opinions fluctuated wildly regarding 'nitrogen balance' between the soil and the plant. Initial investigations² indicated that plants did *not* uptake nitrogen from the air, rather from the soil. Priestley's initial premise was subsequently presumed to be false, and the hypothesis developed by von Liebig that plants absorb carbon from the air, and all other nutrients in the form of salts from the soil (a premise which has been shown to be largely true). Thus many of the early results which indicated perhaps some plants could take nitrogen from the air and assimilate it were literally considered with a grain of salt. In some cases this was justified. Many early investigators came to the right conclusions for the wrong reasons. For example Berthelot^{3,4} observed an increase in soil nitrogen content during soil incubation but later claimed the same observations in the case of organisms that are now conclusively known *not* to fix nitrogen⁵, placing the reliability of the earlier work in question. One author

made the claim that “the assimilation of elementary dinitrogen is distributed all over the plant kingdom where chlorophyll is present”⁶.

Nitrogen fixation associated with leguminous plants was first conclusively demonstrated in 1888 by Hermann Hellriegel and Hermann Wilfarth at Bernberg in Germany. This discovery proved to be a landmark discovery in the field of nitrogen fixation. Around that time in Germany there was considerable interest in finding means for more efficient plant growth and yield from crops, due to a recent increase in population density. One particular Duchy, Anhalt thrived on the cultivation of sugar beets, but was having some problems with low yields. It was hoped that mineral fertilization might ameliorate the situation somewhat, so the government set up a research station to investigate this. This was the environment in which Hellriegel and Wilfarth conducted their research⁷—initially to investigate methods for increasing sugar beet yields. Hellriegel had developed a methodology for investigating the nutrient requirements of plants by cultivating them



Figure 1: Prof Dr Hellriegel (left) and Prof Dr. Wilfarth (right)

on a medium of purified quartz sand, to which various nutrients could be added in carefully controlled amounts. He had some problems applying this approach to the growth of sugar beets, because of problems with too-rapid water loss and an unknown plant disease (later shown to be caused by Boron deficiency). Subsequently, along with his assistant Wilfarth, he began experimenting with barley, oats, peas and lupins, which grew better in sand. They obtained the following results:

- The growth barley and oats was directly proportional to the amount of nitrogen added to the sand, and the amount of nitrogen absorbed by the plant was less than the available amount in the medium.
- Cultivated leguminous plants showed mostly higher nitrogen amounts than the substrate. The effect remained even when plants were grown in glass bulbs from which the air had purged of all nitrogen compounds. They proposed the nitrogen within the plants came from atmospheric dinitrogen gas.
- Growth of the plants on sterile sand, with addition of boiled soil extracts gave the same result as barley and oats.

- The characteristic “root nodules” of legumes were only formed when specific microorganisms were added to the soil.

These results were interpreted at the time with both great enthusiasm and great disdain. Many agronomists, who had previously noticed differences in the nitrogen metabolism of legumes (referring to them as “nitrogen samplers”), were extremely excited by these results. However, a contemporary botanist, B. Frank, was enraged because they contradicted his hypothesis that nitrogen fixation was a property of all green plants. But today they have been clearly confirmed and represent the beginning of the study of nitrogen fixation as a field unto itself.

1.1.2 Consequences of Hellriegel and Wilfarth's Discovery.

The discovery that nitrogen fixation in legumes was enhanced by the presence of microorganisms led to the onset of the inoculum industry. Microbiologists were enlisted and given the task of screening for pure cultures. The first bacteria isolated were given the neutral name *Rhizobium*,⁸ because at that stage it was still not known whether the active species was bacterial or fungal. The problem was complicated when the bacteria isolated from root nodules proved to be incapable of nitrogen fixation on their own. However, the addition of these bacteria to sterile legume cultures elicited the formation of root nodules. This result had obvious agricultural implications, and was capitalized on. Bacteria were sold to the farmer in the form of a liquid suspension, or on agar. Eventually, methodology was developed for the use of peat as a microbial carrier. Inoculation became a very popular technique for the enhancement of leguminous crops, as it is still today.

It took almost a decade for the existence of free-living microorganisms to be recognized. *Clostridium pasteurianum*, a free-living anaerobic organism and friend of nitrogenase biochemists, was discovered in 1893⁹. The aerobic organisms *Azotobacter chroococcum* and *Azotobacter agilis* were also observed to be efficient nitrogen fixers.¹⁰ In addition to the symbiotic and free-living bacteria which have been shown to act as nitrogen fixers: nitrogen fixation has been demonstrated in blue-green algae¹¹ (more correctly referred to as cyanobacteria) and in other photosynthetic bacteria, such as purple and green sulfur bacteria.

In the 1930's, P.W. Wilson studied the biochemistry of nitrogen fixation for the symbiotic system of *Rhizobium trifolii* and red clover. He conducted these using 9 liter serum bottles (which could be evacuated and filled with the gas of choice) and sterilized sand substrates (similar to those used by Hellriegel and Wilfarth). The intention was to monitor growth of the plants in response to varying concentrations of nitrogen, oxygen and carbon dioxide. All the vials were kept at atmospheric pressure using argon or helium. When the investigators tried using hydrogen as a backfill gas, assuming it to be inert, the growth of the plants was inhibited. Initially, they assumed this result to be due to some impurity gas in the hydrogen, but the same result was obtained with hydrogen that had been rigorously scrubbed, as well as hydrogen generated by the action of HCl on zinc metal and other sources. The result was competitive inhibition of growth, which was reversed upon addition of combined nitrogen to the plants. These results clearly showed that hydrogen was inhibiting growth because it was acting as a competitive inhibitor of the process of nitrogen fixation.¹²

The availability of ¹⁵N-enriched N₂ gas in the late 1930's led to the enterprising experiments of Robert H. Burris, who realized that this material would make an ideal tracer gas for studying nitrogen fixation. At the suggestion of Dr. Harold Urey at Columbia University, who supplied the isotope, Burris conducted an experiment with a non-equilibrium mixture of N₂ species of molecular masses 28,29 and 30. He grew cultures of the free-living diazotrophic (nitrogen fixing) bacteria *Azotobacter vinelandii*

on this mixture. If there was any exchange processes taking place, i.e. reversible N_2 dissociation/reassociation reactions, then the gas mixture would approach equilibrium. However, even in cultures vigorously reducing dinitrogen, *no* such equilibration was observed¹³. This indicated that once the nitrogen-nitrogen bond is cleaved (however that occurs), *both* atoms are irreversibly committed to the process of nitrogen fixation.

It took some time before investigators were convinced of the role of ammonia as an intermediate in the fixation of atmospheric dinitrogen. Burris' results with amino acid fractionation of *A. vinelandii* grown on $^{15}N_2$ showed the highest level of incorporation was into glutamic acid. His mass spectrometric analysis entailed the hydrolysis of glutamine to glutamic acid followed by liberation of the amide group as ammonia¹⁴. He proposed that this was indirect evidence that ammonia was the intermediate species between elemental and fixed nitrogen, and that it was incorporated into the cellular metabolism by way of glutamic acid. The premise of ammonia as the intermediate was largely ignored because at the time there was some evidence for hydroxylamine as the "missing link"¹⁵. These results have since been discredited, and ammonia is known to be the initial product of nitrogen fixation, which should serve as a cautionary reminder to those in this field that wrong conclusions can be made, and have been in the past.

The first definitive evidence for the central role of ammonia in nitrogen fixation came with studies of the free-living anaerobe *Clostridium pasteurianum*, which excretes ammonia under certain conditions. Ammonia recovered from cultures of this bacterium grown on $^{15}N_2$ was far more enriched in ^{15}N than any other compound analyzed¹⁶.

In order to more clearly elucidate the details of nitrogen fixation, many groups pursued the goal of achieving this in cell-free extracts. Burris and coworkers¹⁷ achieved measured success in attaining this goal, but reproducible results were not obtained until work by Carnahan et al¹⁸. They used extracts of *Clostridium pasteurianum* obtained through sonication, using pyruvic acid as a substrate at very high (0.15M) concentration. Following on from these experiments, nitrogen fixation has been observed in cell-free extracts from *Rhodospirillum rubrum*, cyanobacteria, *Klebsiella pneumoniae* and various *Azotobacter* species. Success with cell-free extracts opened the door for purification of the enzyme responsible for nitrogen fixation, nitrogenase, which was purified in the 1960's from a number of sources. Nitrogen fixation activity was shown to depend on the presence of not one but two separate component proteins: one containing both iron and molybdenum (the molybdenum-iron, or MoFe protein), and another ferredoxin-like protein containing only iron (the Fe protein). The structure and function of nitrogenase has been studied in great detail for many years now, and these results are summarized in section 1.2.3 below.

Although it was not possible to clearly formulate mechanistic ideas with the limited data available before isolation of nitrogenase, there are some interesting results which allude to the mechanism. The first one is the very early observation that hydrogen acts as a competitive inhibitor of nitrogen fixation. Secondly, Mozen demonstrated that nitrous oxide¹⁹ could also act as a substrate for the (at that time unknown) enzymes responsible for nitrogen fixation. The third, even more interesting result was the observation that incubation of nitrogen-fixing plants in the presence of deuterium (D_2) gas led to formation of HD²⁰. More astonishingly, this exchange process *only* occurred in the presence of nitrogen gas.

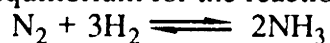
These early foundational experiments have proven enlightening even to this day, and in fact much of the interpretation of today's data hinges upon some of these keystone discoveries.

1.2 Chemistry of Nitrogen Fixation.

1.2.1 Industrial Catalysts.

Around the turn of the century, efforts to find a means of producing ammonia from its elemental components, that is hydrogen and nitrogen gases, were intense. This was motivated by a desire to find an economic route for the synthesis of nitrogenous fertilizers (such as ammonium nitrate) and also for cheap nitric acid, for use in explosives. Haber and Oordt²¹ are credited with the first success in this arena, published in 1905.

It was believed that the decomposition of ammonia at high temperatures might be irreversible, because experiments had shown this reaction to proceed essentially to completion. In order to investigate this possibility more thoroughly, Haber and Oordt intended to investigate the equilibrium for the reaction



They performed the experiments in the presence of a dispersed iron catalyst, and noticed the presence of a small (0.02-0.10%) amount of ammonia was generated at 1000°C and atmospheric pressure. Subsequent equilibrium calculations did not agree with the experimental result, however when the experiment was repeated under conditions of high pressure, the result was consistent with theory.²²

Haber devised an efficient reactor whereby the newly synthesized ammonia could be stripped and replaced with fresh hydrogen and nitrogen in a continuous fashion. His collaborative efforts with Bosch at BASF led to the first industrial scale production of ammonia. Initial pilot scale reactors used osmium as the catalyst, which was found to be extremely effective, yielding 8% NH₃ at 550°C and 20atm pressure continuously for a long period²³. Uranium and uranium carbide also proved to be effective catalysts, however these were susceptible to poisoning by O₂ or water vapor.

Although very effective as catalysts, osmium and uranium were impractical for consideration as industrial catalysts because of their expense. Haber and his coworkers embarked on an extensive search for an effective, cheap catalyst and in so doing investigated some 2500 different potential catalysts. They had surmised that a multicomponent catalyst might be a realistic proposition because of the readiness with which such mixtures form nitrides in the presence of N₂. Finally, they achieved success with a single sample of magnetite from Sweden. Other samples of iron ore proved to be less effective, so it was presumed that the effectiveness in catalysis was induced by impurities. Further studies showed that high-melting point, stable oxides (such as alumina, TiO₂, or magnesia) were effective in increasing the catalytic properties of pure iron; sulfur was extremely poisonous; and alkali metal oxides, when used in conjunction with alumina, were also conducive to effective catalysis. These results led to the introduction of an Fe-Al₂O₃-K₂O composite which reproduced the activity of the Swedish magnetite sample. This is for the most part identical with the catalysts used today in the industrial production of ammonia.

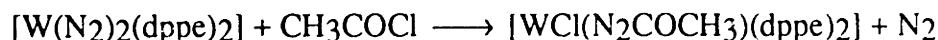
The chemistry of dinitrogen at the surface of the iron catalyst, interestingly enough, may be of some relevance to the solution of the problem of biological nitrogen fixation, as discussed in more detail in chapter four.

1.2.2 Chemical Nitrogen-Fixing Systems.

It has been some thirty years since the first dinitrogen complex, [Ru(NH₃)₅(N₂)]²⁺ was discovered²⁴, and in that time there has been considerably more progress in the field of metal-dinitrogen chemistry. Transition metal complexes have been successfully synthesized with fairly unstable nitrogen species (e.g. diimide, N₂H₂) coordinated. The

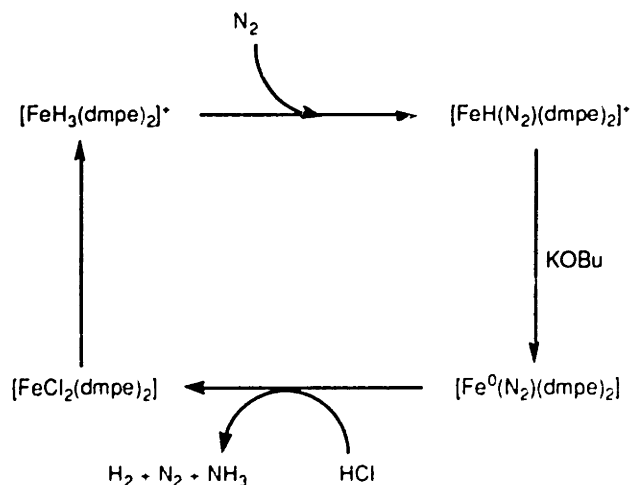
metal serves to stabilize such otherwise transient species. The metal serves to stabilize such otherwise transient species. [more to be written later]. Dinitrogen is isoelectronic to carbon monoxide, and given the plentitude of carbonyl complexes, it was perhaps not surprising that many stable dinitrogen complexes have now been isolated.

But what relevance do these complexes have to the real-life chemistry of nitrogen fixation, when the rubber meets the road? Perhaps the beginnings of the answer to that question begins when one investigates experiments with dinitrogen-binding inorganic complexes which not only bind dinitrogen but are actually capable of reducing it. The first example of such a reaction was observed with a tungsten complex²⁵, which reacts as follows:



While this reaction is not exactly a contender for replacing the Haber-Bosch process, it is nevertheless a very important step in the right direction, because this acetylation reaction is in many ways analogous to protonation. In fact, this work opened the door for studies of the protonation of coordinated dinitrogen²⁶

Until the discovery of the vanadium and all-iron nitrogenases, it was assumed that dinitrogen reduction by nitrogenase probably worked by a mechanism similar to that exemplified by group 6 transition metals. It seems highly unusual to have the reduction of dinitrogen occurring at an all-Fe center, at least when one considers the range of other chemical systems which are capable of reducing dinitrogen. It would seem that in order for Fe complexes to carry out the reduction of dinitrogen, one would have to invoke very reduced states and possibly even formally negative oxidation states in order to accomplish this. There are examples of dinitrogen-reducing chemistry occurring at Fe, but up until very recently it seemed that the role of Fe in such systems was spurious. Examples of the latter type of chemistry are those involving a transition metal complex in cohorts with a strong organic reducing agent, such as FeCl₃ with LiPh. Jimenez-Tenorio and Leigh²⁷ discovered a novel Fe-containing system which is capable of reducing dinitrogen. Their system involves an Fe complex of dimethylphosphinoethane, which is apparently reduced to Fe(0) upon addition of base. This compound releases ammonia when treated with acid.



1.2.3 Molybdenum Nitrogenase.

The isolation of cell-free nitrogen fixing extracts, followed by the purification of the enzyme responsible (for nitrogen fixation) from *A. vinelandii* (1966)²⁸ then from *C. pasteurianum* (1967)²⁹ opened doors to the characterization of this complex process at the molecular level for the first time. Of particular interest and import was the discovery that nitrogenase catalyses the $2e^-$ reduction of acetylene to ethylene, a process that is

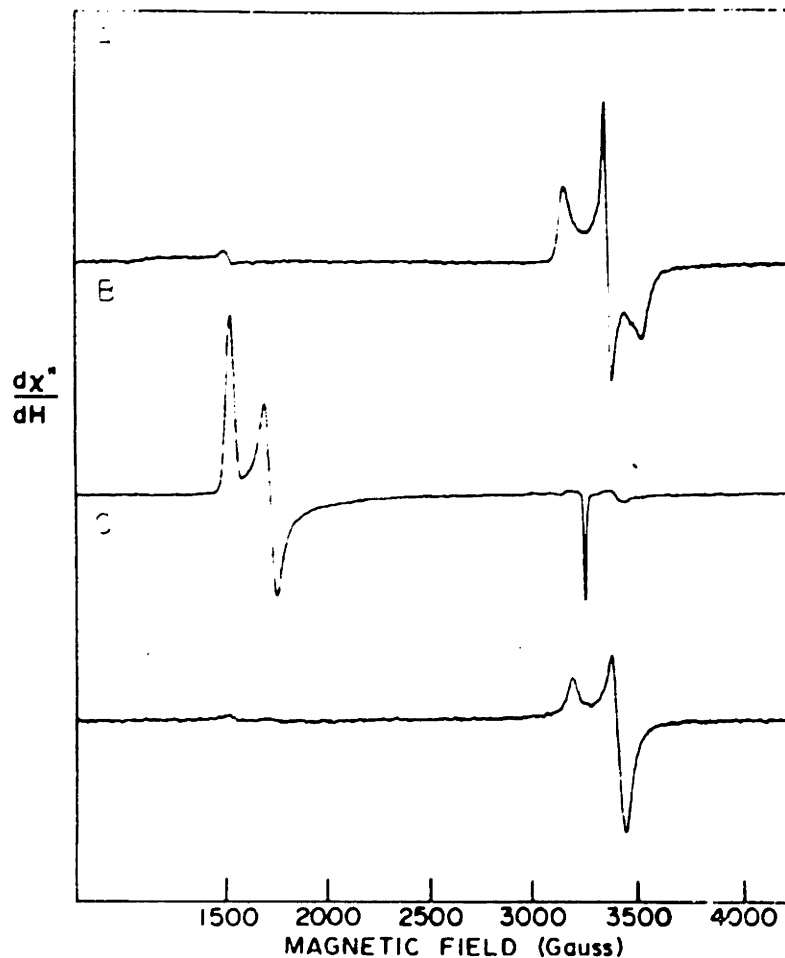


Figure 2: EPR spectra of nitrogenase proteins

a) Nitrogenase Fe protein

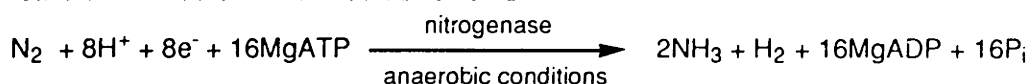
b) Nitrogenase MoFe protein

c) "impurity protein"

[From Palmer et al, Arch . Biochemical Biophysics, 153, 325-332, (1972)]

relatively straightforward to assay by gas chromatography.³⁰

After the advent of cell-free N-fixing systems and the introduction of more rigorous anaerobic techniques (not usually the domain of the biochemist!) that entailed the purification of the enzyme to homogeneity, the baton was passed, so to speak, to the physical biochemists. Both components were found to be similar to ferredoxin, and with the discovery of Molybdenum in one of the components they were labeled as “azoferredoxin” and “molydoferredoxin” respectively. Both components were shown to be paramagnetic by electron paramagnetic resonance spectroscopy (EPR)^{31,32}, and later this same technique was used to conclusively demonstrate that electron transfer proceeded from azoferredoxin (alternatively component two, Fe protein or dinitrogenase reductase) to the molybdoferredoxin component (also known as component one, MoFe protein or dinitrogenase)³³. This electron transfer process was shown to occur with the concomitant hydrolysis of ATP in the presence of Mg²⁺. The transfer of one electron requires the hydrolysis of 2 ATP³⁴; in practice one observes ATP/2e ratios of about 5, thus the process is not completely efficient. In addition, reduction of dinitrogen always proceeds with the evolution of H₂. This reaction cannot be suppressed by increasing the partial pressure of N₂. In the absence of N₂, the enzyme simply produces H₂. The overall reaction can be summarized as follows



Within the cell, the Fe protein in turn receives reducing equivalents. The reductant varies³⁵ from species to species. In *C. pasteurianum*, the Fe protein receives electrons from a specific ferredoxin, while *Klebsiella pneumoniae* has developed an unusual electron transfer system from pyruvate, through a ferredoxin-based pyruvate decarboxylase (the gene product of the *nifJ* gene in the nitrogen fixation or *nif* gene cluster), to a flavodoxin (NifF) and onto the Fe protein. The sequential delivery of electrons to the MoFe protein results in the conversion of this protein into a “super-reduced” form, with concomitant loss of the EPR signal, to which dinitrogen binds and is reduced. This is illustrated schematically in figure 3

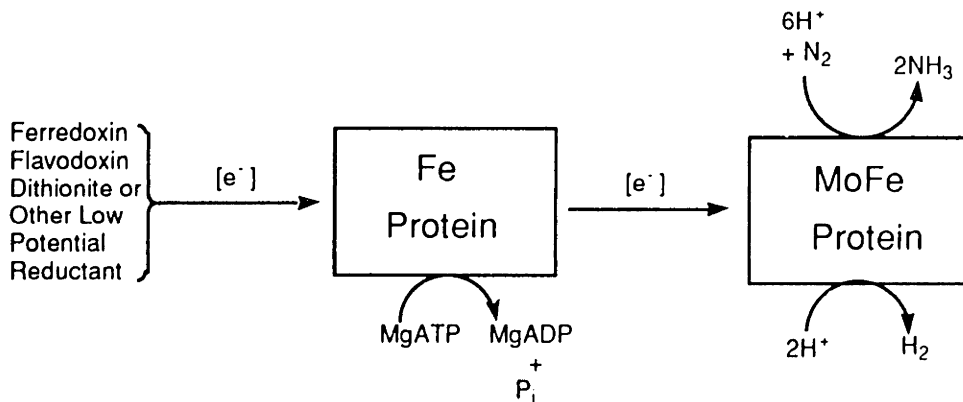


Figure 3: Electron Transfer to Nitrogenase

Further studies have shown that the MoFe protein, which is a large protein (M. wt ~220,000) containing a large number of Fe atoms (24-35) and 2 Mo atoms³⁶, is composed of two types of metal center. Two biophysical techniques—**Mössbauer spectroscopy** (which measures the effect of the electronic environment of ⁵⁷Fe nuclei on a specific nuclear transition) and **Electron Paramagnetic Resonance** (EPR, which looks at the effect of the metal environment on an *electron* spin) proved to be quite illuminating.

Mössbauer studies showed that the large $S=3/2$ EPR signal was associated with at least 12 of the Fe atoms, and in addition there were some 16 Fe atoms in some paramagnetic center. Later it was demonstrated that the Mo-containing cluster could be removed from the protein by treatment with N-methyl formamide³⁷, that it was in a form bound to iron, and that extracts of this "Fe-Mo containing cofactor" could be used to restore nitrogenase activity to extracts from NifB⁻ ("cofactor-less") bacteria. This cofactor (known as FeMoco) was shown to have similar (though not identical) EPR characteristics to the native protein. Biosynthetic studies showed that homocitrate was incorporated into cofactor during *in vitro* synthesis, which implies the cofactor contains homocitric acid as a ligand³⁸. This result was in agreement with earlier genetic complementation studies with non-diazotrophic mutants.³⁹

Isolation of FeMoco from a mutant strain of *A. vinelandii* defective in the *nifV* gene (which reduces acetylene and produces H₂, but does not reduce N₂), and insertion of this into to the apo-MoFe protein from nifB⁻ extracts resulted in transfer of the phenotype⁴⁰. It was surmised on this basis that FeMoco is the site of substrate reduction. Similarly, treatment of partially denatured MoFe protein with certain thiols showed the remaining "P-cluster" Fe could be "extruded" as [4Fe-4S] clusters⁴¹. Thus the MoFe protein was found to contain at least two metal-sulfur clusters: the Iron-Molybdenum cofactor, of unknown structure, and the 'P'-clusters (presumed to be some form of ferredoxin-like [4Fe-4S] cluster).

The precise nature and disposition of the metal centers was revealed more completely with the completion of the crystal structure of *A. vinelandii* MoFe⁴² and Fe⁴³ proteins in 1992. The structure of *C. pasteurianum* MoFe protein has also been solved more or less independently by two groups, Doug Rees and colleagues at CalTech⁴⁴ and Jeff Bolin and colleagues at Perdue⁴⁵. Refinement of the structure of the MoFe protein to 2.2 Å revealed some heretofore unforeseen features in the structure of the "M-center" or cofactor, which are still somewhat mysterious. Surprisingly, the structure of the cofactor revealed the presence of 3-coordinate Fe atoms, approximately trigonal. Such an arrangement has only been observed once, fairly recently⁴⁶, in a complex which contained extremely bulky dimethyl silyl ligands which could not form a more usual 4-coordinate compound for steric reasons. Another somewhat unusual feature was the nature of coordination at the Mo-site—this site was coordinatively saturated, with homocitrate coordinated as a bidentate ligand. Homocitrate and other tricarboxylic acids usually have a propensity to form tridentate complexes^{47,48,49}. In addition, the P-clusters were revealed to be a conglomerate of two [4Fe-4S] clusters in close proximity, rather than two discrete entities as had previously been considered^{50,51}. The limitations of x-ray crystallography become apparent when dealing with proteins, since the resolution is not high enough to discern the exact connectivity of the metal clusters. For example, the possibility of Fe-Fe bonding in the cofactor has not yet been eliminated, and this would eliminate the difficulties posed by the "3-coordinate" Fe atoms. The nature of the P-cluster site is still in contention⁵². Rees and coworkers have settled on a model which contains a disulfide bond within the cluster, which would have very unusual redox properties. Bolin and colleagues, however, seem more determined that the P-cluster contains a bridging hexacoordinate sulfur, at least for the MoFe protein from *C. pasteurianum*.

The discovery of molybdenum in nitrogenase is unusual because of its association with iron and sulfur in a cluster. In most molybdoenzymes, molybdenum is the sole inorganic constituent of the active site associated with a unique dithiol as the molybdenum cofactor. Here the molybdenum plays an obvious central role in the chemistry of the enzyme⁵³. The role of molybdenum in nitrogenase is a little less clear. A Fe/V nitrogenase has been isolated and characterized, and in the absence of both Mo and V, some diazotrophs

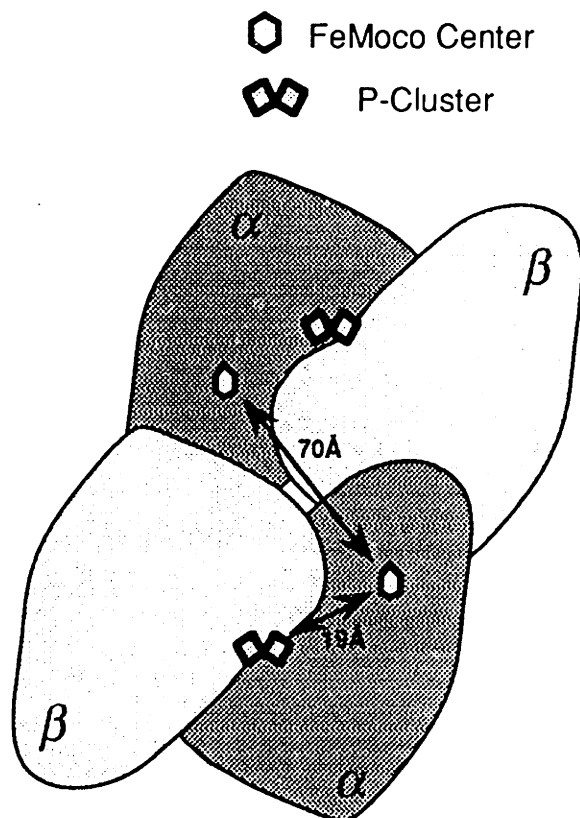


Figure 4: Schematic diagram showing relative positions of clusters relative to the molybdenum-iron protein of nitrogenase.

between postulated modes of binding of dinitrogen to iron in the industrial catalyst⁵⁸ and to iron in the FeMo-cofactor^{59,60}. Model compounds where nitrogen binds to Fe and is subsequently reduced to ammonia⁶¹ illustrate the feasibility of Fe as a scaffold for dinitrogen reduction.

The overall structure of the nitrogenase proteins at the macroscopic level is shown below. The Fe protein is an α_2 dimer, with a ferredoxin-like [4Fe-4S] cluster at the dimer interface⁶², somewhat exposed to solvent (as predicted by spectroscopic studies)⁶³. In the crystal structure there is a single bound ADP^{64,65,66} which bridges the two subunits. Examination of the primary structure revealed the presence of a "Walker motifs", i.e. consensus sequence (GXXXXGKS)⁶⁷ for nucleotide binding sites. These sites (total of two; one per subunit) are believed to be the sites of ATP hydrolysis and are similar in tertiary structure to the GTP-binding motif in ras p21⁶⁸. The nature of ATP-binding, hydrolysis and its coupling to electron transfer (Fe \rightarrow MoFe protein) is still relatively unclear. It is reasonable to submit that the hydrolysis of ATP induces some conformational change in the Fe-MoFe protein complex which enables electrons to be transferred from the Fe protein to the P-clusters (or to the FeMo cofactor, wherever the site of electron deposit is). It is also possible that ATP even facilitates electron transfer between clusters *within* the MoFe protein. The precise details are still mysterious.

express an alternative nitrogenase which contains all Fe, no Mo and no V⁵⁴. Although the all-Fe and Fe/V nitrogenases are not as effective as the Fe/Mo nitrogenase, their very existence is puzzling and throws a wrench in the machinery of many proposed "molybdocentric" (with nitrogen binding at Mo and subsequently reacting) mechanisms of dinitrogen reduction. The existence of alternative nitrogenases does *not* preclude mechanisms of substrate reduction at the molybdenum, however⁵⁵. There is still the possibility of 8 or 9-coordinate intermediates (which are feasible with Mo and small ligands, particularly hydrides)^{56,57}, or alternatively one could postulate mechanisms where the homocitrate moiety momentarily dissociates to allow substrate binding. Nevertheless it is exciting to consider enzymatic models where substrate is reduced at the "3-coordinate" irons. In fact, there is striking similarity



Figure 5: Ribbons diagram of the polypeptide fold of the Fe-protein [From Howard & Rees, *Annu. Rev. Biochem.* 63, 235-64, (1994)]

The second component of nitrogenase, the MoFe protein, is an $\alpha_2\beta_2$ tetramer, consisting of two $\alpha\beta$ subunit pairs. The placement of the metal clusters with respect to the polypeptide scaffolding is likely to be of some importance in terms of how the oxidation state of the clusters effects the conformation of the protein. Each $\alpha\beta$ -subunit pair appears to contain all the machinery required for dinitrogen reduction, thus the nitrogenase molecule can be thought of as two identical functional units related by a C_2 axis of symmetry. The FeMo-cofactor is located almost entirely within the α -subunit, at the junction of three domains of the α -helical/ β -sheet type. There is some interaction with the β -subunit; this type of interaction may be important in communicating local changes in the oxidation state to more distant metal centers, or to the ATP-binding site, in a "molecular relay" of sorts. Significantly, the P-cluster pair is located at the interface between the two subunits, a locale which enables the protein scaffold to be used to amplify small changes in local structure. These small "tweakings" become larger changes in protein conformation, which in turn could be used to trigger ATP hydrolysis, electron transfer and substrate reduction, events which must be exquisitely coupled in order for nitrogenase to function effectively.

Of particular interest and relevance to the study of nitrogenase are the techniques of Mössbauer and EPR spectroscopy. The former uses gamma radiation to probe ^{57}Fe nuclei and the effect of the electronic environment on the transition to higher nuclear spin

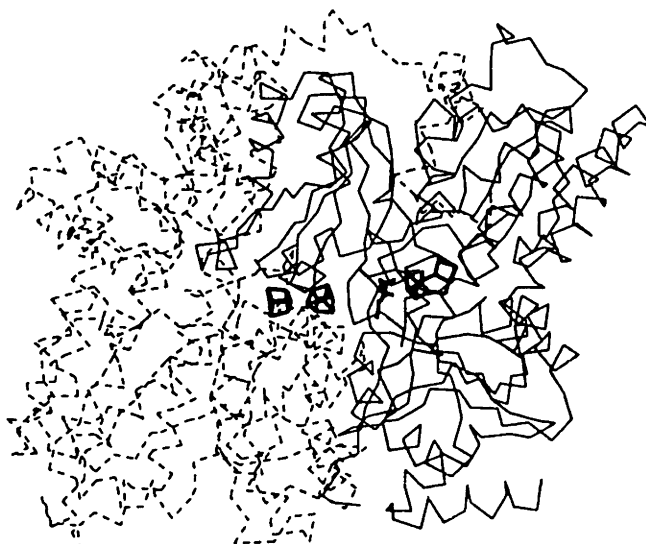


Figure 6: Alpha carbon trace of an $\alpha\beta$ subunit pair of the MoFe protein. The α and β subunits are shown as solid and dashed lines, respectively, with the metal clusters shown.

states. In an electric field gradient, the nuclear spin state is split by quadrupole effects, and this is observed in the gamma absorption spectrum⁶⁹. The latter investigates the effect of the structure and bonding on the electron spin state transition of unpaired electrons in the active site. The specialized EPR techniques of ENDOR⁷⁰ (electron nuclear double resonance) and ESEEM⁷¹ (electron spin echo envelope modulation) are particularly useful. These utilize the hyperfine coupling interaction between the electron spin and the neighboring nuclei to characterize the environment around the metal center. Mossbauer spectroscopy has enabled the metal centers within the MoFe protein to be classified^{72,73,74}. EPR spectroscopy of wild-type MoFe protein from several species (designated Av1 for *A. vinelandii*, Cp1 for *C. pasteurianum* and Kp1 for *Klebsiella pneumoniae*) shows the existence of a $S=3/2$ system⁷⁵.

This is evident in Mössbauer spectra as a paramagnetic component (labeled M). In the resting state, the Fe atoms can be further divided into D, Fe^{2+} and S environments. A protocol has been devised in this laboratory for the selective labeling of either the M-centers or the P-clusters with ^{57}Fe , so that the metal centers can be more completely characterized. Studies of MoFe protein labeled at the P-clusters alone^{76,77} has confirmed that the Fe environments within nitrogenase can be divided as follows: M (12Fe), D

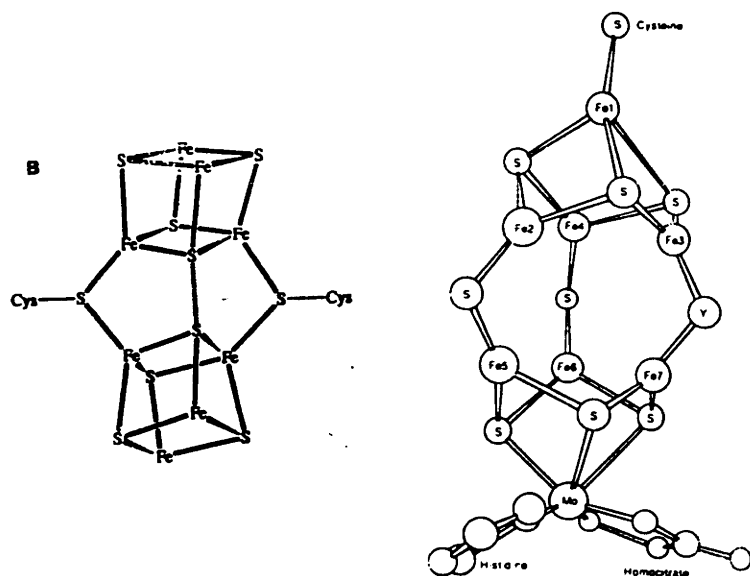


Figure 7: Structure of the metal centers of nitrogenase.
 (left) the P-clusters [from Chan et al. *Science* **260**, 792 (1993)]
 (right) the FeMo-cofactor [by S. Selengut]

(10Fe) Fe^{2+} (4Fe) and S (2Fe). Since this accounts for the Fe in the complete MoFe protein, which can be considered as two $\alpha\beta$ subunit-pair functional units, one can assign 6 (or perhaps 7) Fe atoms per FeMo-cofactor, and 8 Fe atoms to each P-cluster pair. It can be seen from the crystal structure of the MoFe protein that there is a serine residue that approaches one of the Fe atoms in the P-cluster pair. It is tempting to assign this atom as the 'S' Fe, although there is no experimental evidence for this as of yet. Perhaps the use of site-directed

mutagenesis to produce a mutant protein with the serine replaced by some other residue could help answer this question. Mössbauer studies have continued, and have more recently focused on the oxidized proteins, reinterpreting the 'POX' state (see below) as a non-Kramers (even number of electrons) system.⁷⁸

The mechanism of substrate reduction by nitrogenase has been thoroughly investigated over the last two decades. However, knowledge of the exact molecular details are still the subject of conjecture. Much of the work on this subject to date has been carried out in the laboratories of Roger Thorneley and David Lowe, at the A.F.R.C. Unit of Nitrogen Fixation in Sussex, England. Intensive studies in the 1970's and the early 1980's led to the proposal of the following mechanism for nitrogenase; a mechanism based upon the concept of two interlinked cycles: (1) the hydrolysis of MgATP and the concomitant process of electron transfer, essentially the role of the Fe protein, (2) the progressive reduction of the MoFe protein by the Fe protein to states which are capable of releasing hydrogen gas, and of binding substrates for further reduction⁷⁹. Both of these processes are cyclic in nature, as shown by the following scheme.

The precise mechanism of coupling between MgATP hydrolysis and electron transfer remains elusive. These two processes are known to be tightly coupled in most circumstances, however uncoupling is observed at $\text{pH} < 6.4$ or > 8.5 , at temperatures $< 20^\circ\text{C}$ or $> 30^\circ\text{C}$, and at low electron flux⁸⁰ (when the concentration of Fe protein in molar terms is substantially less than the concentration of MoFe protein). The "uncoupling" is due to a process called "reductant independent ATP hydrolysis". This is occurs because of the ability of oxidized Fe protein to react with ATP and MoFe protein, causing hydrolysis of ATP *without* any electron transfer. MgATP hydrolysis is a property of Fe protein complexed with MoFe protein; neither of the proteins hydrolyze MgATP in isolation⁸¹. The binding of both MgATP and MgADP to the Fe protein is well documented and characterized⁸². MgATP binding to the Fe protein causes changes to a number of physical properties of the enzyme^{83,84}. For example: (1) the $S=1/2$ EPR signal that is characteristic of the [4Fe-4S] complex of reduced Fe protein changes to a

more axial form; (2) the [4Fe-4S] becomes susceptible to degradation by chelators, such

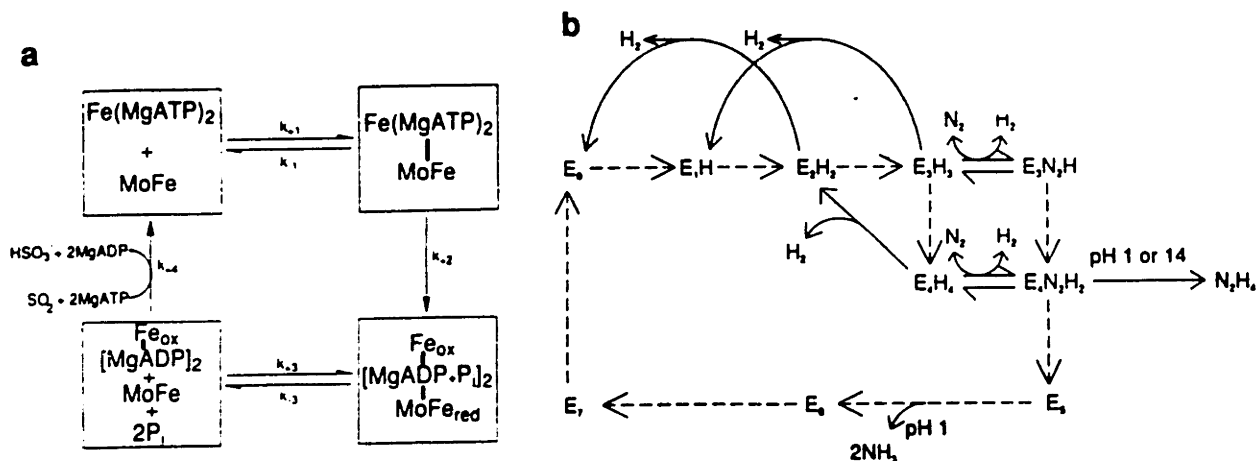


Figure 8: (a) the Fe protein cycle, where MgATP hydrolysis and electron transfer occur. (b) cycling of the MoFe through progressively reduced states through those that are capable of causing dinitrogen reduction.

as α, α' -dipyridyl; (3) the reduction potential of the FeS cluster changes, becoming ca. 100 mV more negative.⁸⁵

Various site-directed mutagenesis studies⁸⁶ have produced Fe proteins which exhibit some of these properties but are deficient in others. These studies have added credence to the hypothesis that MgATP binding effects changes in the cluster via amino-acid side-chain mediated conformational change. For example, replacement of Asp25 (a residue which is believed to interact directly with Mg^{2+} during ATP hydrolysis) with Glu results in a protein which releases Fe to chelators not only in the presence of MgATP but also in the presence of MgADP, although not active at all in nitrogenase catalysis.

The exact nature of the “P-cluster” iron in the MoFe protein was initially the subject of much debate; there were several factors which contributed to the confusion. One point was the lack of conserved cysteine residues between MoFe proteins of different species. Sequence comparison studies showed there were 20 conserved cysteines per $\alpha_2\beta_2$ tetramer (10 per $\alpha\beta$ functional unit)⁸⁷. Cluster extrusion studies⁸⁸ had shown that 4 [4Fe-4S] clusters were extruded for each molecule of MoFe protein. If these clusters were ligated to the protein in the usual way for [4Fe-4S] clusters, that would account for 16 of the cysteines, leaving only two conserved cysteines to bind each FeMo cofactor to the protein. This was considered at the time to be unlikely, so various arguments were put forward for the existence of the P-clusters as discrete clusters in unique coordination environments (containing ligands other than cysteine) Though it was suggested that perhaps the P clusters resided between subunits in a bridging position⁸⁹. Oxidative titrations of the MoFe protein, achieved either potentiometrically or by the use of the dye thionine, have led to the characterization of states of the P-clusters with up to 4 electrons removed (per cluster)⁹⁰. The oxidation can be described as two redox processes in sequence⁹¹: first, two electrons are removed from the P-cluster ($E_m \sim -300\text{mV}$) followed by the removal of the unpaired electron from the cofactor, with concomitant loss of the

$S=3/2$ EPR signal ($E_m \sim 90\text{mV}$). Further oxidation of the P-clusters is believed to produce a second oxidized state with a mixture of $S=7/2$ and $S=1/2$ spin states. At still higher redox potential, a "super-oxidized" P-cluster is reported, however the mechanistic significance of this state is in question because at potentials this high, degradation of the MoFe protein is significant.

Recent work by Lowe et al⁹² showed that the pre-steady state absorbance changes (due to FeS clusters) occurring in the first second of the reaction of nitrogenase were consistent with the mechanism they had proposed in 1984. An initial, rapid increase in absorbance in the first 25 ms of reaction could be ascribed to the oxidation of Fe protein. Studies of

Summary of absorption coefficient changes associated with MoFe protein during the pre-steady-state phase of substrate reduction.

Reaction	$\Delta\epsilon_{430}(\text{mM}^{-1}\text{cm}^{-1})$
$E_0 \rightarrow E_1$	<0.5
$E_1 \rightarrow E_2$	-2.2
$E_2 \rightarrow E_3$	<0.5
$E_3 \rightarrow E_4$	+6.7

Typically, a decrease in absorbance indicates a reduction for an FeS cluster. These results indicate an oxidation takes place at the E_4 level of reduction.

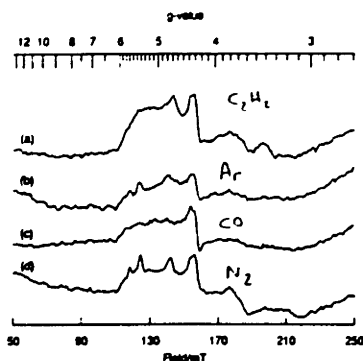


Figure 9: Results of Lowe et al, showing cluster oxidation occurs at the E_3 stage of nitrogenase catalysis. The EPR results are for: (a) C_2H_2 , (b) Ar, (c) CO and (d) N_2 - Note the sharp peaks in the cases of Ar and N_2 .

reduction under N_2 and Ar, but not under C_2H_2 . This was consistent with the observation that the increase in absorbance as $E_3 \rightarrow E_4$ occurred for the first two substrates, but not the latter. They postulated that oxidation of the P-clusters was responsible for the sharp EPR signal. This signal is not unlike those observed by Hagen et al⁴⁸, and proposed to be due to oxidized P-clusters. Also, under CO, there was no indication of the sharp $g=5.4$, 5.7 signals during turnover and H_2 production.

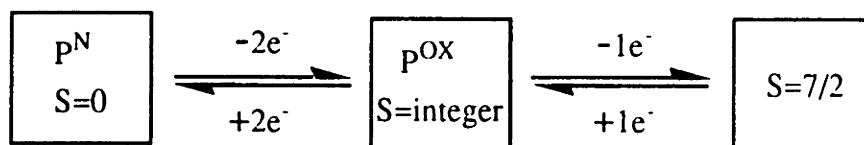
It seems that the P-clusters are required for reduction of dinitrogen, but not of acetylene. Barry Smith presented data at the recent Gordon conference on N_2 fixation (New

the 1-electron reduced MoFe protein showed there is no significant change in absorption during this reduction step, at least on the MoFe protein. Between 100 and 200 ms, a steady increase in absorbance is predicted as a result of the continued increase in concentration of oxidized Fe protein. In fact, what is observed is a plateau in the absorbance-time trace, indicative of decrease in absorbance as the MoFe protein proceeds from state E_1 to E_2 , due to a reduction of one of the metal centers (either FeMoco or the P-clusters). There is no change in absorbance due to MoFe protein as the reduction continues, moving the enzyme from state E_2 to E_3 . The significance of this is not clear. More significant is the observation that as the enzyme proceeds from state E_3 to E_4 there is an increase of absorbance, indicative of an oxidation of the P-clusters. This oxidation step may represent the delivery of a $2e$ pulse from the P-clusters to the M-center, perhaps occurring concurrently with the irreversible commitment of N_2 to reduction. This was postulated by Thorneley and Lowe by analysis of the pre-steady state production of ammonia, and is consistent with the kinetics. Lowe et al noted the appearance of the EPR spectrum of the steady state of nitrogenase under various gases and one could observe

sharp signals at $g=5.4$ and $g=5.7$ for

London, NH; July 1994) which showed that replacement of one or both of the bridging cysteines of the P-clusters with alanine resulted in mutant nitrogenases that were still capable of reducing acetylene, albeit at a slower rate than for the wild type. He made the somewhat bold claim that perhaps the P-clusters are not needed at all, but possibly increase the efficiency of substrate reduction somehow.

One particularly fascinating observation of the P-clusters is unusual spectroscopic and redox behaviour. As discussed above, the oxidation of the P-clusters can best be thought of as occurring in two steps, as shown in the equation:



This spectroscopic data is consistent with the P^N state being considered as an all-ferrous $[4Fe-4S]_0^2$ system. This is an extremely highly reduced state, and has not been observed before in native proteins, however it can be produced in synthetic systems at very low that this state is stabilized by protonation⁹³.

The chemistry of molybdenum nitrogenase, even after some 30 years of intensive study, continues to be an enigma. Research in this field has become more exciting as the prospect of honing in on some of the finer details of the mechanism becomes a greater possibility. The work outlined in this thesis represents a small step in that direction, here, with the observation, for the first time, of a ligand bound to nitrogenase the possibility of observing nitrogen species bound to the enzyme during turnover becomes slightly more tenable.

1.2.4 Alternative Nitrogenases.

Early experiments by Bortels⁹⁴, working with *A. chroococcum*, showed that the process of nitrogen fixation was enhanced by the addition of Mo to the growth media. His later experiments with legumes⁹⁵ showed that V as well as Mo stimulated diazotrophic growth. After the discovery and characterization of nitrogenase, it was assumed that this result was due to the chemical similarity of V and Mo, that perhaps under certain conditions V could substitute for Mo in the MoFe protein. In the mid-1970's it was suggested that the Vanadium stimulated nitrogen fixation by interacting with and stabilizing the MoFe protein⁹⁶ (!) or perhaps by effecting the more efficient utilization of the small amount of Mo available to Mo-starved cells. The possibility of the existence of a totally different nitrogen-fixing system was first addressed by experiments (in the early 1980's) with Nif^- strains of *A. vinelandii* that were unable to fix nitrogen in the presence of Mo, but that were able to grow diazotrophically in media that was rigorously stripped of Mo⁹⁷. Although these initial reports were met with skepticism, it was not long until the purification of a vanadium-containing nitrogenase showed that such an alternative nitrogenase did indeed exist. This nitrogenase (called V-nitrogenase, or nitrogenase-2) was demonstrated to have different catalytic properties to the Mo-nitrogenase. It was much less active than Mo-nitrogenase, did not exhibit the EPR spectrum typical for MoFe protein ($g=4.5, 3.7$ and 2.1) but rather had an EPR signal around $g=2.0$, and also (in the case of *A. chroococcum* V-nitrogenase) was shown to catalyze the production of a small amount of ethane during acetylene reduction⁹⁸. Recent kinetic studies show that ethane production is temperature-dependent, and further that hydrazine is produced by the enzyme during dinitrogen reduction (which is not the case for MoFe protein under similar conditions)⁹⁹. Alternative nitrogenase is shown to be an $\alpha_2\beta_2\delta_2$ hexamer. Interestingly, the bacteria uses an alternative Fe protein as the reductant¹⁰⁰.

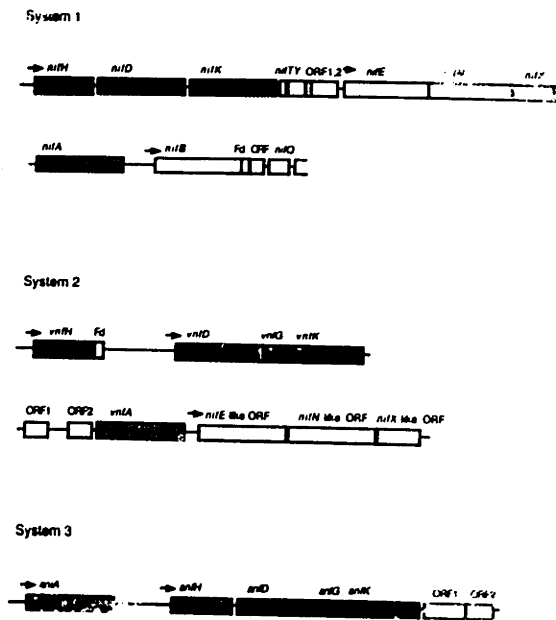


Figure 10: Gene clusters for the three nitrogenase systems: (1) Mo-nitrogenase (2) V-nitrogenase and (3) Fe-nitrogenase. [From Bishop and Premckumer in Biological Nitrogen Fixation, Chapman and Hall, New York, London (1992) Chapter19]

Growth of *A. vinelandii* on media that was depleted of both Mo and V led to the purification of an enzyme (nitrogenase-3) that does not appear to contain any Mo or V, but rather an all-Fe nitrogenase. The catalytic properties of this enzyme are different again to Mo- or V- nitrogenase, with the enzyme exhibiting much lower specific activity.

The genetics of alternative nitrogenase will not be discussed in depth here, however it is interesting to note that each alternative nitrogenase also has a set of genes similar to the *nif* genes. Vanadium nitrogenase is transcribed from the *vnf* cluster, while for the all-Fe nitrogenase (or nitrogenase-3) transcription occurs from the *anf* cluster. These genes are analogous to the *nif* genes which correspond to conventional nitrogenase and its associated proteins.¹⁰¹

1.3 Genetics of Nitrogen Fixation: The *nif* Genes—Overview.

The process of nitrogen fixation is carried out, in the final analysis, by the enzyme nitrogenase. However, coordinating the overall process requires a battery of enzymes and gene products that together work in concert to ensure that the process takes place at the right time (i.e. when the cell actually needs fixed nitrogen), in the right place (an anaerobic environment with the correct “energy charge”, i.e ATP/ADP ratio) and in the right manner (so that the process of nitrogen fixation does not negatively effect the other processes of the cell). The genes which are responsible for this are the *nif* genes. These have been most rigorously studied in the organism *Klebsiella pneumoniae*,¹⁰² because this was the bacteria from which they were first isolated. *K. pneumoniae* was chosen because it is fairly closely related genetically to the workhorse of molecular biology, *Escherichia coli*. In the last 15 years, much progress has been made in studying the genetics of other bacteria including members of the *Azotobacter*¹⁰³ genus, and to a lesser extent *Rhodobacter capsulatus*¹⁰⁴ and *Clostridium pasteurianum*,¹⁰⁵ amongst others.

In the case of *Klebsiella pneumoniae*, cloning and sequencing studies have shown that the structure of the cluster, comprising 21 genes, is *nifICHDKTYENXUSVMZFLABQ*. The function of each of these genes and gene products, in as much as is known, is discussed below. This subject has been reviewed recently.¹⁰⁶

Electron Transfer Proteins (NifJ and NifF): In *Klebsiella pneumoniae*, there are two proteins that are involved in electron transfer to nitrogenase. Nitrogenase ultimately obtains its reducing equivalents from pyruvate, at least in *K. pneumoniae*. This organism uses a ferredoxin-like protein (pyruvate oxidoreductase; encoded by *nifJ*) to convert pyruvate to acetyl coenzyme A, and transfer this energy to a flavodoxin (encoded by *nifF*), which is reduced in the process. This electron transfer chain is specific for nitrogenase. Strains with mutations in either *nifF* or *nifJ* have reduced nitrogenase activity *in vivo*, but activity can be restored *in vitro* by the addition of dithionite¹⁰⁷. The gene products for both *nifF* and *nifJ* have been purified by assaying fractions for their ability to restore nitrogenase to crude extracts of appropriate mutant strains (missing the applicable gene), using pyruvate as the electron donor¹⁰⁸. These proteins can also be used *in vitro* to constitute an assay system for coupling the oxidation of pyruvate to acetylene reduction, using these proteins to channel electrons from pyruvate to nitrogenase. Electron transfer processes in other diazotrophs remain uncharacterized but appear to differ markedly from *K. pneumoniae*. For example, no gene analogous to *nifJ* has been found in the azotobacters. Shah et al¹⁰⁹ observed that the flavodoxin isolated from *A. vinelandii* was considerably less efficient at transferring electrons from pyruvate to nitrogenase via pyruvate oxidoreductase (from *K. pneumoniae*) than *K. pneumoniae* flavodoxin was. However, pre-reduced flavodoxins from either source were equally efficient at driving nitrogenase catalysis. There is significant sequence homology at the amino acid level between *A. vinelandii*¹¹⁰ and *K. pneumoniae*¹¹¹ flavodoxins.

Fe protein (NifH): The *nifH* gene encodes the polypeptide which, upon folding dimerization and acquisition of the [4Fe-4S] cluster (which requires the gene product from the *nifS* gene), forms active Fe protein of nitrogenase. Site-directed mutagenesis studies have added insight to mechanistic speculation. Nitrogenase Fe protein from *Rhodospirillum rubrum* is subject to a reversible inactivation process, which was shown to be due ADP-ribosylation of a surface arginine residue (arg-101)¹¹². A mutant Fe protein isolated from *Klebsiella pneumoniae*, which behaved in many ways similarly to wild-type protein (activity in reductant-independent ATP-hydrolysis; MgATP dependent conformational change), but was inactive in transferring electrons to MoFe protein was shown to contain histidine in place of arginine-101¹¹³. Apparently this residue is required for correct orientation of Fe protein with respect to MoFe protein during electron transfer. Peters et al¹¹⁴ demonstrated that a loop of residues (59 through 67) is very important in component-protein interaction. They constructed a hybrid (nicknamed AvCp2) in which this group of residues in *A. vinelandii* Fe protein (Av2) was replaced with the corresponding loop from the *C. pasteurianum* protein (Cp2). Av1 and Cp2 form a tightly bound, inactive complex. Av1 and AvCp2 did not form an inactive complex, however this hybrid Fe protein did bind to the MoFe protein considerably more tightly than the wild-type protein.

It has also been shown that functional copies of the *nifH* gene are required for FeMo-co biosynthesis. Mutants of both *A. vinelandii*¹¹⁵ and *K. pneumoniae*¹¹⁶ unable to synthesize FeMo-co turned out to have defective *nifH* genes. This result has been confirmed in vitro assays of cofactor biosynthesis¹¹⁷, which have demonstrated that functional, active Fe protein is an absolute requirement for formation of FeMo-co. More recent studies have indicated that the Fe protein may have an active role in the maturation of MoFe protein from its precursor apoprotein¹¹⁸, in addition to its function in the formation of FeMo-co.

MoFe protein (NifDK): Before the crystal structure of MoFe protein was published, site-directed amino-acid substitution studies were initiated with the hope of understanding the role of the polypeptide environment in dinitrogen reduction¹¹⁹. The rationale behind this work was that a description of spectroscopic and catalytic changes elicited by amino acid

substitutions would likely lead to an understanding of the function of individual amino acids within the FeMo-cofactor environment. This entails functions such as transfer of protons and electrons, and correct orientation of the FeMo-cofactor within the polypeptide matrix. Prior to the establishment of the crystal structure of the MoFe protein, Dean et al¹²⁰ concluded that the FeMo-cofactor resided entirely within the α -subunit of the MoFe protein, and established the region α -Gln-191 \rightarrow α -His-195, and α -Cys-275 as being the likely locale. Subsequently, they proceeded to generate site-directed mutants in this region, utilizing new technology that enabled genetics to be carried out in *Azotobacter vinelandii*. They obtained several interesting results, that have become more understandable with the publication of the crystal structure of the MoFe protein. Substitutions at α -Cys-275, with either Ser¹²¹ or Ala¹²² resulted in a Nif⁻ phenotype. Apparently these substitutions induce a disruption in the FeMo-cofactor binding domain, as evidenced by changes in the EPR spectra (broadened signals, with g values of 4.5 and 3.5, cf wildtype 4.3 and 3.7). It is interesting to note how the protein environment around the cofactor effects the S=3/2 EPR signal. For example, even though FeMo-co extracted from *C. pasteurianum* is identical with cofactor extracted from *A. vinelandii*, the EPR spectra¹²³ and catalytic properties of Cp1¹²⁴ differ slightly from Av1. A mutant Av1 protein, with residues in the vicinity of cofactor modified to approximate the M-center environment of Cp1, showed markedly different EPR characteristics (more rhombic spectrum; g values 4.5. and 3.7).

Other amino acid substitutions that induce pronounced changes in the properties of the MoFe protein are alterations of residues α -Gln-191 and α -His-195. Comparison of the sequence of *nifDK* (i.e the genes for MoFe protein) with *nifNE* (a tetrameric protein involved in FeMo-co biosynthesis¹²⁵, homologous to the MoFe protein) revealed that the latter contained a similar domain of amino acids to the (then putative) MoFe protein cofactor binding site. It was proposed that this was the site of cofactor synthesis. In NifNE, the residues in positions homologous to both α -Gln-191 and α -His-195 were altered to Lys and Asn, respectively, in NifE. Other residues were the same or else the differences were conservative. Scott et al¹²⁶ proposed that perhaps these residues were different in the MoFe protein because of some function critical to the proper functioning of nitrogenase. Replacement of α -Gln-191 with Lys did in fact lead to the loss of diazotrophic growth, as did the replacement of α -His-195 with Asn. Both of these mutants exhibited fairly normal S=3/2 EPR signals; although inactive in terms of dinitrogen reduction, both mutants were active in the reduction of both acetylene and H⁺, albeit at a slower rate. It is interesting to note that these mutant proteins catalyze the 4e⁻ reduction of acetylene to ethane—some 15-40% of the reduced electron flux is devoted to this process. Although (as mentioned above) vanadium nitrogenase also catalyzes ethane production from acetylene, this occurs by a totally different mechanism. Unlike the case of vanadium nitrogenase, there is no time lag for ethane production, and the process is not temperature dependent¹²⁷. The His195Gln mutant is especially interesting, as seen in a recent study by Kim et al¹²⁸. Nitrogen gas acts as an inhibitor of both hydrogen production and acetylene reduction, by simple occupancy of the same site on the cofactor. It is clear that dinitrogen is able to bind to this enzyme, but for reasons that remain unclear, it is unable to be reduced. This fact causes ATP hydrolysis to become significantly uncoupled from electron transfer in the case of the mutant (23ATP/2e⁻ vs. 5.5 ATP/2e⁻ in wild-type). The researchers also noted that carbon monoxide (CO) acts as an inhibitor of N₂ binding (as measured by the ability of N₂ to suppress H₂ evolution). CO inhibition of acetylene reduction by the mutant was much greater than for wild-type (K_i=0.00004 atm for mutant vs K_i=0.00033 atm for wild-type).

NifV: This gene product is a requirement for all three nitrogenase systems¹²⁹, although *nifV* mutants remain capable of slow diazotrophic growth¹³⁰. Nitrogenase isolated from *nifV*⁻ strains of *K. pneumoniae* was shown to be extremely poor at reducing dinitrogen,

though it catalyzed H₂ evolution and the conversion of acetylene to ethylene at a reasonable rate¹³¹. Hydrogen evolution by the *nifV*⁻ MoFe protein was substantially inhibited by CO, unlike the wild-type enzyme. These results indicated that the absence of functional NifV protein was causing some structural (and subsequently functional) change at the active site of nitrogenase. FeMo-co isolated from a *nifV* mutant and used to reconstitute extracts of a *nifB*⁻ strain of *K. pneumoniae* produced MoFe protein with the same characteristics as that isolated from the *nifV*⁻ bacteria. Thus the unusual substrate reduction properties of nitrogenase from these mutants were a function of the cofactor itself. The *in vitro* cofactor biosynthesis system of Shah et al was used to demonstrate that the *nifV* gene product was required for homocitrate synthesis, and to show that homocitrate is a component of FeMo-co¹³². The implications of this result are that NifV is a homocitrate synthase. The fact that there is significant sequence homology between this protein and the LEU4 gene product from *Saccharomyces cerevisiae* (an α -isopropyl malate synthase) supports this hypothesis.

Assembly of the Fe protein (NifM, NifU, NifS): Expression of the *K. pneumoniae nifH* gene in *E. coli* did not result in expression of active Fe protein. However when constructs including both *nifH* and *nifM* were prepared and introduced into *E. coli*, the extracts contained active Fe protein. This result complements microbiological studies which showed that strains of *K. pneumoniae* and *A. vinelandii* containing mutations in the *nifM* gene were devoid of Fe protein activity. The function of the NifM protein is still mysterious.

Although it appears that NifM is the sole additional *nif* protein required for generation of active Fe protein in *E. coli*, synthesis of normal levels of active Fe protein in *A. vinelandii* requires the presence of two other gene products: NifU and NifS. The second of these protein NifS has been purified and characterized. It is a pyridoxal 5'-phosphate-containing protein which has been shown to catalyze the removal of sulfur from cysteine, via a enzyme-bound cysteinyl persulfide¹³³. NifS has recently been shown to effectively catalyze activation of apo-Fe protein (formed by the chelating action of α,α -dipyridyl on Fe protein in the presence of MgATP).¹³⁴ The *nifU* gene product has also been purified and characterized, and has been shown to be an unusual [2Fe-2S] protein. The novel spectroscopic properties of this enzyme have been ascribed to an unusual coordination environment. Thorough spectroscopic characterization of this protein failed to address the question of its function, though it is believed that its redox character is essential to some aspect of Fe protein assembly. Perhaps it is needed as a repair enzyme for damaged Fe protein; the specific activity of "purified" Fe protein frequently varies wildly, and it is known that in almost all preparations there is a not-insignificant amount of poorly characterized inactive material.

Cofactor Synthesis, NifB and NifNE: The involvement of NifB in the process of FeMo-co biosynthesis was implied by the observation that *nifB*⁻ mutants accumulate a cofactorless MoFe protein (apo-MoFe protein)¹³⁵. As previously discussed, extracts of *nifB*⁻ bacteria can be made competent for nitrogen fixation by the addition of FeMo-co. Also, it was shown that these extracts would synthesize FeMo-co with the addition of partially purified NifB protein. Recent purification of the NifB protein (an accomplishment that had earlier been prevented by difficulties in solubilization of this membrane-bound protein), enabled the fortuitous discovery of "NifB-co"¹³⁶, a detergent solubilized FeS cluster (structure currently unknown). The NifB requirement for FeMo-co biosynthesis in *nifB*⁻ extracts was fully satisfied by the addition of NifB-co. NifB-co shares many physical properties with the iron-molybdenum cofactor: in solution, it is a greenish-brown color; it is stable in *N*-methylformamide; it is extremely

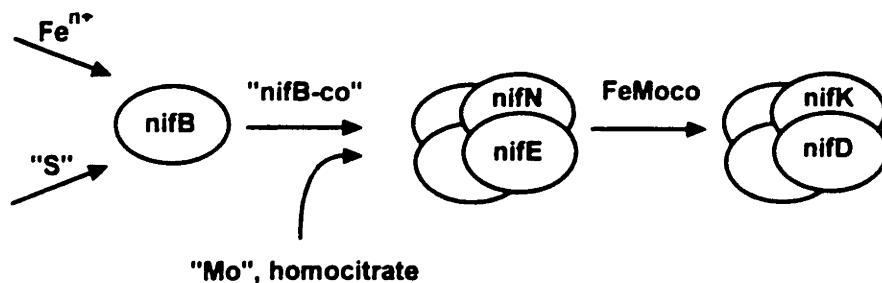


Figure 11: Simplified schematic representation of the biosynthesis of cofactor [from Müller & Krahn, Argev. Chem. Int Ed Engl., 34 1047-1078 (1995)]

sensitive to atmospheric oxygen. Unlike FeMo-co, however, NifB-co contains no molybdenum, and (as isolated) exhibits no EPR activity.

The NifNE polypeptide is structurally homologous to the MoFe protein. It is an $\alpha_2\beta_2$ tetramer, there is significant sequence homology, and has a molecular mass of 200,000 Da. It has been purified, and was shown to contain 4.6 mol Fe, 1.2 mol Zn and 0.63 mol Cu per mol of protein. The protein is oxygen-labile, and absorbs light in the UV-visible region ($\lambda_{\max}=425$ nm), which indicates the probable presence of an FeS cluster in the protein. Brigle et al suggested that the NifNE polypeptide functions as a molecular scaffold for the synthesis of FeMo-co¹³⁷. In vitro biosynthesis experiments indicate that NifNE is indeed involved in the synthesis of FeMo-co, however apparently it's function is not catalytic—NifNE is *consumed* during cofactor biosynthesis. One mole of NifNE is required for every two moles of FeMo-co produced. It is possible to synthesize the FeMo cofactor from NifB-co, with the addition of homocitrate, molybdate and active NifNE protein. As of yet, however, it has not proven to be possible to synthesize FeMo-co *in vitro* using only purified proteins and constituents, apparently some factor or factors is missing.

Assimilation of Molybdenum (NifQ): Mutants of *K. pneumoniae* lacking the *nifQ* gene show subnormal levels of FeMo-cofactor biosynthesis¹³⁸. Normal growth can be restored by the addition of elevated levels of Mo; this observation also holds true in *Rhodobacter capsulatus*¹³⁹ and *A. vinelandii*.¹⁴⁰ Apparently NifQ is not so much involved in the uptake and accumulation of Molybdenum in the cell, as *nifQ* mutants function normally in synthesis of Mo-cofactor (a separate molybdenum-only cofactor found in many metabolic enzymes)¹⁴¹. Use of ⁹⁹Mo as a radioactive tracer showed that Mo uptake in *nifQ* mutants was normal¹⁴². It was noted that addition of cysteine to the growth medium of *nifQ*⁻ bacteria (*K. pneumoniae*) had the same effect as elevated Mo¹⁴³, i.e. full

diazotrophic growth was restored. On the basis of this result, it appears reasonable to assert that perhaps NifQ is involved in the conversion of Mo (absorbed as MoO_4^-) to a Mo/S compound, which is a precursor to the synthesis of cofactor at satisfactory rates. It was asserted that perhaps this occurs to a small extent in the cell non-enzymatically, but at non-optimal rates for full growth.

Effectors of Nitrogenase Activity—NifA and NifL: Derepression of nitrogenase (i.e. the process by which nitrogenase is activated in the cell), caused ultimately by the lack of a suitable fixed nitrogen source, is a complicated process involving the interaction of the *nif* and *ntr* (nitrogen regulation) genes. Here the process that occurs in *K. pneumoniae* is described (see descriptions by Buck¹⁴⁴ and Merrick¹⁴⁵). Activation of the *nif* genes is caused by the *nifA* gene product, which binds to an upstream activator sequence (UAS). This process facilitates transcription of the *nif* genes. The repression of nitrogenase caused by sufficient cellular ammonia or high intracellular O_2 levels is effected by NifL which antagonises the ability of NifA to activate *nif* gene transcription. It appears that NifL achieves this by binding to NifA¹⁴⁶. Apparently, the ability of NifL to bind to NifA is regulated in some way by the presence of fixed nitrogen or dissolved O_2 .

Transcription of the *nifLA* operon occurs separately from the transcription of the other nitrogen fixation genes. It is induced when NtrA, an RNA polymerase σ -factor, activates RNA polymerase to transcribe the *nifL* and *nifA* genes from a downstream promoter element (DPE). This process is activated by the binding of phosphorylated NtrC (dephosphorylated NtrC does not bind) to an activator sequence upstream from the *nifLA* operon. The phosphorylation of NtrC is conducted by NtrB (which is in turn activated by a lack of fixed nitrogen in the cell). If the level of free NifA is high enough, the UAS for the other *nif* genes comes into effect (at the *nifH* and *nifJ* promoters), and the RNA polymerase is able to transcribe these as well. In the absence of active NifA, these genes are not transcribed.

To recap, in the presence of fixed nitrogen, NtrB apparently dephosphorylates the NtrC protein¹⁴⁷, which makes it bind to the activating sequence for the *nifLA* operon less readily. Subsequently, cellular levels of NifL and NifA drop. In addition, the NifL protein becomes activated to bind to NifA, thus inactivating it. The UAS near the other *nif* genes loses bound NifA, and transcription of nitrogen fixation genes ceases. In contrast, when the level of cellular fixed nitrogen drops for some reason, NtrB is converted somehow into a form that now phosphorylates the NtrC protein, thus enabling it to bind the *nifLA* enhancer sequence. Cellular levels of NifL and NifA increase, but NifL is in a form that does not bind NifA. Thus NifA is free to interact with the UAS, causing the initiation of *nif* gene expression.

NifY and NifX: The function of the *nifY* gene remained completely unknown until fairly recently. Theresa White (PhD, MIT, 1991) was conducting native (anaerobic) gel electrophoresis experiments with *K. pneumoniae* extracts, and noticed three immunoreactive species migrating on gels (using anti-MoFe antibodies)¹⁴⁸. The uppermost band comigrated with apoMoFe protein and the lowermost with MoFe protein. The intermediate band was initially unidentified. Two-dimensional gel electrophoresis experiments (second direction SDS-PAGE) revealed that the apo-protein and intermediate species (which was also MoFe protein) both had a small (21kDa) polypeptide associated with them. Judging from relative intensities of this band upon staining, it appeared that there two of these peptides associated with the apo-MoFe protein, and one with the intermediary protein. Titration of apo-MoFe protein with FeMo-co revealed that the 21kDa peptide was released concomitantly with addition of cofactor. Protein sequencing of this peptide (after extraction from gels) revealed that it was the NifY gene product. It appeared that this protein was acting as a “molecular

doorstop", serving to maintain the apo-MoFe protein in such a conformation that the cofactor-binding sites are accessible (which, as seen in the crystal structure, are fairly well buried). Strains of *K. pneumoniae* and *A. vinelandii* with *nifY* deleted appear to be capable of diazotrophic growth. Perhaps this means NifY has some other function than to be a "doorstop", or alternatively perhaps some other peptide is able to substitute for NifY in these mutant strains. It is interesting to note that while *nifY* and *nifX* mutants are fully capable of diazotrophic growth, *nifXY* double mutants show severely stunted growth in the absence of fixed nitrogen.¹⁴⁹ Perhaps NifX is a surrogate NifY. In any case, the functions of both of these proteins are still incompletely known. The existence of NifY associated with apo-MoFe protein was confirmed by Homer et al¹⁵⁰, who purified apo-MoFe protein from both *K. pneumoniae* and *A. vinelandii*, and found that in both these cases apo-MoFe was oligomeric ($\alpha_2\beta_2\gamma_2$). The γ -subunit in the case of *K. pneumoniae* this protein was NifY (confirming T. White's results), while for *A. vinelandii* this protein was found to be a previously unsequenced peptide. This latter result is especially confusing, given the fact that the *nif* gene cluster of *A. vinelandii* a copy of the *nifY* gene!!

NifX, NifT, NifW and NifZ: These proteins have yet to have clear functions ascribed to them. The *nifT* gene, the smallest of the open-reading frames of the *nif* cluster, has yet to be identified at the protein level. It is not known whether or not this protein is expressed in either of the organisms in which it has been studied (*A. vinelandii* or *K. pneumoniae*).

Neither *nifW* nor *nifZ* has been shown to be essential for diazotrophic growth, however the presence of mutations in either of these genes causes reduction of MoFe protein activity. There was no increase in activity upon addition of FeMo-co to either of these extracts¹⁵¹. These results suggest that neither of these gene are involved in FeMo-co biosynthesis but do not necessarily exclude this possibility. In the absence of other clear evidence, there seems to be no reason to suggest that these polypeptides do not have a regulatory role, perhaps to moderate levels of nitrogenase synthesis.

1.4 The Future of Nitrogen Fixation Research.

The relationship between structure and function of such a large and complex molecule as nitrogenase is likely to be somewhat difficult to dissect. Up until recently, studying this enzyme could be likened to probing around in a dark room with a very small flashlight: occasionally finding something of interest but never grasping the interconnectedness and beauty of the room as a whole. However, the publication of the three-dimensional structure of nitrogenase—an incredible feat considering its size—"turned on the light" and at long last we were able to behold the room in its entirety. As impressive as that sight was, we were soon forced to admit this was but a beginning to understanding "how it works".

What is missing is of course an understanding of the *dynamics*. The crystal structure is simply a single frame in a far more extensive movie: a "freeze frame". The action is missing. Kinetic studies have provided some insight into this, and as edifying and informative as these results have been, they have still been restricted to the somewhat colorless "black box" methodologies of analysis and spectrophotometry. This is somewhat like watching a movie blindfold. What is needed is a more effective chemical probe to monitor the status of the enzyme itself during turnover. Freeze quench EPR, while by no means exhausted as a method for studying this enzyme, has been less informative than one would have liked, because most of the reduced states of nitrogenase have metal centers in integer spin states. Perhaps freeze quench Mössbauer studies will provide exciting opportunities in nitrogen fixation research, because they will reveal more

of the chemistry occurring at the metal centers during turnover, and will nicely compliment Lowe's recent spectrophotometric study. There are potentially other avenues for freeze quench work, which need to be explored. There is no reason, other than sheer experimental difficulty, that one cannot conduct freeze-quench EXAFS experiment. With the technology for X-ray absorption improving all the time, requiring less sample and shorter acquisition times than ever before. New techniques such as L-edge X-ray absorption spectroscopy, solid state NMR, and FTIR have yet to be explored, and once again there is no real reason that freeze quench technology cannot be applied to these systems. Whatever the future of nitrogenase research is, there is no doubt that there are a multitude of exciting possibilities, and until the details of dinitrogen reduction have been completely elucidated the question should not really be "where to now?", but perhaps "where to... how?"¹⁵²

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Chapter Two: ENDOR Studies of Nitrogenase.

2.1 Electron Nuclear Double Resonance Spectroscopy.

Electron paramagnetic resonance spectroscopy is often of use in studying biological molecules because many important proteins contain moieties upon which unpaired electrons reside. Significant examples of unpaired electrons in biology are those found in metalloproteins: at heme centers (e.g. cytochromes, hemoglobin, and peroxidases) at Fe-S centers, and at Mo. Unpaired electrons can also reside in organic components, such as at flavins or at aromatic amino acids. Much information can be derived about the environment in the vicinity of the spin from the hyperfine coupling constants of the neighbouring nuclei. Unfortunately, in systems such as metalloenzymes, the hyperfine coupling splitting is frequently very small in comparison to the line-width of the EPR signal, and so this information is not readily obtainable directly from the EPR spectrum. However, through Electron Double Nuclear Resonance (ENDOR) spectroscopy, the "lost" hyperfine coupling information is recovered. The ENDOR experiment can be thought of as a Nuclear Magnetic Resonance (NMR) experiment of those nuclei whose spins interact with the unpaired electron density.

The technique was first established in 1959 by Feher¹. The experiment is conceptually fairly simple: the EPR signal (at a specific g value) is observed while the NMR frequencies are swept in a continuous wave experiment (at fixed magnetic field). As the nuclear spins absorb radiofrequency radiation and precess rapidly, the interaction of the nuclear spin with the electron spin is markedly perturbed. The result is a change in EPR signal intensity. This change in EPR intensity occurs precisely as the nuclei interact with the applied radiation. The net result of this experimentally is doubly valuable: one obtains hyperfine coupling information which EPR would not, and one obtains information about the nuclear spin with much greater sensitivity than through NMR alone.

The hyperfine coupling constant is not just an abstruse physical constant of no real applied value: it contains information of much value to the biochemist. For example, EPR by itself often yields absolutely no information about the atoms in the neighbourhood of the electron spin. The existence of a characterized hyperfine coupling interaction confirms the existence of a certain atom, which may be a ligand or an unforeseen cofactor. The hyperfine interacting nucleus need not be bonded directly to the metal center—it is possible to measure interactions with nuclei that are two or three bonds removed from the unpaired electron.

The NMR interaction is characterized by the response of a nucleus n to an external static magnetic field \mathbf{B}_0 (magnitude $B_0 = |\mathbf{B}_0|$), which causes the nucleus to precess with a frequency ν_n (the Larmor frequency):

$$\nu_n = \frac{g_n \beta_N B_0}{h}$$

where β_N is the nuclear Bohr magneton, and h is Planck's constant. The nucleus in an NMR experiment does not interact with the external magnetic field alone, but with the "internal" magnetic fields induced by the surrounding atoms. These produce small perturbations in the value of the Larmor frequency, of the order of parts per million of the frequency (which is why these "chemical shift" effects in NMR are referred to in values

of ppm). If the neighboring atom harbors an electron spin, however, the additional magnetic field it induces is *not* trivial, in fact the internal magnetic field in such a case may be significantly larger than the external magnetic field. The intensity of the internal magnetic field B^n experienced by the nucleus is related to the hyperfine coupling constant A^n :

$$B^n = \frac{h|A^n|g_n\beta_n}{2}$$

The internal and external magnetic fields can interact in a constructive, or a destructive way depending upon the orientation of the internal field (i.e. the direction of the electron spin, up or down relative to the external field). The result is that the shift in the Larmor frequency induced by the internal magnetic field may be either positive or negative, and the NMR signal appears as a doublet:

$$\begin{aligned} \nu_{\pm}^n &= \frac{g_n\beta_n}{h} |B_0 \pm B^n| \\ &= \left| \nu_n \pm \frac{A^n}{2} \right| \end{aligned}$$

The ENDOR signal appears as either a doublet centered around ν_n , with splitting of magnitude A^n (if the external field is larger than the internal field; this is the case for proton ENDOR) or alternatively a doublet centered around $A^n/2$ and split by $2\nu_n$.² This is illustrated schematically in figure 1.

The intrinsic hyperfine coupling constant A_{int} can be separated into two components: an isotropic (A_{iso}) and an anisotropic component (A_{aniso}). These components, and the degree to which the nucleus exhibits anisotropic or isotropic behaviour, reveal something

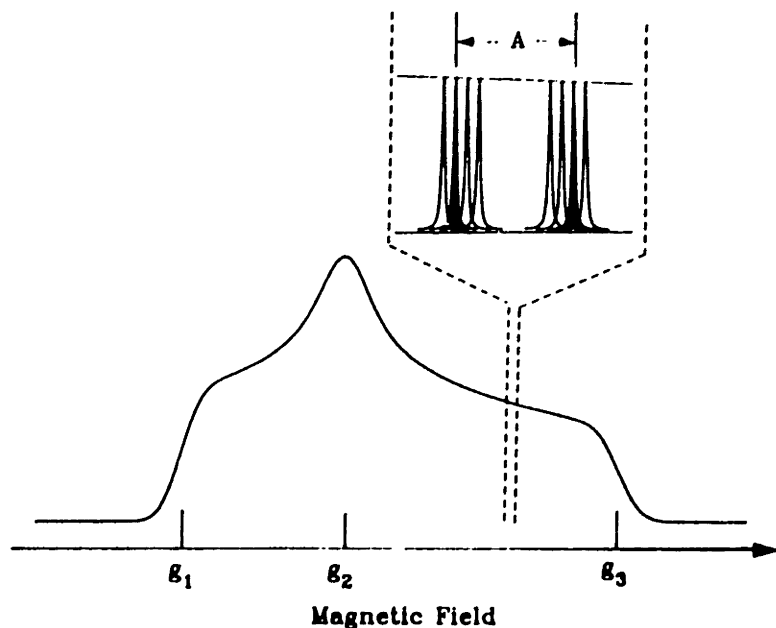


Figure 1: Rhombic EPR absorption envelope illustrating how the broadness of the EPR signal hides the hyperfine coupling information.

about the nature of the bonding between the spin system and the nucleus in question. Isotropic interactions indicate the presence of electron spin density at the nucleus, which is indicative of covalent bonding between spin cluster and the nucleus. The magnitude of the isotropic contribution is related to the degree to which the odd-electron orbital is derived from the s orbital. Anisotropic interactions have two contributions: “local” resulting from spin density residing on p and d orbitals, and “nonlocal” resulting from through-space spin-spin interactions between atoms that are not

physically bonded together.. Anisotropic interactions have two contributions: “local” resulting from spin density residing on *p* and *d* orbitals, and “nonlocal” resulting from through-space spin-spin interactions between atoms that are not physically bonded together.

The magnitude of the hyperfine coupling (and the *g*-value) for the unpaired electron depends on the orientation of the electron spin with respect to the applied magnetic field. In a frozen solution, such as that of a metalloprotein, there will be many orientations of molecules in a uniform random distribution. In frozen solutions, however, the set of molecules exhibiting a specific *g*-value represent a population of molecules with precisely one orientation. Thus by taking ENDOR spectra at different positions across the EPR envelope, one can obtain information about the angular dependence of the hyperfine coupling constant—whether the interaction is isotropic or anisotropic. This is illustrated schematically in figure 2.

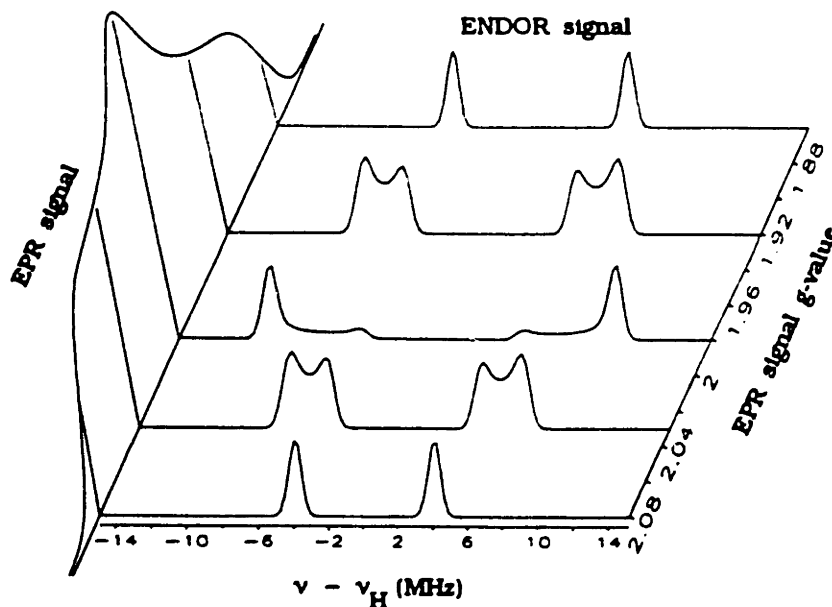


Figure 2: Representation of angle-dependent ENDOR spectra, showing a representation of how different ENDOR line shapes are obtained at different points across the spectrum.

There are two generalized approaches to the problem of acquiring ENDOR spectra: the continuous wave (cw) method, and pulsed ENDOR methods. Data shown in this chapter has been collected in both ways, illustrating the facility of both methodologies. Generally, the resolution is better for pulsed techniques, however this advantage must be weighed against the problem of much lower signal/noise associated with pulsed techniques. Continuous wave experiments are conceptually somewhat simpler. One method, the steady-state technique, is to deliberately set the microwave power so that the EPR signal is partially saturated. Under such conditions, any decrease in the electron spin relaxation time will cause the system to become partially desaturated, resulting in an increase in the signal. The resonance of nuclear spin caused by appropriate rf excitation during a continuous wave sweep facilitates electron spin relaxation, and thus one can monitor ENDOR by observing the increase in EPR signal seen under saturating conditions. Pulsed ENDOR techniques are closely related to ESEEM (electron spin echo envelope modulation) and utilize pulse sequences to monitor the response of spin echo intensity to rf pulses of various frequencies. There are two kinds of pulsed ENDOR experiments, devised by Mims³ (Mims ENDOR) and Davies⁴ (Davies ENDOR). The choice of technique depends upon the system which one is looking at; the size of hyperfine coupling is significant. Small hyperfine couplings are better resolved by Mims ENDOR, while a long electron spin relaxation time can render collection times for such experiments prohibitively long.

2.2 ENDOR Studies of Nitrogenase.

Nitrogenase has been extensively studied by ENDOR spectroscopy. ENDOR spectroscopy has provided several key results that have helped in the characterization of the FeMo center of nitrogenase in the absence of the crystal structure, and have in some ways been confirmed by the publication of the structure of the MoFe protein in 1992.

¹H ENDOR: Data collected from ¹⁰⁰Mo-enriched Av1, Kp1 and Cp1⁵ collected at very slow scan rates (in order to achieve very high spectral resolution) showed the existence of several sets of magnetically equivalent protons, with coupling constants ranging from 0.14 to 3.6 MHz. Despite subtle differences in EPR for the MoFe proteins between species, all three proteins gave similar ¹H ENDOR spectra. Exchange of the proteins into D₂O revealed that at least one of the many protons seen in the resting state enzyme was exchangeable (see figure 3). Recent ESEEM data showing interaction between the spin of the M-center and His195 indicates that the imidazole N-H proton could conceivably be the exchanging species. Examination of NifV⁻ nitrogenase shows there is an additional proton doublet for this species.⁶

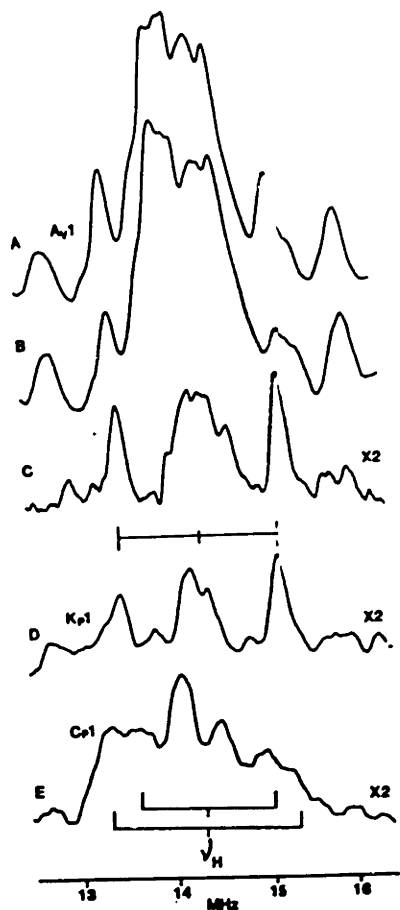


Figure 3: Exchange of protons coupled to the MoFe $S = 1/2$ signal. C shows the result of subtracting the signal of MoFe protein in D₂O from the signal in H₂O. There is one clearly resolved exchangeable proton in the case of *A. vinelandii* nitrogenase. This is less noticeable for *K. pneumoniae* (D) or *C. pasteurianum* (E), but nevertheless the result still holds for these proteins.

⁵⁷Fe ENDOR: Growth of bacteria in media containing ⁵⁷Fe enables enriched proteins to be produced with ⁵⁷Fe at the clusters. ⁵⁷Fe ENDOR showed the presence of 6 clearly distinguishable resonances: a result that held true for proteins from

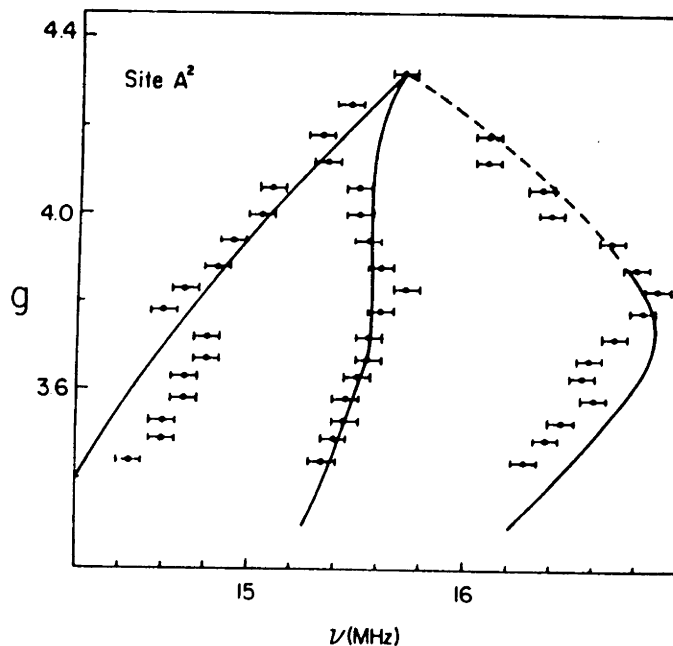


Figure 4: ENDOR calculated vs theoretical peak positions, showing the variation of hyperfine coupling constant (and ENDOR spectra) with g value.

A. vinelandii, *K. pneumoniae* and *C. pasteurianum*., as well as in NifV- nitrogenase from *K. pneumoniae*. Closer examination of the single-crystal-like ENDOR spectra of *A. vinelandii* MoFe protein revealed for the five clearly resolved ^{57}Fe resonances, the hyperfine coupling tensors were all different⁷(figure 4). This is apparently related to constraints placed on the M-center by the protein environment, rather than any innate structural features, as X-ray crystallography reveals a high degree of symmetry for the FeMo-cofactor.

^{95}Mo ENDOR: ^{95}Mo is an $I=5/2$ nucleus, so the hyperfine interactions typical of most ENDOR spectra are complicated by quadrupole effects. Examination of ^{95}Mo ENDOR across the EPR envelope (figure 5) revealed the existence of a ^{95}Mo m_I ($\pm 1/2$) doublet that is most clearly resolved near g_y and is significantly broadened as one proceeds to higher fields ($\rightarrow g_z$). This broadening can be attributed to "satellite" transitions due to quadrupole effects. ^{97}Mo results supported this hypothesis, since the quadrupole moment of this nucleus (also $I=5/2$) is some 10 times larger than that for ^{95}Mo , and this effects the ENDOR spectra, which are significantly more broadened for ^{97}Mo than ^{95}Mo . Examination of the field dependence of the apparent quadrupole splittings, and comparison with experimental data obtained by ^{95}Mo NMR of Mo model compounds revealed that the quadrupolar splitting P^{Mo} of approximately 1.6 MHz was more consistent with an oxidation state of IV ($^{95}\text{Mo}^{\text{IV}}$ has $P^{\text{Mo}} \sim 1$ MHz in model compounds) than VI (for $^{95}\text{Mo}^{\text{VI}}$ the corresponding splitting is < 0.1 MHz).

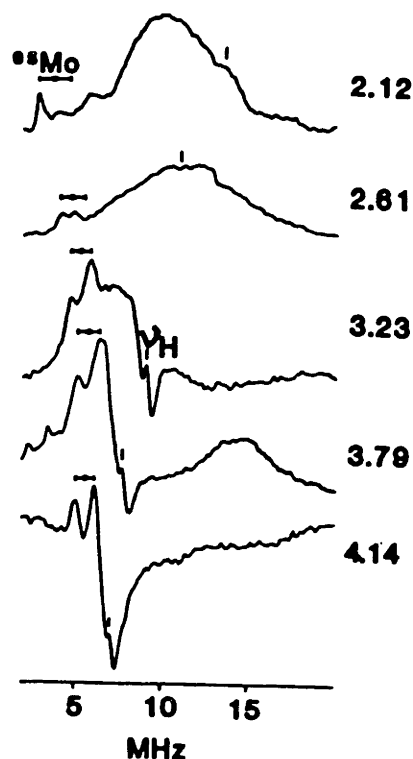


Figure 5: ^{95}Mo ENDOR of CpI at various g values across the EPR absorption envelope.

2.3 ^1H and ^2H ENDOR Studies of the MoFe protein of Nitrogenase after turnover in $^2\text{H}_2\text{O}$.

2.3.1 Experimental Procedures.

Sample Preparation: Nitrogenase MoFe and Fe proteins were purified from *Azotobacter vinelandii* frozen cell paste as described in the appendix. All buffers were degassed thoroughly by stirring under vacuum and backfilling with Ar (several cycles). The reactions were carried out under high dilution conditions, and the reactions monitored (figure 6) to ensure that nitrogenase was reacting with substrate (as described in the table below). Each reaction contained the following: 3.3 μM MoFe protein, 0.7 μM Fe protein, 3.7 mM ATP, 4.2 mM Mg^{2+} , 800 μM $\text{Na}_2\text{S}_2\text{O}_4$, 25mM Tris (pH = 7.4) and 0.1M NaCl (to prevent protein precipitation). Upon completion of the reaction, the solution was brought into a glovebox ($\text{O}_2 < 2\text{ppm}$) and concentrated in an Amicon[®] ultrafiltration cell using YM100 membranes (M. wt. cutoff 100,000) which would allow Fe protein to pass through but would withhold MoFe protein. The backpressure for the ultrafiltration cell was provided using He gas that was scrubbed of O_2 using an Oxisorb[®] cartridge. Protein was concentrated to as small a volume as possible, which gave a final MoFe protein concentration of ca. 100mg/ml.

Table 1: Turnover Reactions Performed

Sample	Substrate	Reaction Monitored by
H ₂ O turnover	H ⁺ under Ar gas	H ₂ evolution (GC)
D ₂ O turnover	D ⁺ under Ar gas	D ₂ evolution (GC)
D ₂ O turnover, N ₂	N ₂ gas, D ⁺	D ₂ evolution (GC)
D ₂ O turnover, C ₂ H ₂	10 % C ₂ H ₂ /Ar gas	C ₂ H ₄ production (GC)
D ₂ O turnover, CN ⁻	NaCN	H ₂ evolution, CH ₄ production.

ENDOR Measurements: These were conducted by V.M DeRose at Northwestern University, using continuous wave and pulsed (Mims ESE-ENDOR) techniques.

2.3.2 Results

When the structure of the FeMo cofactor was first made known to the scientific community, one of the greatest shock waves was the revelation that six of the Fe atoms were trigonal. This unusual coordination pattern had only been seen once before, in the case where the ligands were extremely bulky and the trigonal disposition was the result of extreme steric constraint⁸.

Immediately the proposition was put forward that perhaps there was a fourth ligand for each of the six trigonal ions, but that it was coordinated hydride. The fact that extensive exchange of MoFe in D₂O failed to remove most of the protons coupled to the FeMo cofactor spin seemed highly unusual, and it was thought that perhaps some of these unusual non-exchangeable protons were in fact bound up as hydrides. It was thought that if that was indeed the case, then they would probably be released upon turnover of the enzyme, that somehow such a redox process would "wash out" the some of the extra protons. The first result (figure 7) shows that in fact there was no dramatic loss of protons: rather, the identical result of exchange of one (or possibly two) protons was observed. This is discernable in figure 7 as the accentuation of "shoulders" due to the loss of the coupling that was causing smoothing.

When it became clear that there were (apparently) no hydride ligands in the resting state, it occurred that perhaps some of the "non-exchangeable" protons might be exchanged during substrate reduction at the

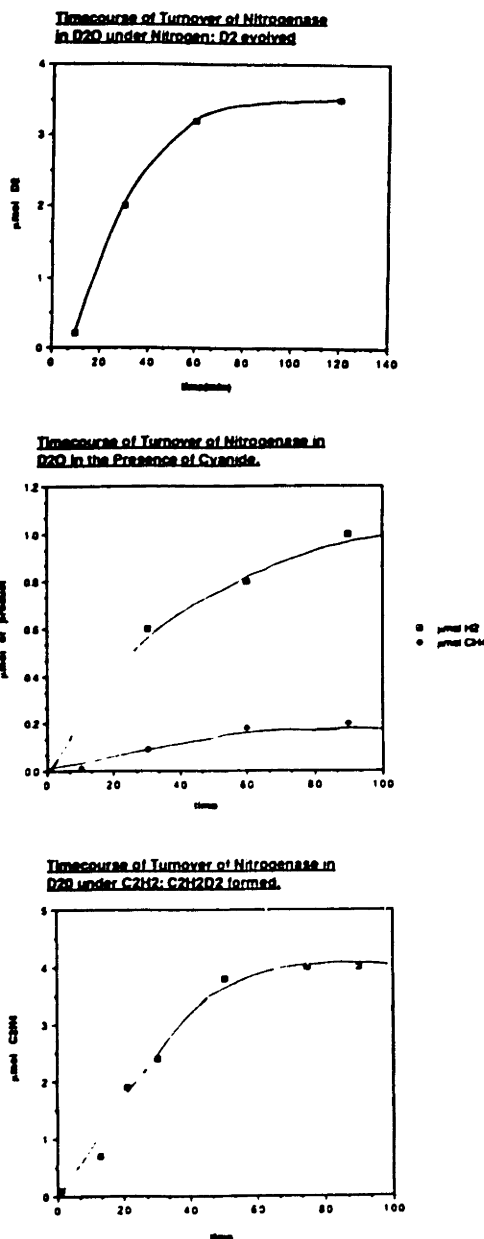


Figure 6: Analysis of nitrogenase turnover products with time.

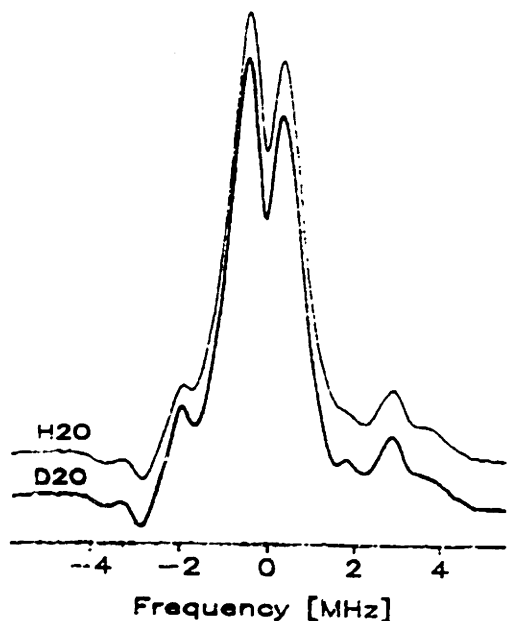


Figure 7: ^1H ENDOR of *A. vinelandii* MoFe protein turned over in H_2O and D_2O .

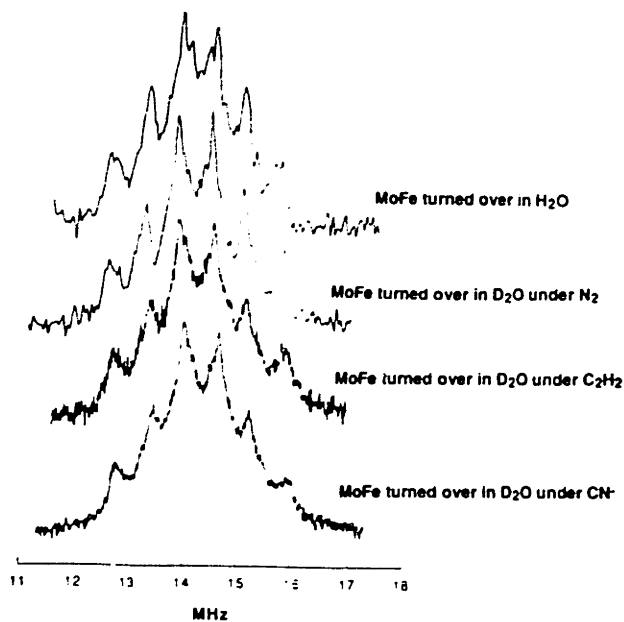


Figure 8: Mims pulsed ENDOR (^1H) of *A. vinelandii* MoFe protein after turnover.

cofactor. Since the enzyme is capable of generating carbon-hydrogen bonds (e.g. methane gas formed by reduction of cyanide), it seemed that it could be conceivable that the enzyme breaks and regenerates a carbon-hydrogen bond in the vicinity of the active site during turnover. Turnover of the enzyme in D_2O under various substrates was attempted. Mims pulsed ENDOR studies of samples post-turnover showed *no* change for the substrates acetylene, dinitrogen and cyanide. This indicated that, at least for those residues close enough to the cofactor for these nuclei to undergo hyperfine interactions (see figure 8). Finally, ^2H -ENDOR of samples of the MoFe protein in D_2O showed clearly not one but three deuterons coupled to the electron spin of FeMo-co in the resting state. These results are shown in figure 9.

2.3.3 Conclusions.

Enzymatic turnover of nitrogenase in D_2O failed to induce any significant loss in the ^1H ENDOR of nitrogenase over that which is lost upon simple D_2O -soaking of the enzyme. Secondly, changing the substrate present in the D_2O , and thus altering the chemistry occurring during

turnover, failed to cause any significant loss of ^1H ENDOR response.

Apparently these non-exchangeable protons are associated with the carbon backbone of the nitrogenase MoFe protein polypeptide chains.

Interestingly, although it is difficult to clearly resolve more than one exchangeable proton through ^1H ENDOR, Mims pulsed ENDOR of ^2H shows the presence of three deuterons in the vicinity of the FeMo-cofactor.

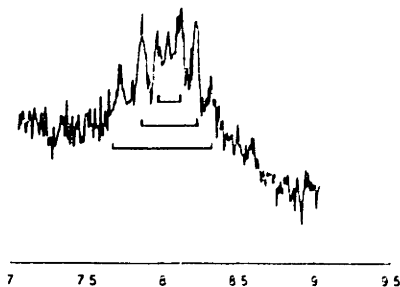


Figure 9: Pulsed ^2H ENDOR of *A. vinelandii* MoFe protein in $^2\text{H}_2\text{O}$.

2.4 ^{13}C ENDOR Studies of CO-inhibited Nitrogenase:

Carbon monoxide has been shown to inhibit the reduction of dinitrogen non-competitively in studies carried out in this laboratory several years ago.⁹ Almost no inhibition of electron flux through the MoFe protein (as monitored by production of H_2) was observed, however acetylene reduction is completely inhibited by an 80-fold molar excess of CO in 15s. Of interest here are the EPR active species which are observed to form upon turnover of nitrogenase. Firstly, it is interesting that the protein must be undergoing turnover in order for the EPR signal to appear. Figure 11 shows, at top, a mixture of Fe protein and MoFe protein in the presence of CO. The unusual shape of the signal at ca $g=2.0$ is caused by a super-imposition of Fe and MoFe protein resonances. Here, since there is no ATP, no turnover is possible and the EPR is essentially that of native protein. At center, we see the appearance of the EPR spectrum (CO present) after turnover for 45 seconds. The $g=3.78$ signal of the M-centers has all but disappeared. However, there is a new signal present around $g=2.0$. This is a paramagnetic species that can be studied by ENDOR spectroscopy. The bottom spectra is the post-turnover situation. The signal for Fe protein has disappeared, as Fe protein is now in the spin zero oxidized state. In fact the appearance of the EPR is now that of isolated MoFe protein, indicative of the transience of the CO-inhibited state.

Further observations suggest that there are two binding sites for CO: one of high affinity for inhibitor, the other low affinity. In the presence of equimolar (relative to MoFe protein) amounts of CO, one observes the spectrum the bottom of figure 11. As further equivalents of CO were added, the signal as shown at bottom disappeared with the simultaneous appearance of the high-CO signal (see top). Line broadening experiments with isotopically labelled proteins showed that the CO-related signals came from paramagnetic centers in the MoFe, not the Fe protein.

2.4.1 Experimental Procedures

Azotobacter vineland nitrogenase components were prepared according to standard

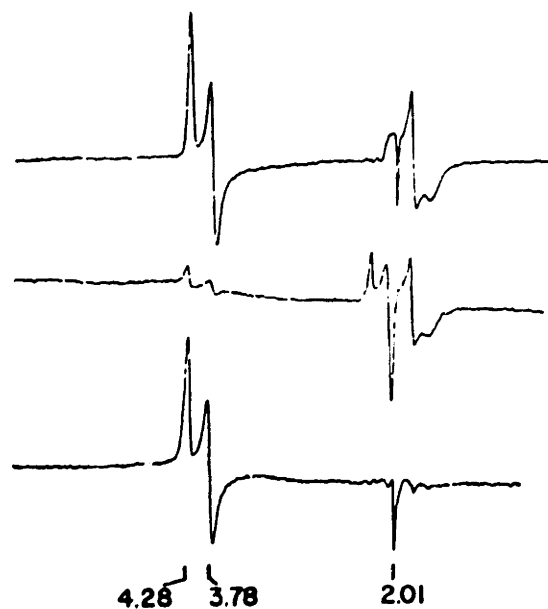


Figure 10: EPR Spectra of CO-inhibited MoFe protein.

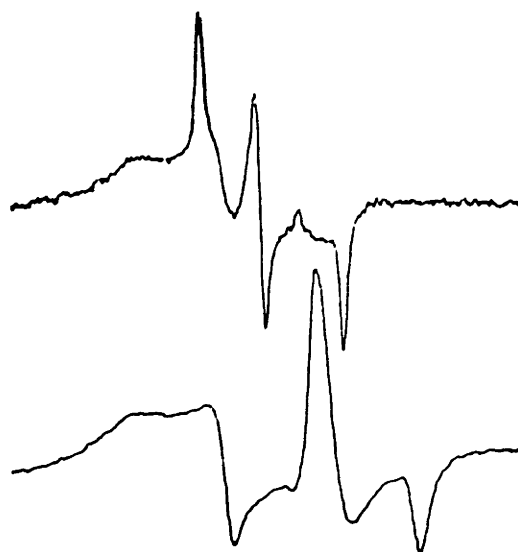


Figure 11: Dependence of the CO-induced EPR signal upon CO concentration.

procedures. All manipulations were carried out anaerobically, either in a glove box (<2ppm O₂) or using syringe and Schlenk line techniques. The turnover experiments were carried out by adding Fe protein (in 25 mM Tris, 0.35 M NaCl, pH =7.4) to a solution of MoFe protein that had been exchanged into "turnover buffer" and equilibrated with CO at a partial pressure of 0.5 atm ("hi CO") or 0.08 atm ("lo CO") in a serum-capped Wheaton vial. This was carried out in a glove box. Samples were then collected at various time intervals using a syringe, injected into Q-band ENDOR tubes, and frozen in the glove box using an isopentane bath (-20 to -80 deg C). The bath was cooled by nitrogen gas flowing through copper tubing, after being precooled in liquid nitrogen, external to the glove box. The initial concentrations of proteins and reagents in the turnover mixture were as follows: 67mg/ml Av1 (0.28mM), 8.3 mg/ml Av2 (0.14mM), 50mM MgCl₂, 100 mM Na₂ATP, 300 mM Na(phosphocreatine), 100mM HEPES, 2 mg/ml creatine kinase, 100mM Na₂S₂O₄, final pH=7.4. Samples were checked by X-band EPR prior to shipping in most cases. Power saturation studies showed that only a small percentage of the protein was in the CO-inhibited form.

2.4.2 Results

¹³C ENDOR spectra were taken for the following four samples (1) Nitrogenase "High CO" signal [¹³CO]; (2) Nitrogenase "Low CO" signal [¹³CO]; (3) Nitrogenase "High CO" signal [¹²CO] and (4) Nitrogenase "Low CO" signal [¹²CO]. These experiments revealed the following: the low CO signal ($g=2.07, 1.98, 1.93$) was associated with a single bound CO, with a mostly dipolar (anisotropic), and fairly weak (~3MHz), coupling. The spectra was present with ¹³CO and absent with ¹²CO, indicating that the ENDOR response originated with bound CO as a ligand. The high CO signal ($g=2.15, 2.07, 1.98$) was associated with two bound CO moieties: one with a very small, ca 1MHz, coupling, and another with a slightly larger and mostly isotropic coupling. The angle-dependent ¹³C-ENDOR spectra for a range of g values are shown in figure 12. The

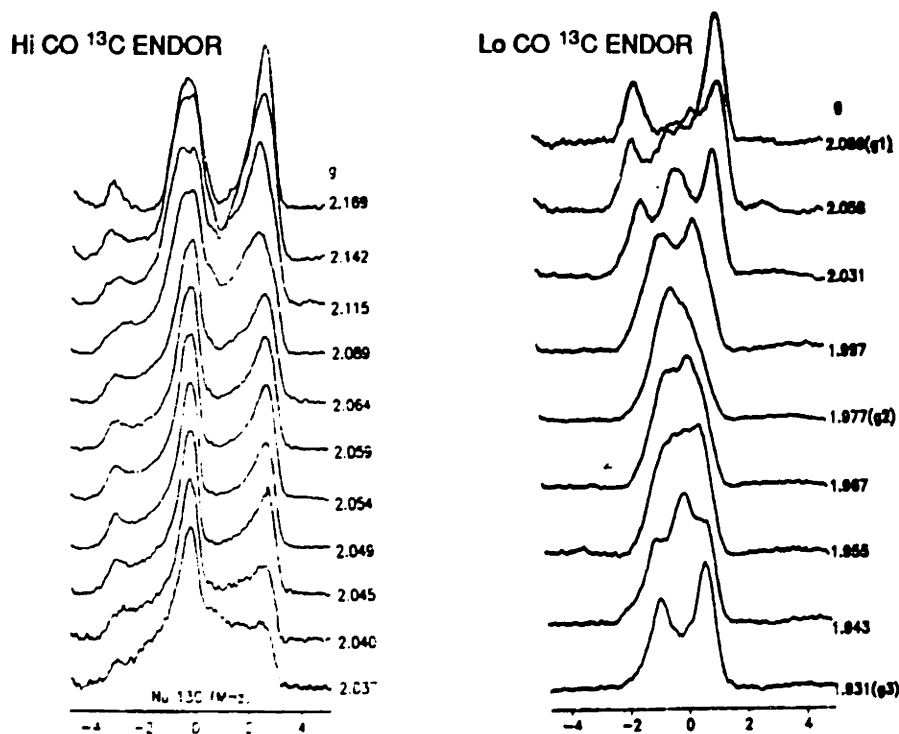


Figure 12: ¹³C ENDOR of CO-inhibited Nitrogenase, showing orientational dependence of spectra.

isotropic nature of the high CO, and the anisotropy of the low CO, hyperfine interactions are clearly seen in this illustration.

2.4.3 Conclusions.

The conclusive demonstration of CO binding at one of the metal centers of nitrogenase is a very exciting result, as it represents the first direct observation of any substrate or inhibitor bound directly to nitrogenase. This work has recently been extended somewhat. Hales and coworkers¹⁰ showed in "pulse-chase" experiments that if the hi-CO form was subjected to vacuum pumping (after treatment with ethylene glycol) it reverted back to the low CO form. They utilized this technique to show that the CO that was associated with the low CO EPR signal was the same CO anisotropically coupled to the high CO signal. This was evident for the following reasons: (1) conversion of ¹³CO-labelled high CO nitrogenase into the low CO form, followed by rapid freezing upon replacement of the headspace above the sample with ¹²CO, showed that residual amounts of ¹³CO (from the low CO form) remained, and gave rise to the normal high CO ¹³C ENDOR response. It is apparent from this result that the low CO and the high CO signal both arise from the same cluster. Patricia Christie¹¹, using MoFe protein labelled with ⁵⁷Fe at the P-clusters (but not at the MoFe cofactor), although it exhibited a high CO EPR signal, did not show hyperfine coupling with ⁵⁷Fe. Uniformly labelled ⁵⁷Fe MoFe protein, on the other hand, did show hyperfine coupling by ENDOR. It is apparent, with this result in mind, that the inhibition of nitrogenase by CO occurs at the nitrogen-reduction site, the FeMo cofactor.

References to Chapter Two

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- ¹¹Patricia Christie, PhD. Thesis, Massachusetts Institute of Technology, February 1996.

Chapter Three: X-ray Absorption Studies of Nitrogenase.

3.1 Principles of X-ray Absorption Spectroscopy.

3.1.1 Overview.

It is in no way intended that this discussion be comprehensive review of the methodology and applications of X-ray Absorption Spectroscopy (for that the reader is referred to an excellent discussion by Vital K. Yachandra, and references therein)¹ However, sufficient information for an understanding of the experiments conducted with CO-inhibited nitrogenase will be discussed here.

Electrons within an atom are constrained by quantum mechanics to occupy orbitals with distinct energy. The Pauli Exclusion Principle constrains electrons to occupy orbitals, and in any given atom some electrons must occupy the inner orbitals in the first shell. These are the “core” electrons. The typical energy difference between core electrons and the “outer” electrons (which occupy the higher-energy levels) is of the order of 6000 eV (for transition metals) which corresponds to electromagnetic radiation in the X-ray region (wavelength ca. 10^{-10} m, or 1Å).

Figure 1 shows the appearance of a typical X-ray absorption spectrum, in this instance for MnO₂. There is no absorbance for X-rays up to a threshold which represents the transition of the core electron to the first of the outer levels, i.e. the 3d orbital. From this point on, there is absorption of X-rays, which is possible because as one progresses higher in energy, the difference in energies between adjacent orbitals gets smaller and smaller, until there is a continuum of allowed energies and quantization becomes negligible. The threshold at which one first observes the absorption of X-rays is called the “edge”. There are in fact two types of “edge”. There is an edge corresponding to the transition of electrons from the 1s orbital to the outer orbitals (the “K-edge”) and a series of three edges corresponding to transitions of electrons from the 2s and 2p orbitals (the “L-edges”). L-edge x-ray absorption spectroscopy is a frontier for the field of nitrogenase research, and while it is not discussed here it is worthwhile to mention that significant progress has recently been made in the methodology required, and now all that remains (!) is for the experiments to be carried out.²

There are two types of X-ray absorption spectroscopy: that which is exemplified by this work, X-ray absorption near-edge structure (XANES) and extended x-ray absorption fine-structure (EXAFS). The EXAFS is, as a rule, more informative than the XANES because (as discussed below) EXAFS provides information regarding atom-atom distances near the element of interest. XANES is, by definition, concerned with the details of the spectrum around the edge, while EXAFS is concerned with the analysis of the sinusoidal modulations (which become less pronounced as the energy increases) beyond the edge.

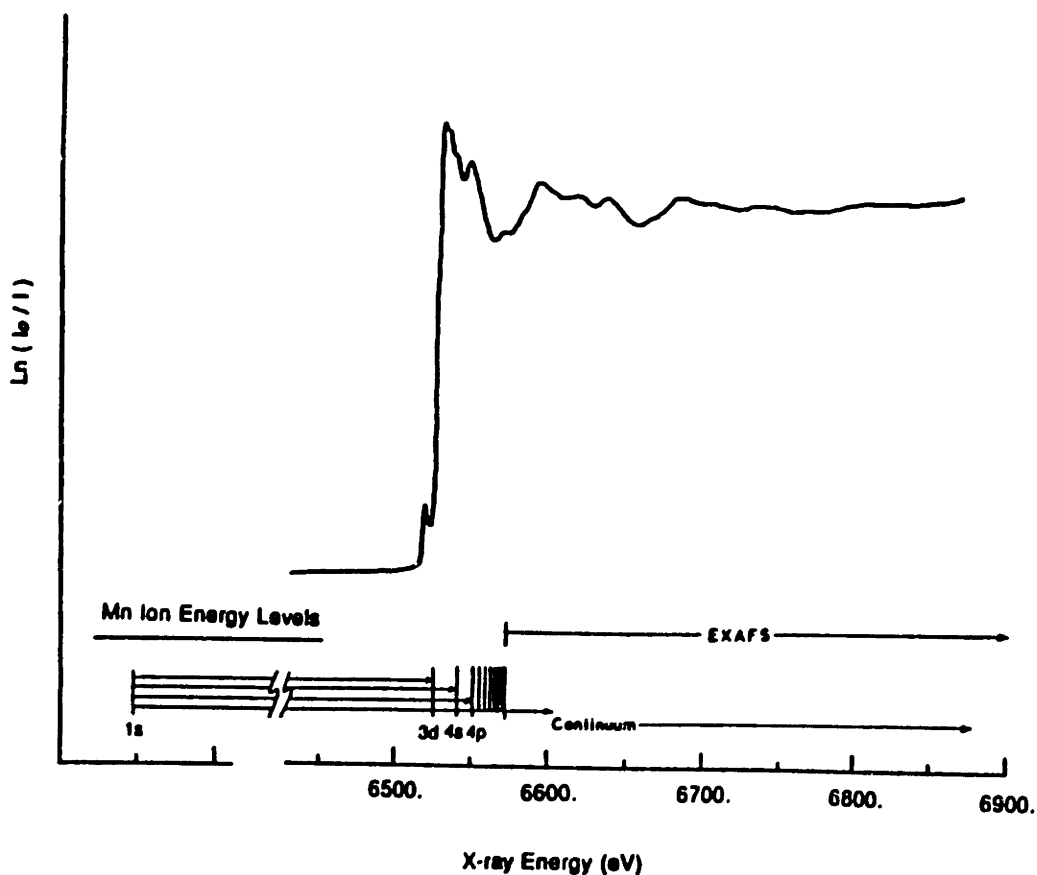


Figure 1: X-ray absorption spectrum of MnO₂(K-edge). [from R.D.Guiles, PLD Dissertation, UC Berkeley (1982)]

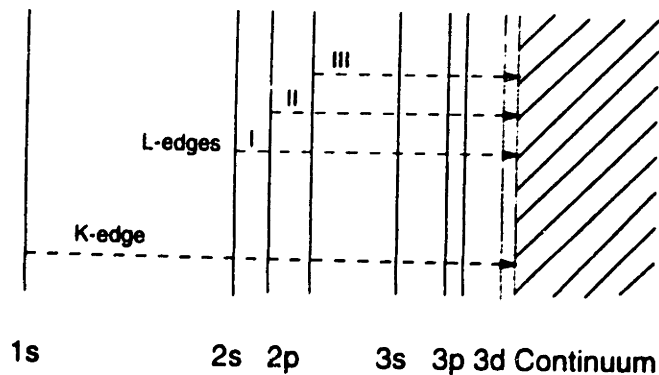


Figure 2: X-ray absorption energy level diagram showing the K and L edge transitions. Energy levels are not drawn to scale, e.g. for Mn the K-edge is at 6539 eV and the L edges are at 769, 650 and 639 eV. [from U.K. tachacdera Methods Ezymol (1995) 246 638]

X-ray absorption spectroscopy is a technique with some attractive features and some significant pitfalls. For the most part the pitfalls have been overcome, but it is important that one is at least aware of the potential difficulties before planning experiments. One of the greatest advantages of this technique over many others is that it is element specific. It is possible to study complicated systems (such as metalloproteins) and focus only on the part of the molecule of interest (often the metal site) without any interference from the surrounding protein. This has proven especially useful in the case of the Mn complex of photosystem two, which of necessity is purified in conjunction with several other polypeptides, lipids, pigments and other metals. X-ray absorption spectroscopy is a very effective technique for looking at this molecule, because it is possible to ignore the many and varied contaminants of typical PS-II preparations and focus on the center of interest. Another advantage is that it is always possible to use X-ray absorption as a probe of the chemistry of a system, no matter what the oxidation state. EPR is a technique that can also be very specific to the center of interest (as shown in Chapter Two) but fails to be useful in many instances because the cluster or metal center is in an EPR-silent state. Finally, there is no requirement for the sample to be any particular state: solid, liquid or gas. It is not necessary for the sample to be crystalline. The only requirement for X-ray absorption samples is that they be "as concentrated as possible" (which is often essential for EXAFS where the signal to noise ratio can be quite high). One of the biggest problems with X-ray absorption spectroscopy is that it involves X-rays! These (as any competent radiographer will inform you) are extremely harmful to biological materials, metalloenzymes included. Serious damage is inflicted upon protein samples by free radicals and hydrated electrons produced by the action of X-rays. However, this problem has for the most part been solved by the use of low temperatures, such as obtainable by the use of a liquid He flow cryostat.

3.1.2 X-ray Absorption Spectroscopy—Practical Considerations

The collection of X-ray absorption data is conceptually fairly simple, the principles involved are similar to those involved in optical absorption/fluorescence. The spectrometer consists of an X-ray source, a monochromator, an incident flux detector (I_0), the sample (which is usually in a cryogenic cavity) a transmitted flux detector (I_1) which detects X-rays that are not absorbed by the sample. Delicate devices, and the parts of the sample mount which for cryogenic purposes must be sealed and/or evacuated, are protected by Mylar or Kapton windows. These materials are tough and fairly transparent to X-rays. The sensitivity of X-ray measurements is greatly enhanced by measuring the intensity of fluoresced X-rays at 90° to the sample (which is inclined at 45° relative to the incident beam). These are generated when an electron from a higher orbital falls into the "hole" in a core orbital left when exposure to X-radiation causes it to be ejected.

Finding a sufficiently powerful and coherent source of X-ray radiation over a range of wavelengths is a non-trivial matter. It turns out that when electrons are accelerated to near the speed of light, and injected into storage rings (where they are contained by magnets which bend the beam of electrons) they emit intense electromagnetic radiation wherever the electron beam is bent, tangential to the direction of motion of the electrons. This radiation is coherent, intense, and covers a wide range of wavelengths. The emitted radiation is most powerful in the X-ray region, and is extremely useful for use in biophysical studies including X-ray absorption spectroscopy and multiwavelength anomalous diffraction (MAD) for X-ray crystallographic studies. Synchrotron radiation greatly facilitates X-ray absorption studies with dilute samples, such as biological materials, simply because of the low signal/noise ratio inherent with such samples.

Finding a suitable monochromator for X-rays is a relatively simple matter. Tunable monochromatic X-rays can readily be obtained through the use of a crystal which is rotated relative to the beam. As the angle of incidence varies, so does the wavelength of the reflected radiation, as made clear by the Bragg relation:

$$n\lambda = 2d \sin\theta$$

As the angle of incidence, θ , varies so does the wavelength. Rotating a crystal to alter the angle of incidence also alters the position of the reflected beam, so in order to maintain the position of the X-ray beam relative to the sample, it is necessary to have a second crystal positioned in parallel which serves to hold the beam in the correct position—focused onto the sample!

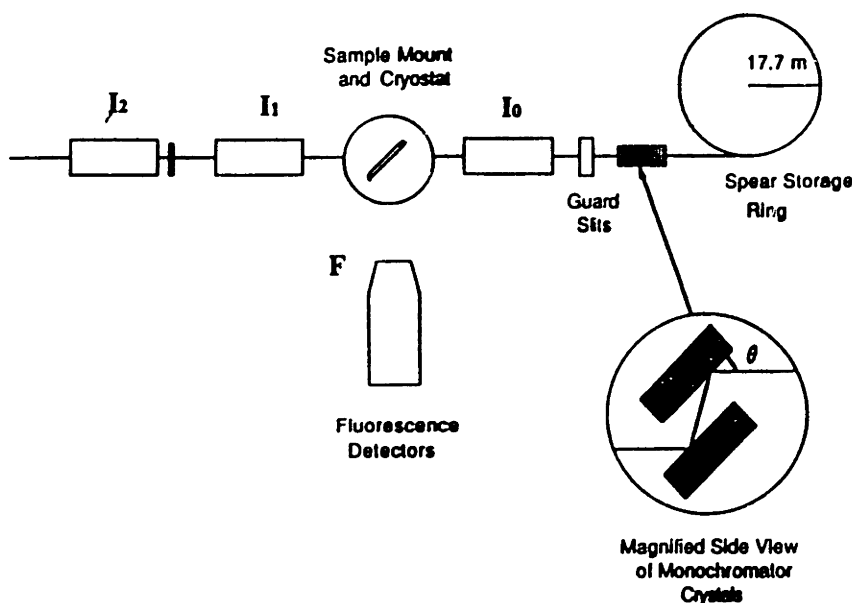


Figure 3: Experimental setup for a typical XAS experiment. [from E.D.Guiles PhD Dissertation, UC Berkeley(1982)]

Detection of X-rays is best accomplished by measuring the X-ray fluorescence from the sample, using solid-state Si or Ge detectors. These have high energy resolution (150eV), can be arrayed as multi-element detectors (large surface area), and respond in linear fashion to X-rays of up to around 40,000 counts per second (cps). The detection of the flux of incident and transmitted photons is less critical (though important) and ion chambers usually suffice for these measurements. One facet of data processing which is critical in XAS measurements is the non-linearity associated with solid-state detectors at high count rates. This is a function of the electronics associated with the detectors, which have a dead-time. One measures the incoming count rate ($ICR=n$) and correlates this with the number of counts measured by the detector (m) after processing, and obtains a saturation curve. The curve is fitted to an equation relating the observed saturation to the dead-time. There are two models for this: the paralizible [$m = \beta n (1 - n\tau)$] and the non-paralizible [$m = \beta n \exp(-n\tau)$]. One determines the deadtime τ and the constant β for each channel, and this enables correction for non-linearity to readily be made.³

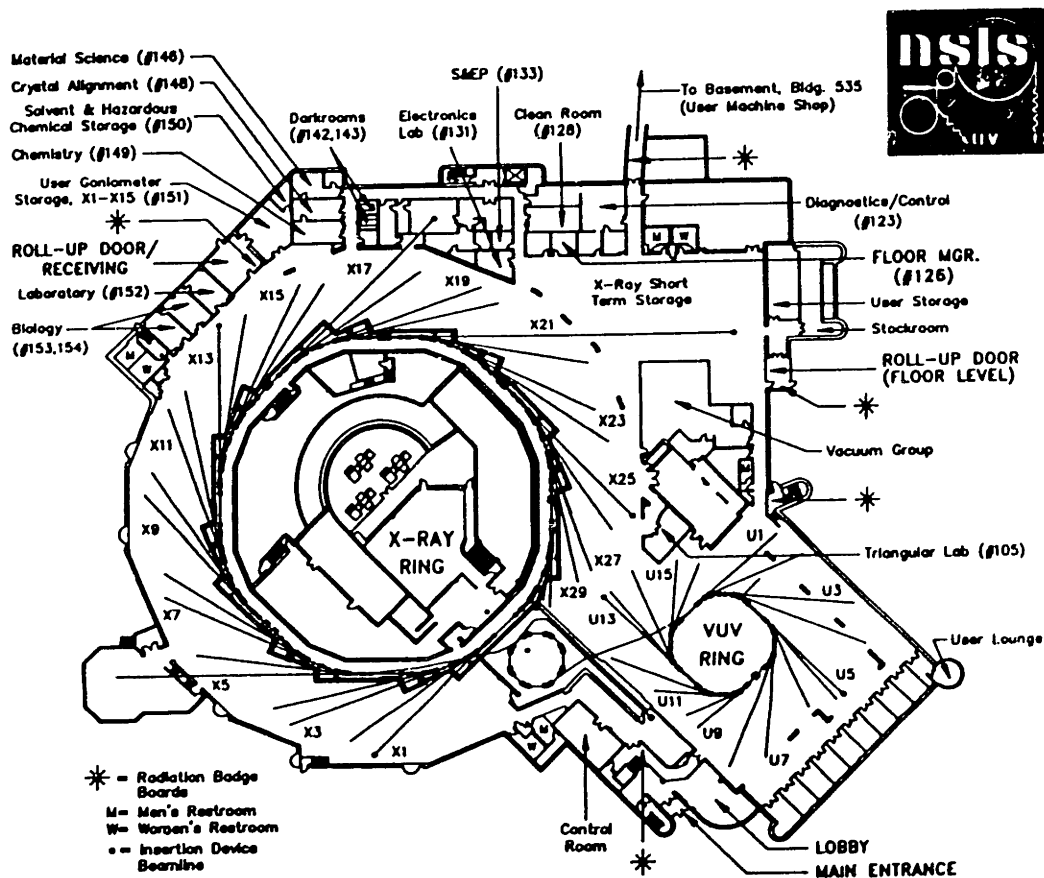


Figure 1. Floor plan of the National Synchrotron Light Source experimental area.

Figure 4: Floor plan of the national Synchrotron Light Source of Brookhaven National Laboratory.

The raw data obtained during an XAS experiment must be corrected to account for pre-edge background and curvature after the edge. This background is the result of scattering processes, for the most part. The pre-edge background is treated as a linear or quadratic polynomial and is subtracted out. Normalization of the data to ensure correct EXAFS analysis is essential. EXAFS modulations, are essentially the deviation in absorbance from that expected for a free metal ion (which would have a featureless flat spectrum after the edge). The actual values referred to are the absorption cross-sections μ , which are the X-ray spectroscopy versions of extinction coefficients (in visible/UV spectroscopy). Formally, the EXAFS response is defined by the following equation:

$$\chi(E) = \frac{[\mu(E) - \mu_0(E)]}{\mu_0(E)}$$

where $\mu(E)$ is the normalized absorption cross section and $\mu_0(E)$ is the absorption cross section for a free atom. Experimentally, one measures *relative* cross sections which are normalized by converting the EXAFS spectra to unit edge height for some arbitrary energy value E' (often the position of the edge maximum).

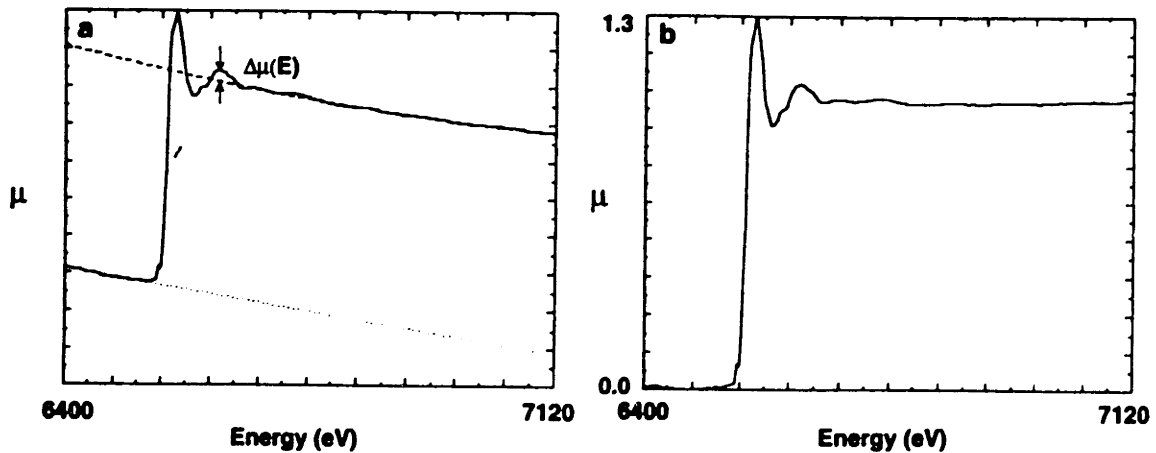


Figure 5: Pre-edge background removal and normalization of an EXAFS spectrum. [from V.K. Yachandra *Methods Enzymol.* 246, 638]

3.1.3 EXAFS—Description and Some Significant Results.

The origins of the oscillatory phenomenon known as EXAFS are quantum mechanical in nature, and are related to the fact that the absorption coefficients, μ , are proportional to the square of the electric dipole transition moment. This value is related to wave functions for the initial and final states of the system. The initial state wave function is the 1s state, i.e. the core orbital, and the final state is that of the photoelectron ejected by the X-radiation. This final state wave function includes contributions induced by the surrounding atoms, which backscatter the electron wave to varying degrees (depending upon the distance from the absorbing atom and the size of the backscattering atoms). It is the effect of this final state wave function on the dipole transition moment which leads to the modulations. Qualitatively, it is best thought of in the following way: The photoelectron wave will have a different “wavelength” depending on the energy of the incident X-ray beam, and will either interact constructively or destructively, as shown in the diagram. Mathematically, it is easier to deal with the data from an EXAFS experiment if one converts the energy terms into “ k space”: k is related to the energy of the photoelectron expressed in terms of the de Broglie wavelength ($k=2\pi/\lambda$). The EXAFS response has been accounted for in this way, and is more rigorously expressed as the following equation:

$$\chi(k) = \sum_j \frac{N_j |f_j(\pi, k)|}{kR_{aj}^2} \sin[2kR_{aj} + \alpha_{aj}(k)]$$

N_j represents the number of backscattering atoms j at a distance R_{aj} from the absorbing atom, $f_j(\pi, k)$ is the backscattering amplitude (which depends upon the atomic number of the backscattering element j) and α_{aj} is the phase shift for both the absorbing and the backscattering atom. This phase shift is a result of the electron experiencing the atomic potentials of the absorbing and neighbouring atoms, and can be calculated theoretically if the scattering atoms are known. The above equation is modified in practise, because thermal effects of the absorbing and scattering atoms lead to a distribution of distance R_{aj} :

$$\chi(k) = \sum_j \frac{N_j |f_j(\pi, k)|}{kR_{aj}^2} e^{-2\sigma_{aj}^2 k^2} \sin[2kR_{aj} + \alpha_{aj}(k)]$$

It is clear from reflection upon the above equation that there is a significant amount of information about the molecular environment surrounding the absorbing atoms. Fourier transformation of the EXAFS data from k space to R space to give information about distances and, from the amplitude of the backscattering ($f_j(\pi, k)$) something about their nature (e.g. atomic number). In order to calculate distances correctly, one needs to have an idea what the phase shift, $a_j(k)$, is. However, this is a function of both absorbing and backscattering atoms, so if the phase shift of the former can be used to give an estimation of the latter. Sometimes, one uses well-characterized model compounds known to be similar to the center of interest to obtain phase information. The deconvolution of EXAFS data is far from straightforward, and there are many techniques for improving the quality of the data. It is rare that quantitative information is obtained from the raw data, data is usually refined by a process of back-transformation of each peak ("shell") in R space into k space (by application of an appropriate filter function). This effectively gives data which can be fit into the EXAFS equation to obtain structural parameters for each shell separately. This process is called "Fourier filtering", and for more information the reader is referred to textbooks on EXAFS.^{4,5}

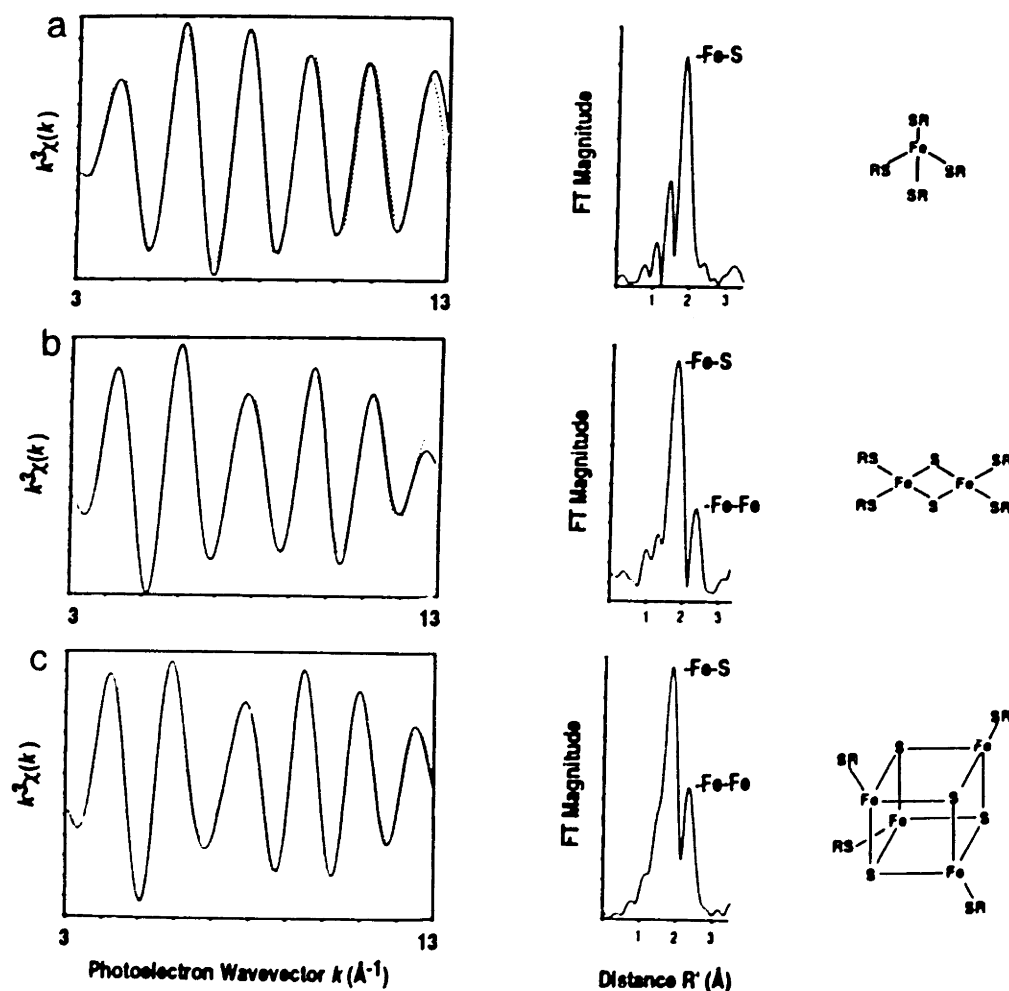


Figure 6: Fe EXAFS of Fe-S proteins. [V.K.Yachandra Methods Enzymol (1995) 246, 638]

EXAFS spectroscopy has been particularly useful in characterizing Fe-S proteins (see figure 6), and has in some cases led to a reconsideration of structures that had been proposed on the basis of X-ray crystallography. The X-ray structure of rubredoxin showed that one of the four cysteinyl ligands was ligated by a shorter bond to Fe than the other four. EXAFS studies showed there was a single Fe-S distance of 2.26Å, and subsequent refinement showed that the “short” bond distance was an artifact! The EXAFS spectrum for rubredoxin shows there is one sine wave, indicative of one set of backscattering atoms (the sulfurs of the ligands). In the spectrum of a [2Fe-2S] cluster of a plant ferredoxin, the presence of a second set of backscattering atom (that of the second Fe) which leads to a “beat pattern” in the EXAFS. The presence of Fe-Fe interactions, as well as the beat pattern effect, is evident in the EXAFS for a [4Fe-4S] bacterial ferredoxin.⁶

EXAFS has been used to attempt to characterize the Mn centers of photosystem II (see figure 7). While these results are extremely interesting, in many ways they illustrate the limitations of EXAFS as a structure-delimiting technique. Mn EXAFS of photosystem II shows the presence of a close set of backscattering atoms at ca. 2 Å, which is interpreted as being two sets of atoms: 1.5-2.5 (bridging) N or O atoms at around 1.8 Å, and another set of 2-4 (terminal) N or O atoms at around 1.95-2.15Å. In addition to these atoms, EXAFS detects a set of atoms at 2.72Å, which is interpreted as being that of 1-1.5 Mn atoms, and another set of 0.5 Mn or Ca atoms at 3.3Å⁷. This set of parameters offers little by way of constraints for interpreting the data structurally. Figure 7 shows one possible structural model that is consistent with the data.

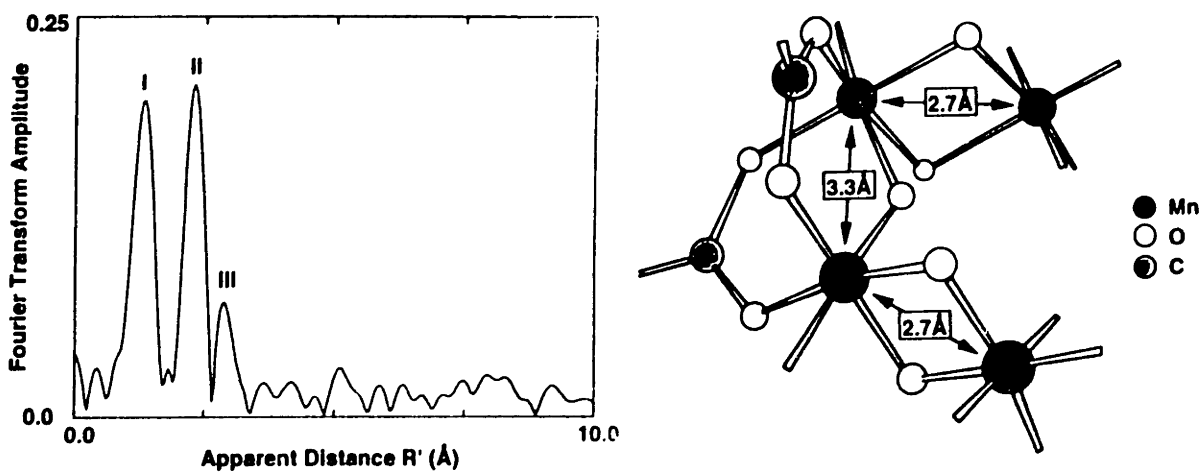


Figure 7: Mn EXAFS of the Mn Cluster of PS-II (left) [from V.K. Yachandra *Methods Enzymol.* (1995) 246, 638] and a possible structure derived from the EXAFS results (right) [from K. Sauer, V.K. Yachandra, R.D. Britt]

3.1.4 Applications and Interpretation of XANES.

Analysis of XANES spectra is relatively simple compared with EXAFS and somewhat less informative. Nevertheless, there is much valuable information to be gained from this technique. The position of the absorption edge depends on the oxidation state of the metal. As this increases, it is correspondingly more difficult to remove an electron from the core orbitals, so the edge shifts to higher values. This is illustrated most clearly in

figure 8. Figure 9 shows this principle applied to a biological system: the Mn center of photosystem II.

Features in the pre-edge region have been assigned fairly unambiguously in many metal complexes by comparing the predicted intensities for various transitions ($1s \rightarrow 3d$, $1s \rightarrow 4s$, $1s \rightarrow 4p$) with those observed experimentally.⁸ As well as the edge shift, there are pre-edge features which are potentially indicative of symmetry-related molecular orbital features. The $1s \rightarrow 3d$ transition is formally forbidden, but is often seen as a sharp "blip" prior to the edge, and is attributed to $d-p$ mixing. Tetrahedral complexes have significant $d-p$ mixing, thus in these complexes the $1s \rightarrow 3d$ transition. The opposite is the case for octahedral complexes, which are highly symmetric and there is little $d-p$ mixing. This is illustrated fairly dramatically with experiments of an Fe-S cluster (the F_x center) from photosystem I. Figure 10 shows that there are four transitions

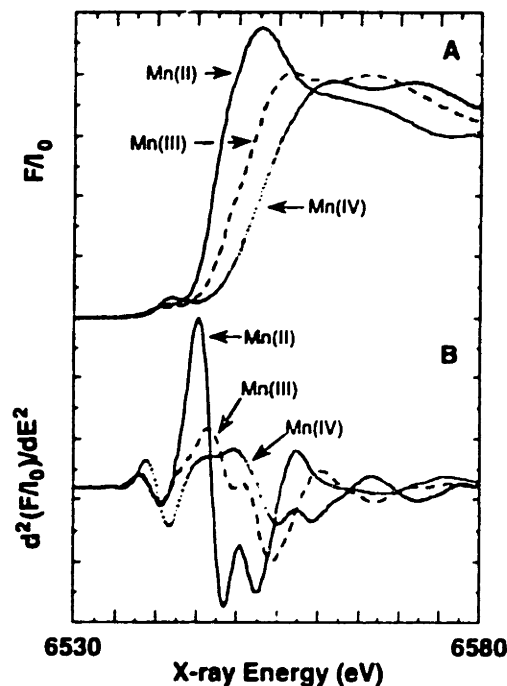


Figure 8: K-edge spectra of Mn compounds of various oxidation states.

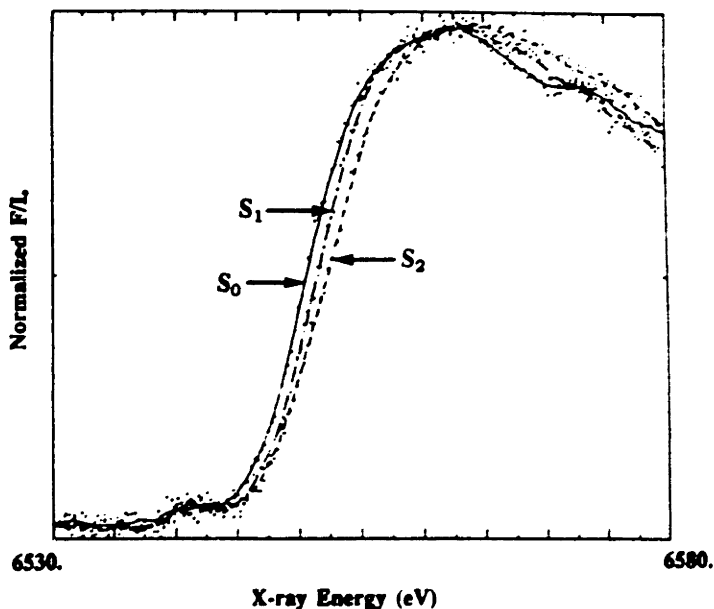


Figure 9: Mn K-edge spectra of photosystem two samples in the S_0 , S_1 and S_2 states.

resolved near the K edge: the first is assigned to $1s \rightarrow 3d$, the second as $1s \rightarrow 4p$ and the last two remain unassigned. The $1s \rightarrow 3d$ transition is all but absent in the heat-denatured sample (which contains mostly octahedral hydrated Fe^{2+}). It is clear that the F_x center (a) exhibits similar transitions to the synthetic $[4Fe-4S]$ cluster $(Et_4N)_2Fe_4S_4(S-benzyl)_4$, (c) so this data is interpreted (along with EXAFS data in the same publication) as being consistent with the F_x center being a $[4Fe-4S]$ cluster.

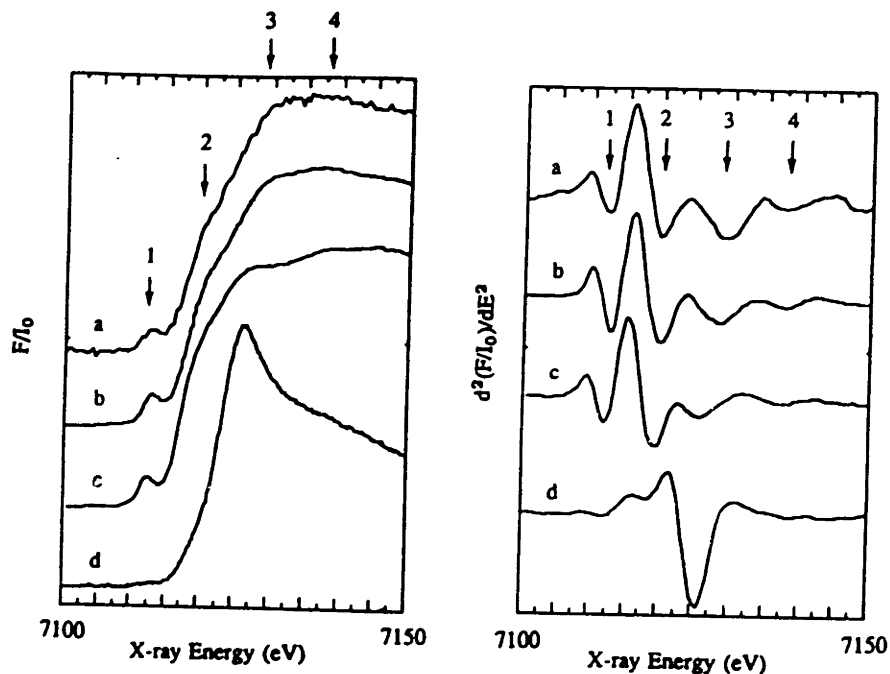


Figure 10: From K-edge spectrum(left) and second derivations(right) for:
 (a) Photosystem I core preparation containing Fx protein,
 (b) $(Et_4N)_2 Fe_4S_4(S\text{-benzyl})_4$,
 (c) Photosystem I complex containing Fa, Fb and Fx cluster [from A.E. McDermott, V.K. Yachandra, R.D. Suites, K. Sauer, M.D. Kleir, K.G. Parrett and J.H. Golbeck Biochemistry 28 8056 (1989)]

3.2 X-ray Spectroscopy Studies of Nitrogenase.

Nitrogenase has been studied extensively by EXAFS spectroscopy: the Fe protein⁹, the MoFe protein (Mo K edge^{10,11}), isolated FeMo-co (Mo K edge,^{12,13,14} Fe K edge^{15,16}), ligand binding to FeMo-co¹⁷, VFe protein (V K edge^{18,19,20}). More recently, the difficult problem of Fe EXAFS in MoFe²¹ and VFe²² proteins (where the solution of EXAFS is complicated by the presence of two FeS centers) has been published. This is by no means a comprehensive list of the EXAFS studies that have been carried out on nitrogenase over the years. Here, the results for the MoFe protein will be summarized, since the results obtained for the Fe protein are less unusual (the Fe protein contains a [4Fe-4S] cluster, which does not change significantly upon oxidation or nucleotide binding). Fe and Mo K edge results are summarized in table 3.1

These results are readily interpreted by reference to the structural models for the metal centers of Rees et al²³, and Bolin et al²⁴. Here some of the more interesting results will be highlighted. Fig 11(a-c) shows how the Mo results are consistent with the structure of the FeMo cofactor. This is exciting when one sees the agreement between what was expected from EXAFS data and what has been confirmed through X-ray crystallography.

Table 3.1: Atom-atom Distances From EXAFS of the MoFe Protein of Nitrogenase.

Component	Amplitude N (N') ^a	Distance R(Å)
Fe-O(N)	0.5 ^b	1.842
Fe-S	3.5 ^b	2.317
Fe-Fe	2 ^b	2.641
Fe-Mo	0.3	2.732
Fe-Fe'	1.3 (2.8)	3.81
Fe-S'	1.2 (1.7) ^c	3.86
Fe-S''	0.8 (1.7)	4.29
Fe-Fe''	0.3 (0.6)	4.7
Mo-O(N)	1.5	2.15
Mo-S	3.5	2.34
Mo-Fe	3	2.70
Mo-Fe'	3	~5

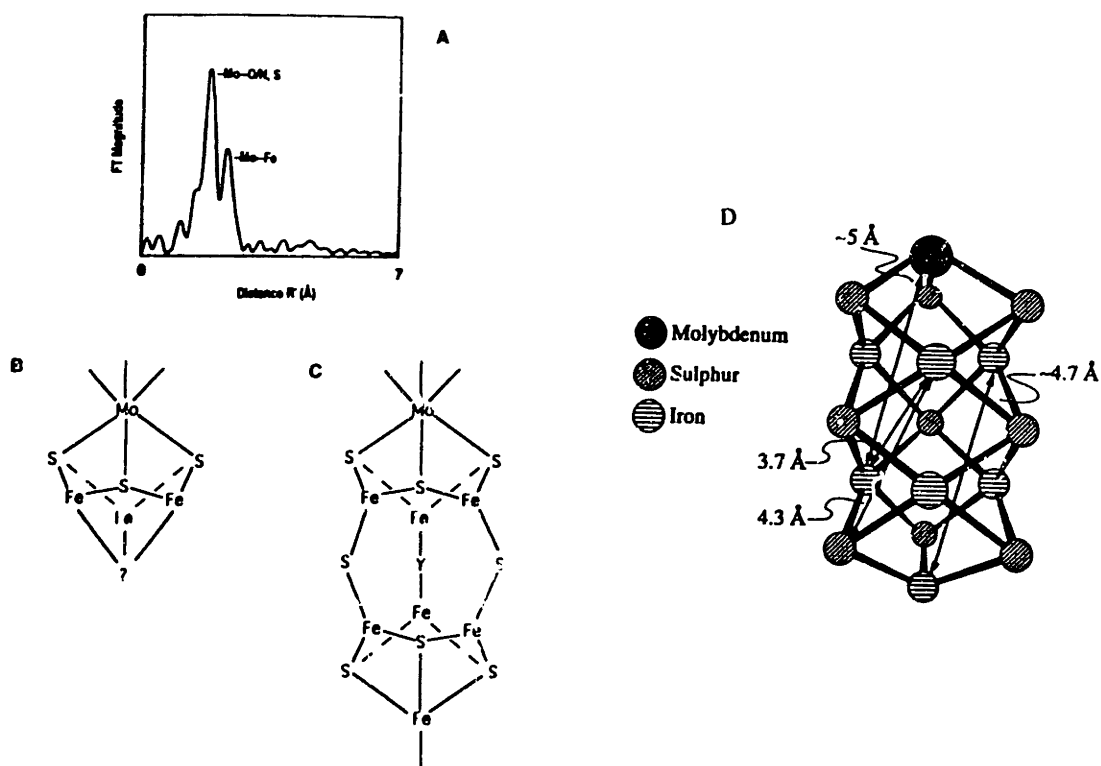


Figure 11: (a) Fourier transform of Mo K-edge EXAFS of nitrogenase MoFe protein. (b) Model derived from EXAFS data. (c) Model of FeMoco from X-ray crystallography [from V.K. Yachandra, *Methods Enzymol* 246, 638 (1995)] (d) Model for FeMo-co consistent with long range Fe-Fe and Mo-Fe interactions observed by EXAFS.

Another particularly interesting set of observations are those seen by the recent Fe EXAFS studies. Here it was noted that there was a set of Fe-Fe distances of ca. 2.61 Å (which was shorter than the previous X-ray crystallographic value of 2.83 Å) and a set of Mo-Fe distances of 2.7 Å (shorter than the X-ray value of 2.83 Å). These experiments led to the refinement of the FeMo-co structure to a more compact and symmetrical model. The further interesting point was the observance of long-range interactions: Mo→Fe of 5.1 Å, and Fe→Fe of ~5 Å. Figure 11(d) shows an unusual model for the MoFe-cofactor and how some of these long-range interactions might be related to this structure.

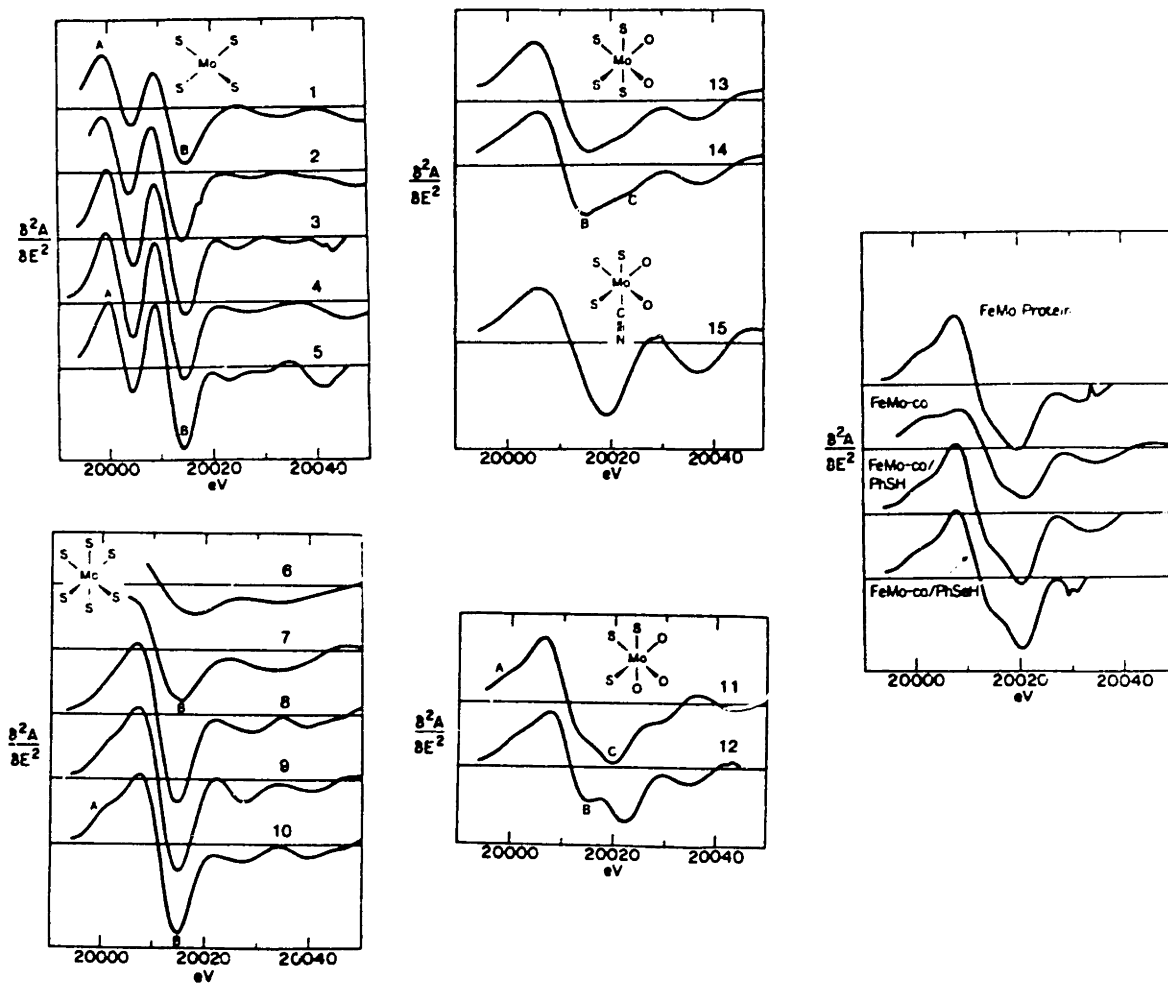


Figure 12: X-ray Absorption Near-Edge Structure (2nd derivative) for a variety of Mo compounds showing strong dependence of the spectra upon Mo co-ordination environment. XANES for MoFe protein, FeMo-co, FeMo-co + PhSH, and FeMo-co + PhSeH shows the environment of Mo in nitrogenase is similar to MoS₃O₃-type compounds.

As well as EXAFS studies, XANES also played a part in the interpretation of the nature of the Mo environment within the MoFe protein, in isolated FeMo-co, and in FeMo-co to which thiophenol had been added. Conradson et al²⁵ looked at the XANES for a range of Mo compounds of known structure and compared these results with those obtained for the FeMo cofactor, and for the native MoFe protein. These results are shown in figure 12. XANES spectra are displayed as the double derivative as this allows fine structure to be more readily determined. It is quite clear from these results that the Mo environment in the MoFe protein most closely resembles that of a 'MoS₃O₃' complex, which is shown to be validated by the crystal structure of nitrogenase. These results confirm the facility of XANES as a technique for analyzing the metal centers within metalloenzymes.

3.3 X-ray Absorption Spectroscopy of the CO-inhibited Forms of Nitrogenase: High vs Low CO.

3.3.1 Experimental Procedures.

Sample Preparation: Nitrogenase MoFe and Fe proteins were purified from *Azotobacter vinelandii* frozen cell paste as described in the appendix. MoFe protein that had previously been concentrated to 120 mg/ml in "turnover mix" (see chapter two: "turnover mix" = 50mM MgCl₂, 100mM Na₂ATP, 300mM Na-[P-creatine], 100mM HEPES, 100mM Na₂S₂O₄, 2 mg/ml creatine kinase, pH=7.4) was diluted to 30 mg/ml (with turnover mix). 3ml serum-stoppered Wheaton vials (containing magnetic stir bars) were purged of O₂ by repeated evacuation and backfilling with Ar, then after final evacuation, the vial was filled with CO gas to the desired concentration. For "Hi CO" samples, [CO] was 0.5 atm; for "Lo CO" samples, [CO] was 0.08 atm, as had previously been determined to be optimal by EPR studies. A "No CO" sample was prepared in an open vial inside the glovebox (with identical protein solutions). The atmosphere in the Wheaton vials was purged of any possible O₂ contaminants inside the glovebox (≤ 2 ppm O₂) by injecting 1ml of 0.1M Na₂S₂O₄ in 0.2 M Tris buffer (pH=7.4) and allowing the solution to equilibrate for ~5min. This solution was removed by syringe, and the MoFe protein in turnover mix was injected (300 μ l) into the vial, and allowed to equilibrate with the CO gas in the headspace (stirred for ~20min). During this time, the isopentane bath in the glovebox was cooled to -40 $^{\circ}$ \rightarrow -80 $^{\circ}$ C. At the appropriate time, sufficient Fe protein to give a 1:1 ratio of Fe/MoFe protein was added (28 μ l of 80 mg/ml Fe protein). Vigorous frothing due to H₂ evolution indicated that turnover was initiated. After a period (determined in earlier EPR experiments to find the time for optimal EPR signals) of 3 min (for low CO samples) or 4 min (for high CO samples) ~100 μ l samples were removed and quickly transferred to lucite sample holders that were sealed with Kapton tape. Samples were immersed in cold isopentane as rapidly as possible (ca 15s) and dropped through the sample port, out of the glovebox, and placed immediately into liquid N₂.

Measurement of XANES Spectra: X-ray absorption data was collected at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory, Upton, Long Island New York, at beamline X10C. Data was collected as X-ray fluorescence using Ge solid-state detectors. At least 12 scans, each of approximately 45 minutes duration, were made for each sample, and the data was corrected for detector deadtime non-linearity and normalized by Brenda Weiss at UC Davis.

3.3.2 Results

Results at this stage are preliminary: samples have yet to be checked for integrity after exposure to the X-ray beam. However, there does appear to be a significant difference in the edge energy for the two low CO samples. Figure 13 shows the near-edge X-ray absorption spectra for a "hi CO" sample, duplicate "lo CO" samples and a sample of nitrogenase turning over ("reduced") in an inert atmosphere. The high CO sample appears to be similar in X-ray absorption properties to the "reduced" enzyme (steady state), while it appears that the low CO samples are even further reduced.

3.3.3 Conclusions

When it was shown that nitrogenase produces unusual EPR signals during H₂ evolution under CO²⁶, it was postulated that the "low CO" signal ($g_{av} \approx 1.98$) originated at a cluster in a reduced state (somewhat like a reduced ferredoxin), while the "high CO" signal ($g_{av} \approx 2.08$) resulted from a more oxidized form of the same cluster. Results recently published²⁷ show that both signals do indeed appear to originate from unpaired electrons in the same cluster. As seen in chapter two, ¹³C ENDOR shows that there are two CO molecules bound in the high CO state, and one in the low CO state. Patricia Christie²⁸ showed (using MoFe protein selectively labelled with ⁵⁷Fe at the P-clusters) that apparently the CO-induced EPR signals originate at the FeMo cofactor. It seems feasible to interpret these results in terms of a high affinity and a low affinity binding site, at the FeMo cofactor. In order to react with CO, the enzyme must be in the reduced state. *Thus CO only binds the enzyme during turnover.* Brian Hales proposed²⁹, using a kinetic argument, that the state that CO binds to is the E₃ or the E₄ state of Thorneley and Lowe's mechanism. Perhaps both CO molecules bind to the same state of the enzyme. The binding of one CO (to the *high* affinity site, which can occur under low partial pressures of CO) traps the M-center in a highly reduced state (though more oxidized than the state to which the CO bound, since the binding of CO is liable to remove some electron density from the metal cluster due to $d \rightarrow \pi^*$ back-bonding). This would explain why the Fe edge data shows that the low CO state is more reduced than "reduced" (E₁) protein. Binding of a second CO to the *low* affinity site (which can only occur when the pCO is sufficiently high) further withdraws electron density from the M-center, to a slightly more oxidized state. Alternatively, the enzyme cycles around to a more oxidized state, to which the second CO binds. This however, seems less likely (though it is certainly possible) because it seems that CO dissociation would occur during such a cycle. CO does not bind to nitrogenase in a tight fashion, but rather transiently.

3.4 Suggestions for Further Experiments.

The binding of CO to reduced states of nitrogenase MoFe protein appears to keep the enzyme temporarily in a more-reduced state. Evidence from ⁵⁷Fe ENDOR indicates that the CO binds at the M-center, a result that in hindsight is not that surprising when one considers the work of Dean and coworkers, who showed that CO acts as inhibitor of N₂ binding (a situation that is accentuated in the case of the His195Gln mutant). The question remains as to *how* CO inhibits N₂ binding. As discussed in chapter two, it is feasible that CO binds analogously to N₂. Unfortunately, there appears (at this stage) to be no obvious way to test this hypothesis.

If the CO-binding site is the N₂ binding site, then it appears a very short step to answering the age-old question: does N₂ bind and react at Mo, or at Fe? Mo K-edge

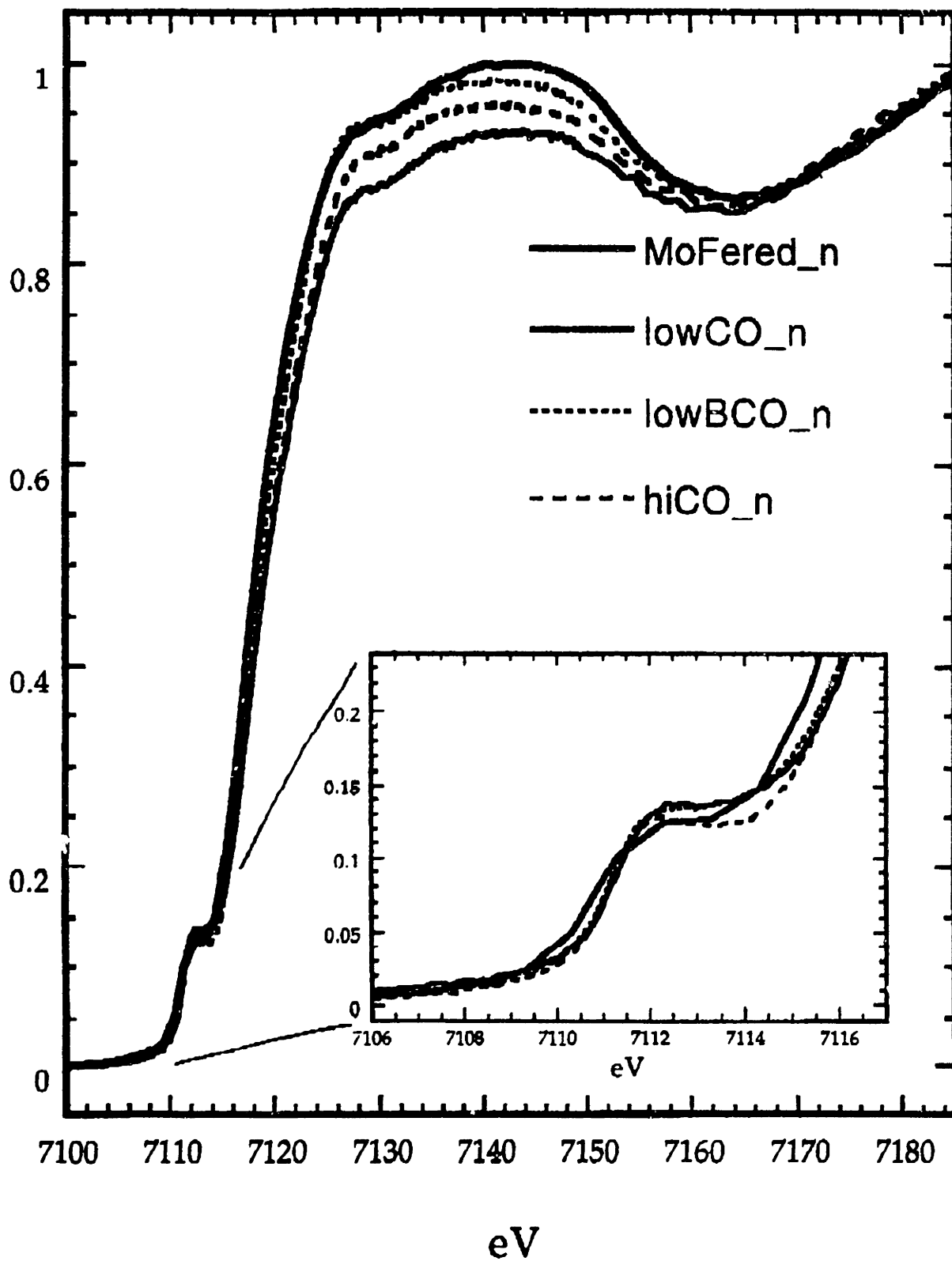


Figure 13: Fe K-edge Absorption Near-Edge Structure of CO-inhibited nitrogenase. Top two curves: “low CO” form; middle curve “high CO” form; bottom curve “reduced MoFe protein” during steady-state turnover.

XANES and EXAFS experiments should be able to clearly discern CO binding at Mo, if indeed this is happening. These experiments are currently being planned.

A further question is related to the strength of CO-binding to nitrogenase. This question can be answered by analysis of the bond-stretching frequency of CO bound to nitrogenase. This can be addressed by looking at the FTIR spectra of CO-inhibited nitrogenase, and conducting isotopic substitutions, e.g. ^{13}CO and C^{18}O . Similar work using FTIR to investigate CO binding has been done with Ni CO dehydrogenase.³⁰

The chemistry of CO-binding to nitrogenase is an extremely useful handle to understand the general N_2 binding chemistry of nitrogenase. It is certain that there is much more to be gleaned from further studies in this area.

If CO inhibits binding by physically blocking the N_2 binding site, then it appears that characterization of the CO-binding site is of utmost importance, because in so doing one would implicitly characterize the N_2 binding site. *However*, it is unfortunately not immediately apparent that CO inhibits N_2 binding in this way. It is *likely*, because CO is isoelectronic with N_2 , and it seems that a state of the enzyme that is predisposed to binding N_2 would certainly be predisposed to binding CO. If the state of the enzyme with N_2 bound is in any way electronically similar to that with CO bound (low CO), it seems that it might be possible to trap (by freeze quench experiments) sufficient quantities of this state (which would be EPR active) to observe nitrogen bound to the enzyme by ^{15}N ENDOR spectroscopy.

X-ray absorption spectroscopy has been intimately connected to the study of nitrogenase for almost 30 years now. The future will hopefully continue to bring forth exciting and novel results through studies of nitrogenase using the technique of X-ray absorption spectroscopy.

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Appendix

Appendix A: Protein Purification

Spectroscopic experiments of the kind outlined in this thesis necessitate the use of large amounts of protein in a relatively pure form. Purification of gram quantities of the MoFe protein of nitrogenase has been routinely carried out in this laboratory. The process of bacterial fermentation and purification of the component proteins has been exhaustively described by David Wright (1) and by Patricia Christie (2). A brief outline of the procedure is as follows:

(a) Growth of Bacteria.

Klebsiella pneumoniae and *A. vinelandii* can both be readily grown in 50 L fermentations. The bacteria is grown to an optical density (absorbance at ~600nm) of around 2.0. At this stage the bacteria are harvested and resuspended in nitrogen-free (i.e. no *fixed* nitrogen) media. The bacterial metabolism is extremely sensitive to the amount of ammonium ion in the cell. In the absence of a fixed nitrogen source, the production of *nif* gene products (including nitrogenase) is stepped up. Bacteria are harvested at an O.D. of ~3. Typical yields are ~200g of packed cell paste for a 50 L fermentation.

(b) Purification

The cells are suspended in buffer (pH = 7.4) and passed through a french press to lyse the cells. At this stage, it is absolutely essential that all procedures be carried out strictly anaerobically, as the two components of nitrogenase are readily inactivated by O₂. After lysing, the cells are transferred to centrifuge tubes (well-sealed!!) inside a glove box. The atmosphere inside the glove box is carefully monitored and kept at or below 2ppm O₂, and all solutions contain Na₂S₂O₄, which scrubs any residual oxygen from the solutions. After removal of extraneous solid material (e.g. cell walls, DNA, etc.) the supernatant solution is loaded onto a column of DEAE-cellulose (DEAE = diethyl-ammonium ethyl), which is an anion exchanger. Elution of proteins by a salt gradient effects a purification, with MoFe protein eluting first (salt concentration around 0.25 M), and Fe protein second (salt concentration around 0.35M).

¹ Wright, D. W., PhD. Thesis, Massachusetts Institute of Technology, February 1994

² Christie, P. D., PhD Thesis, Massachusetts Institute of Technology, February 1996.

A. vinelandii MoFe protein is further purified by “salting in”: lowering the salt content of a concentrated solution of impure MoFe protein causes the MoFe protein to crystallize (at a salt concentration of ca 40mM salt),

The Fe protein can be purified by anaerobic size exclusion (or gel filtration) chromatography. Small volumes of concentrated Fe protein are injected into the top of a ca 2m long column, and eluted slowly overnight. The Fe protein separates from the other colored contaminants: blue flavoprotein, and brown MoFe protein (which elutes first). Usually the first 2 or 3 fractions of Fe protein are contaminated with a “background” of MoFe protein activity and must be discarded (minimal lost) or repurified.

Yields of MoFe protein are typically around 2-3 mg for every g of cell paste. About 50 mg of protein is more than sufficient for an ENDOR or ESEEM experiment.

Appendix B: Freezing Samples in Glovebox

In order to prepare EXAFS and ENDOR samples of CO-inhibited MoFe protein, a protocol was devised that enabled samples to be frozen inside the glovebox and removed quickly and easily before the samples thaw.

A tank of nitrogen gas, with regulator attached, is hooked up to a coil of copper refrigerator tubing which is immersed in a large glass dewar. The nitrogen gas flows from the regulator, through the tubing, into liquid N₂, and becomes chilled. The chilled N₂ gas, flows through insulated tubing into the glovebox, through another coil of copper tubing which is immersed in isopentane. It is essential that this be degassed (by purging with Ar gas) thoroughly before being brought into the glovebox (<2ppm O₂ inside). The isopentane bath takes about 30 minutes to an hour to cool sufficiently to freeze samples, but eventually (as long as one is vigilant to watch the level of N₂ gas in the tank and of liquid N₂ in the dewar) it will cool down to -80°C.

Once samples are frozen, they are removed from the glovebox through a hole in the glove box to which a 1/2" NPT nipple is attached. At the other end, a specially bought ball valve with bore ID exactly that of NPT pipe. Samples are dropped through the hole in EPR tubes (still frozen). With the glovebox overpressurized, the ball valve is quickly opened, and the sample caught in a gloved hand and immediately placed into liquid nitrogen.