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MECHANISM OF NITROGEN FIXATION:
USE OF CARBON MONOXIDE TO PROBE NITROGENASE ACTIVITY

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
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Doctor of Philosophy

in
The Department of Chemistry

by
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ABSTRACT

The inhibitor CO was used to investigate the mechanism of Mo and V nitrogenase.

While low pressures of CO enhanced, high pressures inhibited V nitrogenase-catalyzed C₂H₆ formation. This is not the case for C₂H₄ formation, where the rate of electron flux determined if CO acted as an enhancer or inhibitor. A two-site model is proposed, in which CO binding to one site enhances, and CO binding to both sites inhibits product formation.

Replacement of CO(g) by Ar or C₂H₂ resulted in correlated decay of hi-CO and development of lo-CO EPR signals, suggesting that the two signals arise from one metal cluster. The detection of CO binding to the lo-CO form of the enzyme in the absence of turnover suggests that CO stabilizes a MoFe protein cluster or turnover state and enhances binding of additional CO molecules.

¹³CO and ⁵⁷Fe ENDOR studies of Mo nitrogenase in moderate flux demonstrated that (i) lo-CO Mo nitrogenase has one bound CO molecule and hi-CO enzyme has two; (ii) the second CO binds to the same cluster that has one bound CO; and (iii) CO binds to the FeMo cofactor and not to the P cluster.
CHAPTER 1 INTRODUCTION

1.A NITROGEN FIXATION

1.A.1 Biological Nitrogen Fixation

Plants require nitrogen for growth and structure. Molecular nitrogen (N\textsubscript{2}) is abundant in the earth's atmosphere but it cannot be metabolized by plants. The process in which N\textsubscript{2} is converted into NH\textsubscript{3} (ammonia), a usable form of nitrogen for plants, is called nitrogen fixation.

Industrially, the Haber-Bosch process for nitrogen fixation is highly endothermic. Biological nitrogen fixation occurs at ambient temperatures and pressures. Nitrogenase is the only known biological catalyst for the reaction N\textsubscript{2} → NH\textsubscript{3}. Nitrogenase activity in free-living soil bacteria and other micro-organisms contributes approximately 60\% of the natural world's fixed nitrogen. To understand how nitrogenase converts N\textsubscript{2} into NH\textsubscript{3} remains the goal of investigations across a number of disciplines.

It is worthwhile to examine the relationship of biological nitrogen fixation to human life today.

The human population of the world is projected to double by the early decades of the twenty-first century. One important reason to study nitrogen fixation is to better understand and design strategies for increasing food production (Postgate, 1990). Human food largely derives

1
from plants. Crops deplete the soil of nitrogen; natural, biology-based processes\(^1\) (which recycle existing nitrogen in the biosphere and can supply 80% of plants' needs) do not replace nitrogen fast enough, today, to meet the demand for sustained high yields of crops.

Today, ~60% of new nitrogen comes from biological nitrogen fixation, and ~30% from chemical fertilizers. Use of chemical fertilizers, which are prepared industrially from atmospheric N\(_2\), has resulted in increases in food production. However, soil nitrogen must increase by 12% annually to counter wastage (Postgate, 1990).

Even if traditional methods were re-introduced in places where their use has given way to the use of chemical fertilizers, the gain in nitrogen wouldn't be enough to meet the present demand. Human population growth will depend on creative adoption of strategies that increase the supply of nitrogen-containing compounds in soils.

One approach, transfer of bacterial nitrogenase genes into plants, has a low chance of success at the moment because the nitrogenase gene system is very complex. However, strategies such as selection of nitrogen-fixing strains of bacteria that are capable of growth on the roots

\(^1\) Biology-based methods of supplying nitrogen to the soil include crop rotation, growing of self-fertilizing crops (soya beans, peanuts), intercropping with nitrogen-fixing plants, and recycling of manure composed of nitrogen-fixing plants and cyanobacteria) (Postgate, 1990; Gilliland, 1988).
of cereal crops, for example, may be more feasible at present for improving soil nitrogen content (Gilliland, 1988; Anderson, 1990; Brill, 1995).

1.A.2 Nitrogen-Fixing Bacteria

Biological nitrogen fixation is the process whereby atmospheric dinitrogen \((N_2)\) is reduced to ammonia \((NH_3)\) by certain bacteria and a few plants. Of the \(2 \times 10^8\) metric tons of nitrogen fixed annually on earth by biological and nonbiological processes, nitrogen fixation by bacteria accounts for \(\sim 60\%\) (Postgate, 1982). The bacteria which fix nitrogen include aerobes, anaerobes, symbionts, and free-living species. All known nitrogen-fixing bacteria synthesize nitrogenase, the enzyme responsible for the organism's nitrogen-fixing ability.

Cyanobacteria are free-living nitrogen-fixing bacteria that live by nitrogen fixation and photosynthesis; nitrogen-fixing soil bacteria such as Azotobacter and Klebsiella use organic food. Rhizobia (and a few genera of soil bacteria) are nitrogen-fixing bacteria which live in a symbiotic relationship with leguminous plants (such as peas, lupins, and clover) and a few other plants. Rhizobia cause the roots of a host plant to form nodules, and inside the nodules the bacteria live by nitrogen fixation. Nitrogen fixing plants include the water "fern" Azolla and trees such as alder and Leucaena (Postgate, 1990).
1.A.3 Brief History of Research on Nitrogenase

Biological nitrogen fixation was identified empirically over a century ago (Hellriegel & Wilfarth, 1888). In the first half of the twentieth century, researchers studied biological nitrogen fixation in living organisms. For example, in one study, the growth of rhizobia-inoculated plants in a controlled N\textsubscript{2} atmosphere was compared to the growth of non-inoculated, nitrogen-supplemented plants. Growth was measured as the total nitrogen content of the plants. Nitrogen fixation increased linearly with increasing P\textsubscript{N\textsubscript{2}} up to ~10 kPa of N\textsubscript{2}, beyond which there was no relationship; these results implied saturation of a nitrogen-fixing system (Wilson, 1936). Similar studies of rhizobia-inoculated plants in CO-containing N\textsubscript{2} showed that nitrogen fixation was significantly inhibited by 0.01% of CO (Lind & Wilson, 1941).

The microbiologist Bortels observed that Azotobacter vinelandii required Mo and, to a lesser extent, V, for growth and nitrogen-fixing ability (Bortels, 1930, 1936).

Research on nitrogen fixation was restricted to study of living cells because the isolated nitrogen fixing enzyme system is labile. One attendant problem, however, was the difficulty of isolating intermediates and products of nitrogen fixation from the plants (Wilson, 1936).
In 1960 Carnahan and associates published details of methods for obtaining cell-free extracts of a nitrogen fixing enzyme system (Carnahan et al., 1960). For the first time, N₂ fixation could be studied reproducibly in vitro. The researchers stressed that critical attention must be paid to the method of cell breaking, addition of a source of energy such as pyruvate, control of pH, and maintenance of strictly anaerobic conditions.

The utility of the method was soon proven. At that time the end-product of nitrogen fixation was widely believed to be ammonia. Evidence that supported this hypothesis came with the observation that cell-free extracts of the nitrogen-fixing bacterium Clostridium pasteurianum quantitatively converted ^15N₂ into ^15NH₃ (Carnahan et al., 1960).

Soon after, the requirement for ATP (Bulen et al., 1964) and the suitability of dithionite (S₂O₄²⁻) (Bulen et al., 1965) or reduced ferredoxin (Mortenson, 1964) as a source of electrons for nitrogen fixation in cell-free systems, was established.

1.B NITROGENASE STRUCTURE
1.B.1 Nitrogenase Structure, Overview

All nitrogen-fixing organisms have a Mo- and Fe-containing nitrogenase. Its synthesis, functioning and regulation are directed by a complex genetic system; in Klebsiella pneumoniae, for example, the nitrogen-fixation
gene cluster (nif) consists of 21 distinct genes. Some nitrogen-fixing organisms also have genetic systems for alternative nitrogenases. In Mo-limiting conditions, in these organisms, the genes for V nitrogenase (vnf) are expressed, and in Mo- and V-limited conditions the Fe nitrogenase genes (anf) are expressed (Eady, 1991; Lowe, 1996). Each type of nitrogenase has its own genetic machinery.

Nitrogenase consists of two component proteins, the Fe protein and the MoFe protein. Both are metalloproteins. The Fe protein, the smaller of the two, transfers electrons to the MoFe protein; the MoFe protein has the active site (Burris, 1991; Newton, 1992). X-ray crystal structures of the proteins and their metal clusters (Georgiadis et al., 1992; Kim & Rees, 1992b; Chan et al., 1993) stir the imagination as to how the enzyme might work. Studies of mutant nitrogenases with site-specific alterations in critical amino-acids have aided in defining catalytic roles for the metals and proteins (Dean et al., 1993).

Nitrogenase catalysis is characterized by these unique features: (i) the MoFe protein is activated when the Fe protein, MgATP and a source of electrons are present; (ii) donation of an electron by the Fe protein to the MoFe protein depends on hydrolysis of MgATP bound to the Fe protein; and (iii) H₂ evolution accompanies substrate reduction. Two major questions are: (i) how is MgATP
hydrolysis coupled to electron transfer, and (ii) how does substrate reduction occur?

The following section describes the structure of the two nitrogenase proteins and how the proteins interact to catalyze a reaction.

Reviews of the structure and functioning of nitrogenase are available (Burgess & Lowe, 1996; Dean et al., 1993). The alternative nitrogenases have been reviewed recently (Lowe, 1996; Pau, 1989; Eady, 1991).

1.B.2 Fe Protein

The primary function of the Fe protein is to donate an electron to the MoFe protein; no other known substance can induce catalytic activity in the MoFe protein. In addition, the Fe protein is necessary for synthesis of the FeMo cofactor, insertion of the formed cofactor into apoMoFe protein (cofactor-less MoFe protein), synthesis of functional MoFe protein, and possibly, regulation of alternative nitrogenase systems.

The Fe protein is the nifH gene product. The DNA sequences of nifH from different nitrogen-fixing organisms are highly conserved (Eady & Leigh, 1994). In addition, the Fe protein amino-acid sequence is highly conserved, being found in nine Fe proteins from different organisms. This may explain why, in some cases, the Fe protein from one organism can substitute for the Fe protein from another
species and complement the MoFe protein, with little loss of activity.

The Fe protein is a homodimer of $M_r \sim 60,000$ with a single $[\text{Fe}_4\text{S}_4]^{2+/1}$ cluster positioned between the two subunits (like an "iron butterfly" with cluster as head, Georgiadis et al., 1992). The FeS cluster is attached to the protein by sulfhydryl ligands from cys97 and cys132 on each subunit bonding to Fe atoms. The cluster is located near the apical end of each subunit and may be exposed to solution. Nearby on the top surface of the protein are residues arg100 and glu112 which are known to interact with the MoFe protein. ADP ribosylation of arg100 is involved in regulation of nitrogenase synthesis. Chemical cross-linking of the Fe protein to the MoFe protein specifically involves glu112 of the Fe protein (Willing et al., 1989; Willing et al., 1990).

The actual oxidation state of the FeS cluster is not known, but comparison of $S = 1/2$ EPR signals of the Fe protein and other FeS proteins suggests that the cluster has a $+2$ charge in the oxidized protein and a $+1$ charge in the protein as isolated in dithionite. The Fe protein FeS cluster can apparently be reduced to the 0 oxidation state by reductants other than dithionite (Watt & Reddy, 1994). In the absence of added reductant the Fe protein is extremely oxygen sensitive, a property that may be due to
the position of the cluster close to the protein's external surface.

The Fe protein has a MgATP/MgADP binding site on each subunit; the site is located ~20 Å from the metal cluster and ~20 Å from the corresponding site on the other subunit. The X-ray crystal structure of the Fe protein shows that one MgADP molecule may occupy one of the nucleotide-binding sites. Sequence analysis reveals that the Fe protein subunit has core regions of sequence homology to other nucleotide-binding proteins; these regions include the Walker A and B motifs, which bind the phosphates and Mg$^{2+}$ of triphosphate nucleotides (Story & Steitz, 1992). Furthermore, the 3-D structure of the Fe protein is similar to that of the GTPase p21$^{ras}$. The Fe protein shows functional similarities to some nucleotide-binding "switch proteins": (i) the action (change of state) of the protein is assisted by nucleotide hydrolysis, and (ii) nucleotide hydrolysis requires or is aided by formation of a multiprotein complex (Pai et al., 1990).

In active nitrogenase two MgATP molecules bind to the Fe protein. Transfer of one electron from the Fe protein to the MoFe protein is accompanied by hydrolysis of two MgATP molecules [both component proteins are required for ATP hydrolysis (Bulen & LeCompte, 1966)]. The protein also binds MgADP; MgADP is a potent inhibitor of nitrogenase catalysis.
The binding of MgATP apparently alters the conformation of the Fe protein and destabilizes the FeS cluster. The binding of MgATP: (i) lowers the redox potential of the Fe protein from $-294$ to $-400$ mV (NHE); (ii) alters the $S = 1/2$ EPR signal from a rhombic ($g=2.06/2.05, 1.94, 1.84/1.85$) to an axial ($g=2.04, 1.93$) signal; (iii) promotes chelation of the cluster by $\alpha,\alpha'$-bipyridyl (i.e. increases the lability of the Fe); (iv) changes the absorbance at 430 nm; (v) changes the CD of the protein; (vi) increases the sensitivity of the protein to oxygen; and (vii) alters the reactivity of SH groups on the protein.

One useful strategy for examining how communication might occur between the MgATP/MgADP binding site and the FeS cluster in the Fe protein has been to construct mutants that have substitutions in selected amino-acids thought to be located along that route. Mutants with substitutions for lys15 and leu127, for example, show altered MgATP hydrolysis, electron transfer, and Fe chelation properties, supporting the hypothesis of a signal transduction mechanism (Ryle et al., 1995; Lanzilotta et al., 1996).

1.B.3 MoFe Protein

The function of the MoFe protein is to catalyze the conversion of $N_2$ (substrate) into $NH_3$. To do so, the MoFe protein must receive electrons from the Fe protein.
Concomitant with substrate reduction, the MoFe protein produces H₂.

Structural models of the *A. vinelandii* and *C. pasteurianum* MoFe proteins and their metal clusters have recently been determined from x-ray crystallography studies (Kim & Rees, 1992a,b; Bolin et al., 1993; Chan et al., 1993; Kim et al., 1993).

The MoFe protein is a heterotetramer (α₂β₂) of Mr ~220 000; α and β proteins are the gene products of nifD and nifK, respectively. Each αβ pair is considered to be a functional unit. The α and β polypeptides show considerable sequence homology. Two kinds of metal clusters are found in the MoFe protein. The P cluster, [Fe₈S₇-e], is located at the interface of α and β subunits. The FeMo cofactor (M center, FeMoco), MoFe₇S₉:homocitrate, is located in the α subunit ~14 Å from the P cluster. Both metal clusters lie ~10 Å below the protein surface. The proteins lack channels for the passage of electrons, protons, or substrates to the metal centers.

The αβ pairs in the tetramer are related by the molecular two-fold rotation axis. The α and β subunits are also somewhat related by a two-fold rotation axis that passes through the P cluster and between the α and β subunits. Two wide, shallow clefts exist on either side of
this axis. Within the tetramer the αβ pairs are in closest contact between their β subunits. A site for a Mg$^{2+}$ or Ca$^{2+}$ ion is buried between the two β subunits and may stabilize the tetramer. Neither the subunits of the MoFe protein nor those of the Fe protein have been purified separately.

Until recently the structure of the P cluster was not known. Considerable evidence suggested that the eight-iron cluster was, in fact, a pair of [Fe$_4$S$_4$] clusters. The recent x-ray crystal structures show that both models are correct.

The P cluster is composed of two linked [Fe$_4$S$_4$] cubane clusters, one in each protein subunit (Figure 1.1). Currently there are two hypotheses regarding how the two cubanes are linked: (i) by two bridging sulfides plus a disulfide bond between two sulfur atoms of the two cubanes (Chan et al., 1993; Kim et al., 1993); and (ii) by the two cubanes sharing one corner sulfur atom, which would be six-coordinate (Bolin, in Orme-Johnson, 1992). The proposed disulfide bridge is on the same side of the P cluster as the Fe protein binding site (Kim & Rees, 1992b). The P cluster is attached to α and β proteins by six sulfhydryl ligands from cysteines and one oxygen atom from a protein serine; the Fe atom liganded by the oxygen is pentacoordinate. The residues coordinating the P cluster are strictly conserved. The amino-acid residues in the
Figure 1.1  P cluster. Schematic representation of a P cluster in MoFe protein of A. vinelandii. Open circles, S atoms; shaded circles, Fe atoms; solid circle, O atom. Adapted from Eady & Leigh, 1994.
immediate environment of the P cluster are mostly hydrophobic in character. Mössbauer data of the as-isolated MoFe protein indicates that the iron atoms in the P cluster are "all-ferrous", suggesting that the cluster has a net oxidation state of 0. A redox reaction at the P cluster, involving electron transfers and a disulfide-sulfide transition, could result in H₂ evolution (Chan et al., 1993; Kim et al., 1993).

As well as the structural genes nifDK, synthesis of functional MoFe protein involves (i) synthesis of the FeMo cofactor on a protein scaffold (nifN and -E gene products); (ii) synthesis of a cofactor-less MoFe protein; and (iii) insertion of cofactor into apoprotein.

The FeMo cofactor is composed of an [Fe₄S₃] and an [Fe₃S₃Mo] cluster bridged by three sulfide ions and capped by the organic acid homocitrate at the Mo end (Figure 1.2). The football-shaped cofactor is tethered to the MoFe protein at opposite ends of the cofactor: the sulfhydryl moiety of cys275 is a ligand to a tetrahedrally coordinate cluster Fe atom, and the imidazole N of his442 coordinates the Mo atom. All the other Fe atoms in the cofactor are trigonally coordinate; Fe-Fe interactions may occur between them. The Mo atom is octahedrally coordinate, with three sulfur atoms from the cluster and two oxygen atoms from homocitrate occupying the other coordination sites. The cofactor sits in a predominantly hydrophilic environment.
Figure 1.2  FeMo cofactor. Representation of the cofactor in A. vinelandii MoFe protein. Symbols: as in Fig. 1; dark ball with white spot, Mo atom; balls attached to homocitrate, O atoms; ball in His442, N atom. Adapted from Eady & Leigh, 1994.
and has several H-bonding interactions with surrounding amino-acid residues, in particular with his195 and gln191; numerous H₂O molecules surround the cofactor [~25 H₂O molecules (Bolin, 1996)]. The cofactor and the P cluster are ~19 Å apart (~14 Å at their closest points).

Cofactor biosynthesis involves the gene products of nifQ, -B, -V, -N, -E, and -H. The cofactor is assembled on a scaffold of the gene products of nifN and -E. "NifBco", an all FeS cluster, is assembled by the gene product of nifB (Shah et al., 1994). NifBco may be the source of Fe for the FeMo cofactor and for the cofactors in the alternative nitrogenases because nifB is required for the synthesis of all cofactors. On the scaffold proteins, nifBco could become fused with an M:homocitrate intermediate (M=Mo, V) to form the cofactor (Wright et al., 1996). The gene product of nifS may play a role in obtaining the sulfur atoms for an FeS cluster (Zheng et al., 1993). The gene products of nifH and nifY are apparently necessary for the insertion of cofactor into cofactor-less MoFe protein. Cofactor-deficient, inactive MoFe proteins are synthesized by strains with mutations in nifB, -N, -E, or -H. These MoFe proteins can be activated in vitro by addition of isolated FeMo cofactor in N-methylformamide (Shah & Brill, 1977).

ENDOR studies detected the presence of five magnetically-distinct irons in the cofactor. Mössbauer
data suggests that the Fe atoms in the cofactor are of two types. These data may represent ferric and ferrous atoms, which could be antiferromagnetically coupled in the cofactor (Orme-Johnson, 1985).

Considerable evidence indicates that the cofactor is the site of substrate reduction (Hawkes et al., 1984; Scott et al., 1990, 1992; Kim et al., 1995). When apoMoFe protein (from a strain, UW45, that produces MoFe protein lacking cofactor) is combined with isolated cofactor, the resulting MoFe protein is active. MoFe protein from a nifV strain has citrate in place of homocitrate, and this protein shows altered substrate reduction properties. MoFe protein containing the "MoFe cluster", a cofactor that lacks homocitrate, is inactive, but MgATP is hydrolyzed. The cofactor may also be a site for H₂ production.

It is unlikely that a substrate would coordinate directly with the Mo atom unless the Mo ligand environment is changed (Kim & Rees, 1992b); for example, substrate could displace the homocitrate ligands (Leigh, 1995a). Substrates may be more likely to bind to metal atoms of low coordination number, such as the trigonally-coordinate Fe atoms (Kim et al., 1993). Suggested binding modes for substrate molecules with the cofactor include "end-on" to a metal atom, "side-on" between two Fe atoms after displacing a bridging sulfide atom, or on the exterior surface of the cofactor where a substrate molecule could simultaneously
interact with up to four Fe atoms (Kim et al., 1993). The "cage" formed by the cofactor may be too small by 0.5 Å to accommodate N₂ inside it, although a theoretical model predicts this could occur (Stavrev & Zerner, 1996). A side-bound N₂ could bond between two Fe atoms (Orme-Johnson, 1992).

Note that the exact oxidation states of the metal clusters in the MoFe protein (or the cluster in the Fe protein) aren't known. In studies of the MoFe protein with redox-active dyes, six electrons can be removed without loss of activity; the first three electrons are removed from the P clusters, and the next three electrons come from the cofactors. Reduction of dye-oxidized MoFe protein supplies three electrons to the P clusters, two electrons to the cofactors, and one electron to an unknown center. The order in which the metal centers are reduced is disputed (Yates, 1992).

There is a need for greater understanding of these aspects of MoFe protein structure and mechanism of action: (i) sites of substrate reduction, H₂ evolution, and CO inhibition; (ii) mechanism of substrate reduction; and (iii) pathway of electron transfer between P cluster and FeMo cofactor.
1.B.4 Model of Coupled MgATP Hydrolysis and Electron Transfer

A little-understood process is, how is electron transfer (from the Fe protein to the MoFe protein) coupled with MgATP hydrolysis?

Electron transfer from the Fe protein to the MoFe protein requires that the proteins form a complex. Few details of the events leading up to electron transfer are known. According to a current model, described below, the FeS cluster of the Fe protein is brought into a position as close as possible to the P cluster of the MoFe protein by MgATP hydrolysis-driven conformational changes in both proteins (Howard, 1994; Peters et al., 1995).

The "head" surface of the Fe protein appears to make contact with the MoFe protein; evidence for the involvement of this region of the Fe protein includes the chemical cross-linking study that implicates glu112 of the Fe protein as a contact residue, and studies of mutants in which substitutions for arg100 of the Fe protein were associated with a wide range of growth and activity properties (Wolle et al., 1992). Regions of apparent charge complementarity exist on the surfaces of the two proteins in the proposed docking region.

According to the model, the FeS cluster end of the Fe protein docks with the P cluster region of the MoFe protein. This would allow the two two-fold rotation axes
of the two proteins to be superimposed. The two proteins associate by weak but highly specific electrostatic and hydrophobic interactions. Complex formation with the MoFe protein induces a conformational change in the Fe protein and MgATP hydrolysis occurs. This event may serve to drive the conformational change necessary in the Fe protein to optimally align the FeS cluster with the P cluster for efficient electron transfer between the clusters. Electron transfer is followed by dissociation of phosphate, which mis-aligns the clusters and so minimizes back flow of electrons. The Fe protein-MoFe protein complex in this state must be structurally different from the initial complex since it is a tighter complex; Fe protein-MoFe protein dissociation is the rate-limiting step in nitrogenase catalysis (Thorneley & Lowe, 1984a).

In this model, MgATP hydrolysis in the Fe protein induces alignment of the FeS cluster of the Fe protein with the P cluster in the MoFe protein. Alternatively, nucleotide hydrolysis in the Fe protein may trigger a conformational change in the MoFe protein that induces a change in the oxidation state or redox properties of the P cluster (Chan et al., 1993). In either case, it is conformational changes caused by MgATP hydrolysis which are proposed to facilitate oxidation state changes in the metal clusters.
The model assumes that MgATP hydrolysis precedes electron transfer, however, evidence for this is contradictory, and the issue is not settled.

As described in the model, MgATP hydrolysis may serve as a gating mechanism to control electron transfer (Georgiadis et al., 1992). The Fe protein is proposed to function like other nucleotide-binding proteins in using nucleotide hydrolysis to switch between two states. In one, metastable state, electron transfer occurs from the Fe protein while the products of MgATP hydrolysis remain bound. The Fe protein adopts the second conformation upon release of phosphate, which leaves ADP bound and in which reverse electron transfer is prevented.

Dissociation of a protein-protein complex after each electron transfer has long been proposed (Hageman & Burris, 1978). An alternative hypothesis, that the Fe and MoFe proteins remain associated with each other during turnover, has empirical support from studies of nitrogenase in non-dithionite reductants (Druzhnin et al., 1993; Larser et al., 1995).

Any significant increase in the ATP/2e⁻ ratio (moles of P_i formed compared to moles of pairs of electrons transferred, calculated from products) indicates that MgATP hydrolysis and electron transfer have become "uncoupled", and enzyme catalysis is less efficient.
At present, several questions remain: are MgATP hydrolysis and electron transfer linked events? Is the purpose of MgATP hydrolysis to provide the energy needed to overcome an energy barrier and drive electron transfer? Or, does electron transfer depend on the formation of the two protein complex, and MgATP hydrolysis drives another reaction or step (Burgess & Lowe, 1996)?

It is important to note the following features of this process. No MgATP hydrolysis occurs unless the protein-protein complex forms; there is little evidence, however, for direct participation of the MoFe protein in the event. Reports have indicated that MgATP or MgADP binds to the MoFe protein; however, no significant role associated with this has been suggested (Miller & Eady, 1989; Miller et al., 1993). Second, hydrolysis of MgATP occurs whether or not an electron is transferred. Therefore, MgATP hydrolysis in nitrogenase depends on complex formation, and may occur due to a conformational change in the Fe protein induced by the MoFe protein.

Additional features of MgATP hydrolysis in nitrogenase, which also must be accounted for in an acceptable model, include: (i) ATP hydrolysis is reversible; (ii) apparently, MgATP can exchange (dissociate) without dissociation of the protein-protein complex, indicating that the MgATP-binding site is not
covered by the MoFe protein; and (iii) two MgATP are hydrolyzed for every one electron transferred.

1.B.5 Nitrogenase EPR
1.B.5.a EPR of MoFe Protein

As-isolated MoFe protein shows a rhombic EPR signal with $g$-factors of ~4.3, 3.67 and 2.01 (Figure 1.3). The low temperature EPR spectrum has been interpreted as arising from the paramagnetism of the $S'=1/2$ ground state Kramer's doublet of an $S=3/2$ spin system with small rhombicity ($\lambda=0.053$, where $0\leq \lambda \leq 1/3$) and a positive axial zero-field splitting ($D\approx 6.0$ cm$^{-1}$) (Munck et al., 1975). This spectrum originates from the FeMo cofactor. During turnover, the MoFe protein EPR signal declines to between ~40% and 10% of its original intensity (Figure 1.3); upon termination of turnover, the signal returns to its original intensity. The decrease in amplitude of the cofactor signal which occurs during turnover has been shown by Mössbauer studies to be due to a one-electron reduction of the FeMo cofactor to an integer spin, EPR-silent state.

The $S=3/2$ signal integrates to 1 electron spin per Mo atom. Comparison of the spectra of $^{95}$Mo- and $^{57}$Fe-substituted protein revealed that the unpaired electron is more associated with Fe atoms than with the Mo atom.
Figure 1.3  EPR spectra of Mo nitrogenase proteins. A, MoFe protein of *A. vinelandii*, as-isolated (175 μM); B, Fe protein, as-isolated (0.6 mM); C, Mo nitrogenase 2 min after initiation of turnover, Fe protein:MoFe protein 1:5, MoFe protein concentration 40 μM. EPR spectroscopy conditions, 3.0 mW, 12 K (A, C); 15.9 mW, 3.8 K (B). Spectra A and C at same scale.

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While the EPR spectrum of as-isolated MoFe protein is hardly affected by either ATP or dithionite, the $g$-factors are pH dependent.

1.B.5.b Fe Protein EPR

Reduced (as-isolated) Fe protein shows an EPR signal of an $S=1/2$ spin system with $g$-factors of ~2.05, 1.94 and 1.88 (referred to as the $g=1.94$ signal) (Figure 1.3). The signal quantitates to ~0.3 spins per Fe protein molecule, but the concentration of spins represented by the signal depends on the medium: in 0.5 M urea, the $g=1.94$ signal almost disappears, whereas in 50% ethylene glycol the signal integrates to almost 1 spin per molecule. The $g=1.94$ signal changes from a rhombic to an axial shape (with $g$-factors of 2.04 and 1.93) upon addition of MgATP. In addition, signals from an $S=3/2$ system, with $g$-factors of 5.8 and 5.15, can be detected; this signal quantitates to ~0.6 spins per molecule (Lindahl et al., 1985). During turnover, the Fe protein EPR signals almost disappear (Figure 1.3) due to oxidation.

1.B.5.c EPR of Mo Nitrogenase in CO

CO induces two EPR signals, depending on $P_{\text{CO}}$, from turning-over Mo nitrogenase (Yates & Lowe, 1976; Lowe et al., 1978; Davis et al., 1979). Low $P_{\text{CO}}$ (<8 kPa) elicits a rhombic signal (lo-CO) having $g$-factors of ~2.08, 1.975 and 1.93, and greater $P_{\text{CO}}$ (>10 kPa) results in an axial signal (hi-CO) with $g$-factors of ~2.17 and 2.08/2.04 (Figure 1.4).
Figure 1.4 EPR spectra of Mo nitrogenase in CO. A, hi-CO (g-factors of 2.17, 2.08/2.04); B, lo-CO (g-factors of 2.07, 1.975, 1.93). Fe protein:MoFe protein 1:5 (A), 1:1 (B); MoFe protein concentration 40 μM; P_{CO} 101 kPa (A). Mo nitrogenase 14 min after exchange of CO (101 kPa) for C_{2}H_{2} (10%) (B). EPR spectroscopy, 3.0 mW, 12 K.
It appears that the type of signal (lo- or hi-CO) depends on the ratio of the concentrations of dissolved CO and MoFe protein. Insufficient data are available to ascertain if the type of CO signal varies with electron flux. Slight variations in g-factors occur in spectra from Mo nitrogenases of different species. The signals disappear when the supply of reductant is exhausted.

The reciprocal behavior of lo- and hi-CO signal intensities with variation in $P_{CO}$ suggests that they originate from a single metal centre. Both signals appear with a $t_{1/2}$ of $\sim$4 s after initiation of turnover at high flux, whereas the onset of CO inhibition occurs much sooner ($\sim$220 ms). The absence of lo-CO from timed spectra of the developing hi-CO signal may indicate that lo-CO takes longer to develop than hi-CO, and it has been suggested that lo-CO may arise from an EPR-silent turnover state after many one-electron transfer steps.

The CO signals are not broadened by $^{13}$CO or by $^{95}$Mo in labeled enzyme; $^{57}$Fe in the MoFe protein broadens the signals. The signals quantitate to $<0.5$ spins per MoFe protein molecule.

CO signal intensities decrease with decreasing electron flux and lower incubation temperature. CO signals from CO-incubated *C. pasteurianum* Mo nitrogenase are much more sensitive to low temperatures of incubation than the corresponding signals from *A. vinelandii* Mo nitrogenase in
CO. (In contrast, the *C. pasteurianum* enzyme remains active - as indicated by the near-disappearance of the cofactor EPR signal - at far lower temperatures than *A. vinelandii* Mo nitrogenase.)

The characteristics of CO signals (lo-CO: rhombic, \(g\_\text{av}<2\); hi-CO: axial, \(g\_\text{av}>2\)) suggest that the signals arise from a \([\text{Fe}_4\text{S}_4]\) cluster that has net charge of +1 or +2, respectively.

1.C NITROGENASE ENZYMEOLOGY

1.C.1 Nitrogenase Enzymology

1.C.1.a Turnover, Electron Flux, Activity

When the two nitrogenase proteins, MgATP, a low potential reductant, and anaerobic conditions are present, nitrogenase is active (turning over).

Every Fe protein cycle (Figure 1.5) results in transfer of one electron to the MoFe protein, therefore the rate of electron flow through the MoFe protein (electron flux) is a function of the ratio of Fe protein to MoFe protein. A titration of the MoFe protein with Fe protein yields activity that increases linearly to a plateau; maximum Mo nitrogenase activity occurs with a ratio of ~4-5 Fe protein per MoFe protein. The titration curve resembles a classical enzyme saturation curve, with Fe protein acting as a substrate for MoFe protein, and the plateau denotes saturation of the MoFe protein with Fe protein.
Figure 1.5  Fe protein cycle. Reduction cycle of nitrogenase Fe protein showing coupling of MgATP hydrolysis and electron transfer. Fe P, Fe protein; MoFe P, MoFe protein. Adapted from Lowe & Thorneley, 1984a.
Ratios of Fe protein:MoFe protein of $\geq 4:1$ turn over Mo nitrogenase at high flux and presumably, maximum efficiency. Component protein ratios in the range of $\sim 1:1$ and $<1:10$ are considered to be moderate and low flux conditions, respectively.

The opposite titration shows a linear increase in activity to a peak and then a decline. The peak occurs with a ratio of MoFe protein:Fe protein of $\sim 2$; the reason for this optimum protein ratio is not understood. When the proportion of MoFe to Fe protein is increased, free Fe protein becomes limiting; the shortage is exacerbated by the slow (rate-limiting) dissociation of the two protein complex (Table 1.1). The decline in activity may occur because (i) a smaller fraction of total MoFe protein is turning over, or (ii) MoFe protein is binding with Fe protein that has not been re-reduced, forming an unproductive protein complex.

"Specific activity" is determined at high flux, and is a measure of enzyme activity in units of nanomoles of product produced per minute per milligram of protein.

Enzymically-reduced MoFe protein was active even after separation from the Fe protein by column chromatography (reported in Burris & Orme-Johnson, 1976).
1.C.1.b  Theoretical Turnover States

Each oxidation-reduction cycle of the Fe protein results in transfer of one electron to the MoFe protein. The stoichiometry of $N_2$ reduction by nitrogenase at optimum activity is:

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$

therefore eight complete Fe protein cycles must occur for the reduction of one molecule of $N_2$ to ammonia.

A useful model for discussion describes eight hypothetical states in a reduction cycle for the MoFe protein (Figure 1.6) (Lowe & Thorneley, 1984a,b; Thorneley & Lowe, 1984a,b, 1985). $E_0$ represents the MoFe protein in the as-isolated, dithionite-reduced state. The MoFe protein during turnover is in state $E_n$, where $n$ refers to the number of Fe protein cycles (transfer of one electron accompanied by a proton to the MoFe protein) that have occurred. Pre-steady state data (obtained with high flux enzyme) indicate that every step in the cycle involves the same limiting protein-protein dissociation rate ($k_3$=6.4 s$^{-1}$ at 23 °C (Lowe & Thorneley, 1984a)).

The model shows that high flux conditions would drive the enzyme into the higher $E$ states which are favorable for substrate binding and reduction. Furthermore, the greater the flux, the higher the level of reduction of the enzyme-substrate complex. At high flux, more electrons would be
distributed to substrate reduction and fewer into $H_2$ evolution. It is proposed that $N_2$ binds to $E_3$ and $E_4$, and $C_2H_2$ to $E_1$ and $E_2$ (Lowe & Thorneley, 1984a; Lowe et al., 1990). This would account for the observations that $N_2$ is not reduced at low flux (Thorneley & Eady, 1977) and $C_2H_2$ is reduced in preference to $N_2$ as flux is lowered (Davis et al., 1975).

The model also suggests that higher flux conditions should be accompanied by greater formation of the more-reduced product. Evidence for this is slim.

The model predicts that $H_2$ production is inherent in the mechanism of nitrogen fixation; for example, binding of $N_2$ to $E_3$ or $E_4$ displaces $H_2$.

An alternative explanation is that a certain electron flux may not "pump" electrons into the MoFe protein fast enough to convert it into a high $E$ state capable of binding substrate, therefore electrons "leak" into proton reduction (B. J. Hales, personal communication).

The Thorneley-Lowe model predicts very high flux nitrogenase will continue to evolve $H_2$; i.e. $H_2$ production cannot be shut off. If, on the other hand, $H_2$ results from a "leakage" of electrons, then at very high flux no $H_2$ would be expected. New methods for increasing electron transfer to the MoFe protein should allow these predictions to be tested.
Figure 1.6 MoFe protein cycle. Model of theoretical reduction states \((E_n)\) of the MoFe protein during catalysis. Adapted from Lowe & Thorneley, 1984a. Arrows represent transfer of one electron accompanied by a proton, i.e. one complete Fe protein cycle. H atoms not shown, for clarity. Transitions \(E_2 \rightarrow E_0\), \(E_3 \rightarrow E_1\) and \(E_4 \rightarrow E_2\) evolve \(H_2\), and \(H_2\) is displaced when \(N_2\) binds to \(E_3\) or \(E_4\).
Table 1.1 Rate Constants, Fe Protein and MoFe Protein Cycles

<table>
<thead>
<tr>
<th>rate constant</th>
<th>value</th>
<th>comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{+1}$</td>
<td>$5 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$k_{-1}$</td>
<td>15 s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$k_{+2}$</td>
<td>200 s$^{-1}$</td>
<td>coupled MgATP hydrolysis and electron transfer</td>
</tr>
<tr>
<td>$k_{+3}$</td>
<td>$4.4 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$k_{-3}$</td>
<td>6.4 s$^{-1}$</td>
<td>rate-limiting at high flux</td>
</tr>
<tr>
<td>$k_{+4}$</td>
<td>$3.0 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$</td>
<td>reduction by SO$_2$$^-$</td>
</tr>
<tr>
<td>$k_{+7}$</td>
<td>250 s$^{-1}$</td>
<td>H$_2$ evolution rate</td>
</tr>
<tr>
<td>$k_{+8}$</td>
<td>8.0 s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$k_{+9}$</td>
<td>400 s$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$k_{+6}$</td>
<td>$1.2 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$</td>
<td>S$_2$O$<em>4$$^{2-}$ $\leftarrow$ $k</em>{+6}$ - 2SO$_2$$^{2-}$</td>
</tr>
<tr>
<td>$k_{-6}$</td>
<td>1.7 s$^{-1}$</td>
<td></td>
</tr>
</tbody>
</table>

Simulations of *K. pneumoniae* kinetics made use of these rate constants (from Lowe & Thorneley, 1984a).

*K. pneumoniae* @ 23°C; 320 s$^{-1}$, *A. vinelandii* Mo nitrogenase @ 30°C (ibid.)

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The challenge to the spectroscopist is to find evidence of intermediate catalytic states as depicted in the Thorneley-Lowe scheme.

1.C.1.c Substrates, Inhibitors

Lineweaver and co-workers reported that whole cells of Azotobacter show N₂ fixation that follows Michaelis-Menton kinetics, with an apparent dissociation constant of 20 kPa of N₂ for an EN₂ complex (Lineweaver et al., 1934).

Nitrogenase catalyzes the reduction of N₂ to NH₃. Nitrogenase accepts a number of other small molecules as substrates (Table 1.2), including acetylene (C₂H₂) (Burgess, 1985; Burgess & Lowe, 1996). Almost all nitrogenase substrates have a double or triple bond between a C or a N and a C, O, or N atom. The exceptions are protons, and hydrazine (N₂H₄). Substrates are reduced by multiples of two electrons, and, except for N₃⁻ and NO₂⁻, consume an equal number of electrons and protons. CO, NO, CS₂, and CN⁻ are nitrogenase inhibitors that are not substrates (Burgess, 1985; Seefeldt et al., 1995). Substrates inhibit each other because they compete for a limited supply of nitrogenase electrons. MgADP is a nitrogenase inhibitor. H₂ at non-physiological pressures inhibits N₂ but not C₂H₂ reduction.

All nitrogenase reactions are accompanied by the evolution of H₂, produced from protons in the medium. In the absence of added substrate, total electron flux is
Table 1.2 Substrates and Products of Mo Nitrogenase

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Products</th>
<th>comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂</td>
<td>NH₃</td>
<td></td>
</tr>
<tr>
<td>H⁺</td>
<td>H₂</td>
<td>obligatory (Burris, 1991)</td>
</tr>
<tr>
<td>N₂H₄</td>
<td>NH₃</td>
<td>the only substrate without a multiple bond</td>
</tr>
<tr>
<td>N₅⁻</td>
<td>N₂, N₂H₄, NH₃</td>
<td></td>
</tr>
<tr>
<td>N₂O</td>
<td>N₂ (+ H₂O ?)</td>
<td>fate of O unknown</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₂H₂</td>
<td>C₂H₄</td>
<td></td>
</tr>
<tr>
<td>C₂H₄</td>
<td>C₂H₆</td>
<td>A. chroococcum, not A. vinelandii</td>
</tr>
<tr>
<td>CH₃CCH</td>
<td>H₃CCHCH₂</td>
<td></td>
</tr>
<tr>
<td>C₂H₅CCH, H₂CCCH₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyclopropene</td>
<td>cyclopropane, H₃CCHCH₂</td>
<td></td>
</tr>
<tr>
<td>diazirene</td>
<td>H₃CNH₂, CH₄, NH₃</td>
<td></td>
</tr>
<tr>
<td>trans-dimethyldiazine</td>
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<td></td>
</tr>
<tr>
<td>HCN</td>
<td>H₃CNH₂, CH₄, NH₃</td>
<td></td>
</tr>
<tr>
<td>CH₃CN, C₂H₅CN, C₃H₇CN</td>
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</tr>
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</table>

(table con'd.)
<table>
<thead>
<tr>
<th></th>
<th>H₂CNH₂, NH₃</th>
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<tbody>
<tr>
<td>H₂NCN</td>
<td>(cyanamide)</td>
</tr>
<tr>
<td>CH₃NC</td>
<td>(methyl isocyanide)</td>
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<tr>
<td>C₂H₃NC</td>
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</tr>
<tr>
<td>H₂CCHNC</td>
<td></td>
</tr>
<tr>
<td>COS</td>
<td></td>
</tr>
<tr>
<td>CO₂</td>
<td></td>
</tr>
<tr>
<td>(H₂ + D₂O + N₂)</td>
<td>HD</td>
</tr>
<tr>
<td>(D₂ + H₂O + N₂)</td>
<td>HD</td>
</tr>
</tbody>
</table>

- (adapted from Burgess, 1993)
directed to H$_2$ production. (It is generally assumed that H$_2$ evolution under Ar represents the maximum total number of electron pairs that the enzyme can transfer.) In the presence of added substrate, this total electron flux is distributed between substrate reduction and H$_2$ evolution; i.e. total electron flux is independent of substrate.

Nitrogenase catalyzes the formation of HD from H$_2$ + D$_2$0 or from D$_2$ + H$_2$0 if N$_2$ is present; the mechanism of HD formation is not understood (Jackson et al., 1968; Guth & Burris, 1983).

1.C.1.d Distribution of Electrons

Nitrogenase activity depends on electron flux. Electron flux influences the distribution of electrons to products: (i) whether substrate or protons are reduced (Table 1.3), and (ii) whether 2-, 4-, or 6-electron reduction products are released and in what proportion. An example of this is N$_2$ reduction. N$_2$ reduction is favored over H$_2$ evolution as the ratio of Fe protein to MoFe protein is increased. At optimum flux and elevated P$_{N2}$, electrons are distributed in a 3:1 ratio between N$_2$ reduction and H$_2$ evolution. This has been interpreted as evidence that N$_2$ binds to a highly reduced state of the MoFe protein, a state capable of completely reducing the substrate. N$_2$ is reduced to NH$_3$; there is no evidence of intermediate reduction products such as hydrazine (N$_2$H$_4$).
Table 1.3 Percentage of Electrons to Substrate Reduction

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mo</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>H⁺ in Ar</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>N₂</td>
<td>70-75</td>
<td>50</td>
</tr>
<tr>
<td>C₂H₂</td>
<td>95</td>
<td>15-50, 30</td>
</tr>
</tbody>
</table>

*A. vinelandii*  
*A. chroococcum*
In most nitrogenase reactions, as electron flux is lowered, \( \text{H}_2 \) evolution is favored over substrate reduction (Silverstein & Bulen, 1970; Davis et al., 1975). However, the opposite trend, in which high ratios of Fe protein:MoFe protein shift more electrons into \( \text{H}_2 \) formation and away from substrate reduction, is seen with the substrates HCN, \( \text{CH}_3\text{NC} \), and \( \text{N}_2\text{H}_4 \). With *K. pneumoniae* nitrogenase, \( \text{H}_2 \) evolution is maximal from nitrogenase with component proteins in a ratio of 1:1, with a lower proportion of \( \text{H}_2 \) produced by lower and higher fluxes.

It is thought that a higher electron flux favors formation of the more-reduced product. However, a different trend occurs with HCN reduction; a low electron flux shifts the distribution of electrons in favor of the 6-electron reduction pathway (formation of \( \text{CH}_4 + \text{NH}_3 \)) over the 4-electron formation of \( \text{CH}_3\text{NH}_2 \) (Li et al., 1982).

The above examples illustrate that each reaction catalyzed by nitrogenase is unique in the proportion of electrons allocated to substrate reduction or \( \text{H}_2 \) evolution, whether the 2-electron or 4-electron reduced product is formed and in what proportions are products formed. Nitrogenases from different species show different reactivities. Finally, the Mo, V, and Fe nitrogenases show unique activities.

Little attention has been given to investigations of one enzyme activity as performed by nitrogenases from
different species. More effort has been directed into research on mutant proteins and has yielded insights into the details of the mechanism of catalysis.

Temperature (Miller & Eady, 1988; Dilworth et al., 1993) and pH (Pham & Burgess, 1993) also affect nitrogenase activity.

Small changes in electron flux can result in a shift of electrons into different routes of substrate reduction. For substrate where more than one pathway for substrate reduction is possible, each path may function most effectively at a unique optimum electron flux. For nitrogenase reduction of CH₃NC, the formation of CH₄ + CH₃NH₂ reaches a maximum when the Fe protein: MoFe protein ratio is ~3, and the same occurs for the products C₂H₄ and C₂H₆. The formation of CH₃NHCH₃ is at a maximum at a protein ratio of ~2, and H₂ evolution is maximized when the protein ratio is ~4 (Rubinson et al., 1983). A similar phenomenon occurs with HCN reduction (Li et al., 1982). In contrast, H₂ evolution, N₂ reduction, and HD formation show their maximum rates of formation at the same protein ratio (Wherland et al., 1981).

1.C.1.e Acetylene as Substrate

Acetylene (C₂H₂) is an alternative substrate for nitrogenase. Acetylene reduction is a popular method of detection of nitrogenase activity because of the assay's sensitivity and convenience. Mo nitrogenase allocates 95%
of electrons to C₂H₂ reduction. At lower fluxes the percentage of electron flux allocated to C₂H₂ reduction decreases (Eady & Postgate, 1974; Lowe et al., 1990). Only the 2-electron reduced product is detected. In the presence of C₂H₂ + D₂O, cis-C₂H₂D₂ is produced (Burgess, 1985). Higher pressures of C₂H₂ inhibit nitrogenase (Shah et al., 1975), therefore the standard assay is performed in saturating but noninhibiting P_{C₂H₂} (~13 kPa). For comparison, nitrogenase is saturated by P_{N₂} of ~10 kPa N₂ and is not inhibited by elevated pressures of N₂. An infinitely high P_{C₂H₂} would completely suppress H₂ evolution (Rivera-Ortiz & Burris, 1975). In contrast, with infinite P_{N₂} ~25% of total electron flux would be directed to H₂ evolution (Simpson, 1984). C₂H₂ is reduced in preference to N₂ when MoFe protein is in excess (Davis et al., 1975).

A search of the literature was undertaken for data on the Kₘ of C₂H₂→C₂H₄ by nitrogenase (Table 1.4). It must be remembered that early studies (pre- ~1975) were performed with bacterial cell crude extracts (cell-free extracts) or partially-purified nitrogenase components.

The source material are diverse but the data show remarkable agreement and cluster around an apparent Kₘ of 1 kPa of C₂H₂. Some of the data suggest that the apparent Kₘ depends on electron flux (Orme-Johnson & Davis, 1977; Davis et al., 1979); this was shown by C. pneumoniae.
<table>
<thead>
<tr>
<th>$K_m$ (kPa of $\text{C}_2\text{H}_2$)</th>
<th>comment</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>Schöllhorn &amp; Burris, 1967</td>
</tr>
<tr>
<td>0.02–0.9</td>
<td>A. v. *</td>
<td>Hardy et al., 1971</td>
</tr>
<tr>
<td>1</td>
<td>cell lysate; purified proteins</td>
<td>Davis et al., 1975</td>
</tr>
<tr>
<td>1.5</td>
<td></td>
<td>Hwang et al., 1973</td>
</tr>
<tr>
<td>1.2</td>
<td></td>
<td>Rivera-Ortiz &amp; Burris, 1975</td>
</tr>
<tr>
<td>~0.2, 0.3</td>
<td>$\text{C. p.}$, when Fe protein is limiting</td>
<td>Orme-Johnson &amp; Davis, 1977; Davis et al., 1979</td>
</tr>
<tr>
<td>~23</td>
<td>$\text{C. p.}$, when MoFe protein is limiting</td>
<td>&quot; ; &quot;</td>
</tr>
<tr>
<td>0.3–2</td>
<td></td>
<td>range reported by Hardy, 1979</td>
</tr>
<tr>
<td>1.25</td>
<td>A. v., value used for data-fitting</td>
<td>Davis et al., 1979</td>
</tr>
<tr>
<td>~0.1 b</td>
<td></td>
<td>reported in Davis et al., 1979</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>Kim et al., 1995</td>
</tr>
</tbody>
</table>

* A. v., A. vinelandii; C. p., C. pasteurianum

b observed in experiments using very short reaction times and very low substrate concentrations (unpublished results, Davis & Wang)
Mo nitrogenase but not by the *A. vinelandii* enzyme.

However, the authors said that the use of $P_{C_2H_2}$ that varied $>10$-fold from the reported $K_m$ of 1 kPa, as well as use of a method of data analysis which assumed the presence of two forms of the enzyme enabled them to detect two different $K_m$ values.

Their data suggest that Mo nitrogenase in low flux shows a lower $K_m$ for acetylene reduction than does the enzyme in high flux. It is surprising that lower flux would be associated with greater affinity of Mo nitrogenase for $C_2H_2$; one might expect that, at high flux, the enzyme would be operating more efficiently, which would include having a greater affinity for substrate. Apparently, either the binding site or the mode of reduction of $C_2H_2$ is enhanced in low flux conditions. The improved affinity for $C_2H_2$ in low flux may serve to partially counteract the enzyme's low activity in this condition.

Additional evidence for the dependence of $K_m$ on electron flux comes from an earlier report. The $K_m$ increased with increasing Fe protein concentration but not with increased concentration of MoFe protein (when the concentration of the complementary protein was held constant) in assays of cell-free nitrogenase (Bergerson & Turner, 1973). Because of that finding, the experiments with V nitrogenase described in the present work (see section 3) were performed using fixed concentrations of
Fe protein and varied concentrations of VFe protein to prepare different flux forms of the enzyme. Fe protein concentration was kept constant to ensure that different forms of nitrogenase would show the same affinity for substrate in studies investigating CO inhibition of substrate reduction.

1.C.2 CO Inhibition

1.C.2.a CO and Nitrogenase Activity

Lind and Wilson showed that carbon monoxide (CO) is a potent inhibitor of N$_2$ fixation by rhizobia-inoculated red clover plants; inhibition occurred with P$_{CO}$ of ≥0.01 kPa of CO (Lind & Wilson, 1941). Low pressures of CO (0.2 and 0.097 kPa of CO) inhibited N$_2$ fixation by cell-free extracts of C. pasteurianum; the inhibition was reversed when the N$_2$/CO atmosphere was replaced by N$_2$ (Lockshin & Burris, 1965).

CO is a potent inhibitor of all nitrogenase-catalyzed reactions except H$_2$ evolution (Hwang et al., 1973; Burgess 1985). CO inhibits N$_2$ and C$_2$H$_2$ reduction and HD formation by nitrogenase and directs those electrons into H$_2$ evolution, i.e. CO does not inhibit total electron flux (Dilworth et al., 1965; Lowe et al., 1990; Burgess et al., 1981). CO does not inhibit dithionite (S$_2$O$_4^{2-}$) utilization or ATP hydrolysis (Hardy et al., 1965; Hwang et al., 1973; Davis et al., 1979). The inhibition is reversible.

There is at least one report of enhanced $H_2$ formation under a CO atmosphere compared to under Ar (Mortenson & Upchurch, 1981). Utilization of ATP was unchanged (the calculated MgATP/2$e^-$ ratio decreased), therefore, CO increased the efficiency of the nitrogenase system (Burgess, 1985).

1.C.2.b CO Inhibition: Type; $K_i$

Most studies have concluded that CO is a non-competitive inhibitor of $N_2$ and $C_2H_2$ reduction, although the inhibition has also been interpreted to be competitive, or mixed-type (Table 1.5). CO is a tight-binding inhibitor (Morrison, 1969). Tight-binding of CO to Mo nitrogenase is reflected in the low $K_i$ of CO inhibition of acetylene reduction (Table 1.5). When solution concentrations of a tight-binding inhibitor are much less than $K_i$, the inhibitor will be pulled out of solution by the enzyme, disturbing the equilibrium concentration of dissolved gas. This is not a concern in the present work because the experiments reported here made use of pressures of CO (in the range of 0.1 to 101 kPa) in excess of $K_i$.²

² Pressures of 0.1 to 101 kPa of CO correspond to concentrations of about $10^{-6}$ to $10^{-3}$ M of dissolved CO in aqueous solution (Dean, 1985). In all experiments described here, the pressure of dissolved CO exceeded the concentration of MoFe protein.
### Table 1.5 CO Inhibition of C$_2$H$_2$ or N$_2$ Reduction

<table>
<thead>
<tr>
<th>$K_i$ (x10^{-2} kPa of CO)</th>
<th>type of inhibition, comments</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>both C$^a$ and NC</td>
<td>Lockshin &amp; Burris, 1965</td>
</tr>
<tr>
<td>4</td>
<td>C; no change in $K_i$ with change in flux</td>
<td>Bergerson &amp; Turner, 1973</td>
</tr>
<tr>
<td></td>
<td>NC of N$_2$ and C$_2$H$_2$ reduction</td>
<td>Hwang et al., 1973</td>
</tr>
<tr>
<td></td>
<td>NC of N$_2$ reduction</td>
<td>Rivera-Ortiz &amp; Burris, 1975</td>
</tr>
<tr>
<td></td>
<td>NC of HCN$\rightarrow$CH$_4$</td>
<td>&quot;</td>
</tr>
<tr>
<td>$\approx$3 (7)</td>
<td>in excess Fe protein A. v.$^b$ (C. p.)</td>
<td>Orme-Johnson &amp; Davis, 1977</td>
</tr>
<tr>
<td>$&gt;&gt;$3 (5.5)</td>
<td>limiting Fe protein A. v. (C. p.)</td>
<td>&quot;</td>
</tr>
<tr>
<td>1, 4 (6, 8.5)</td>
<td>mixed-type A. v. (C. p.)</td>
<td>Davis et al., 1979</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>Hardy, 1979</td>
</tr>
<tr>
<td>3.3</td>
<td></td>
<td>Kim et al., 1995</td>
</tr>
</tbody>
</table>

$^a$ C, competitive; NC, non-competitive

$^b$ A. v., A. vinelandii; C. p., C. pasteurianum
CO has a low solubility in aqueous solution, as does N2; C2H2 is highly soluble (Table 1.6) (Dean, 1985).

1.C.2.c Where Might CO Bind?

Many studies have attempted to determine if CO has the same binding site in nitrogenase as substrates. Non-competitive inhibition of N2 and C2H2 reduction suggests that CO binds some distance away from N2 or C2H2. Furthermore, CO does not prevent (substrate) inhibition by C2H2 (Hardy, 1979). CO has a more direct influence on a CN− binding site, because low pressures of CO completely relieve the CN− inhibition of total electron flow (Li et al., 1982).

CO is a less potent inhibitor of V than of Mo nitrogenase (see section 3) (Dilworth et al., 1988).

CO inhibition of various mutant nitrogenases implicate the cofactor or its immediate protein environment as the CO binding site. For example, nitrogenase from a nifV strain of K. pneumoniae showed H2 evolution that was sensitive to CO (McLean & Dixon, 1981). CO inhibited total electron flux and H2 evolution, in proportion, and did not affect MgATP hydrolysis (McLean et al., 1983). The degree of inhibition increased with increasing pH, suggesting the involvement of a pH-sensitive group.

The basis for CO sensitivity of H2 evolution was investigated further with a hybrid MoFe protein composed of cofactor-less protein and isolated cofactor from the nifV−
Table 1.6  Solubilities of Gases in Water @ 30 °C

<table>
<thead>
<tr>
<th>gas</th>
<th>concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>8.0x10^-4</td>
</tr>
<tr>
<td>C₂H₂</td>
<td>3.4x10^-2</td>
</tr>
<tr>
<td>C₂H₄</td>
<td>3.9x10^-3</td>
</tr>
<tr>
<td>C₂H₆</td>
<td>1.5x10^-3</td>
</tr>
<tr>
<td>N₂</td>
<td>5.4x10^-4</td>
</tr>
</tbody>
</table>

Values of concentrations were calculated from the values of α, a solubility factor (Dean, 1985).
strain; the hybrid nitrogenase showed the nifV- phenotype (Hawkes et al., 1984). The control nitrogenase, composed with isolated cofactor from the wild-type strain, showed the wild-type phenotype. These results suggest that the cofactor is likely to be the site of CO binding. The data also support the hypothesis that substrate reduction directly involves the cofactor.

MoFe protein from nifV has citrate in place of homocitrate next to the Fe,Mo-containing cluster (Liang et al., 1990). Homocitrate is integral to the cofactor (Hoover et al., 1989). MoFe protein activated with FeMo cofactor synthesized using homocitrate analogues show altered substrate reduction properties (Madden et al., 1990). With particular analogue substitutions, nitrogenase-catalyzed H₂ evolution is inhibited by CO. These studies suggest that the CO sensitivity of H₂ evolution by nifV nitrogenase may be due to CO binding to, or in the vicinity of, the MoFe protein cofactor at its homocitrate end.

Site-directed mutagenesis is a technique that has been used to construct nitrogenases that contain defined amino-acid substitutions in the coordination environment of the cofactor. The response of these mutant nitrogenases to CO may yield information which will allow the structural basis of CO inhibition to be better defined. For example, certain mutant strains of A. vinelandii contain
substitutions for his195 in the cofactor pocket.

Sensitivity of proton reduction to CO was shown by lys195, thr195 and gln195 mutants (Scott et al., 1992; Kim et al., 1995). The gln195 enzyme shows several interesting properties: N₂ binds but is not reduced; N₂ binding inhibits H₂ evolution (in the mutant and in wild-type) and CO reverses that inhibition. In the mutant, MgATP hydrolysis is unchanged. The Kₘ for C₂H₂ reduction is unchanged, but the Kᵢ of CO inhibition of C₂H₂ reduction is smaller by an order of magnitude compared to the wild-type enzyme. The proximity of his195 to the cofactor suggests that the gln195 mutant has an additional binding site for CO that may be on the cofactor. The different sensitivities to CO shown by different his195 mutants suggest that there may be more than one binding site for CO in the vicinity of the cofactor.

The influence of CO upon a pH-titratable moiety in the wild-type Mo nitrogenase is suggested by the observation that CO shifts the pH profile of H₂ evolution at pH>7.5, by A. vinelandii Mo nitrogenase (Pham & Burgess, 1993).

1.C.2.d Two Binding Sites for CO?

CO inhibition is detectable with the onset of turnover; in contrast, CO EPR signals develop slowly (t₁/₂ ~4 s). Given that EPR spectroscopy is a sensitive technique and therefore the absence of EPR signal is not due to technical limitations, the simplest explanation for
the different times of occurrence of inhibition and EPR signal is that there is more than one binding site for CO. Also, the two CO-induced EPR signals could result from CO binding to two sites that differ in their affinity for CO. These hypotheses will be discussed further in the present work.

1.D THEMES OF THIS RESEARCH

CO can be used as a probe of nitrogenase (structure and activity) undergoing turnover. The known effects of CO on nitrogenase, modulation (inhibition) of activity and generation of new EPR signals, suggest that CO may bind to nitrogenase as a ligand to a nitrogenase metal cluster. Studies of nitrogenase with CO may lead to insights into the nature of CO inhibition, and may provide information about transient states of nitrogenase that occur during turnover.
CHAPTER 2 MATERIALS & METHODS

2.A NITROGENASE

2.A.1 Protein Purification

Azotobacter vinelandii cells were obtained from the University of Wisconsin. A. vinelandii Mo-nitrogenase component proteins were isolated, purified, and analyzed according to published procedures (Burgess et al., 1980) as described below. All procedures were performed anaerobically and all buffers contained 2 mM Na$_2$S$_2$O$_4$. (sodium dithionite). All chemicals were of standard commercial grade.

Frozen cells, cell extracts, and purified nitrogenase proteins were thawed in an Ar-filled glove box (Vacuum Atmospheres) fitted with a copper catalyst to remove contaminating O$_2$ and moisture from the inert gas atmosphere. Trace oxygen levels were monitored by an O$_2$ sensor (Teledyne Analytical Instruments, series 316 trace oxygen analyzer). All procedures with nitrogenase in the glove box were performed in an atmosphere of <2 ppm of O$_2$.

Cells were lysed by osmotic shock in a glycerol buffer followed by treatment with DNase, RNase and lysozyme; the cell lysate was concentrated by use of a Minitan concentrator. Component proteins were separated and partially purified on a DEAE-cellulose column that had been pre-equilibrated with 0.025 M Tris-HCl pH 7.4 buffer.
containing 0.1 M NaCl, and that was washed with a linear
gradient of 0.1 to 0.4 M NaCl in 0.025 M Tris-HCl pH 7.4.
MoFe protein and Fe protein eluted at ~0.12 M and ~0.22 M
NaCl, respectively. Component proteins were concentrated
(see below) and further purified by application of 5 mL of
concentrated DEAE eluate (70-75 mg/mL) onto a Sephadex S-
300 (Pharmacia) gel filtration column (3 cm x 100 cm) pre­
equilibrated and washed continuously with 0.3 M NaCl, 0.025
M Tris-HCl pH 7.4.

Nitrogenase protein-containing fractions of gel
filtration eluate were concentrated, in the glove box, in a
concentrator (Amicon) fitted with a YM 30 membrane (for
retention of solution components of $M_r > 30,000$) under
positive Ar gas pressure with continuous stirring,
typically to 30-45 mg/mL (concentrations of MoFe protein as
high as 100 mg/mL were achieved). The Ar cylinder was
fitted with an Oxysorb cartridge (Messer Griesheim). Small
volumes of protein solutions were concentrated in
concentrator cells (Amicon, models B15 {0.5-5.0 mL} or A25
{<0.75 mL}) in an inert gas atmosphere. The concentrator
wall acts as a wick and absorbs filtrate. Purified,
concentrated nitrogenase component proteins were frozen and
stored in liq N$_2$. 

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2.A.2 Protein Concentration Assay

Protein concentrations were determined by the biuret method (Gornall et al., 1949; Lowry et al., 1951). A volume (1-3 μL) of concentrated nitrogenase component protein was diluted to 200 μL with distilled water, combined with 1 mL of biuret reagent (0.75 g CuSO₄·5H₂O, 3.0 g sodium potassium tartrate, and 0.5 g potassium iodide, dissolved in 500 mL of 0.75 M NaOH), vortexed for 5 s, and let stand for 20 min. Protein concentration was determined by comparing the absorbance at 540 nm against a standard curve obtained with bovine serum albumin as the protein standard. Duplicate assays agreed within 5%. Molarities of protein solutions were calculated on the basis of \( M_r 64 000 \) (Fe protein) and 240 000 (MoFe protein).

2.A.3 Nitrogenase Activity Assays

2.A.3.a Acetylene Reduction

Nitrogenase activity was monitored by the acetylene reduction assay (Burgess et al., 1980), as follows.

A rubber-stoppered serum vial (13.8 mL) containing 1.0 mL of a MgATP-regenerating solution was fitted to a Schlenk line and the headspace gas was evacuated for 3-5 min and then filled ("charged") with Ar to a \( P_{Ar} \) of \( \sim 110 \) kPa; after this evacuation-charge cycle was repeated at least once the contents of the vial were considered "degassed". Next, the
vacuum-charge cycle was performed at least two times, charging with 10% C₂H₂ in Ar instead of Ar.

The MgATP-regenerating solution was composed of 2.5 mM ATP, 30 mM phosphocreatine, 0.125 mg/mL creatine phosphokinase, and 5 mM MgCl₂ in 38 mM TES-KOH pH 7.4.

Immediately before addition of nitrogenase proteins, dithionite (20 µL of a 1 M solution in 0.25 N NaOH) was added to the degassed MgATP-regenerating solution under C₂H₂. MoFe protein (for example, 2 µL of a 40 mg/mL solution) was added to the reducing solution. Lastly, Fe protein (for example, 4 µL of a 40 mg/mL solution) was added, which initiated nitrogenase catalysis. The turnover mixture was shaken @ 100 r.p.m. in a water bath @ 30 °C for 15 min. The incubation was terminated by addition of 0.1 mL of 30% TCA. An aliquot (0.1 mL) of headspace gas was transferred by means of a lockable syringe (Pressure-Lok) into a gas chromatograph (Varian, Model 3700) fitted with a Porapak T column and flame ionization detection. Acetylene and the reduction product ethylene (C₂H₄) eluted at ~1.0 and ~0.8 min, respectively. The ethylene peak height was measured relative to the acetylene peak height in order to normalize for variation in the volume of gas injected. The C₂H₄/C₂H₂ peak height ratio was converted into nanomoles of
ethylene by comparison to a standard curve prepared from serial dilutions of C\textsubscript{2}H\textsubscript{4} (Scott Specialty Gases).

2.A.3.b Detection of Evolved H\textsubscript{2}

Nitrogenase activity assay vials contained degassed MgATP-regenerating solution under Ar. The reaction initiation, incubation, and termination were performed as described (section 2.A.3.a). An aliquot (0.5 mL) of headspace gas was transferred in a lockable syringe into a gas chromatograph (Shimadzu, Model GC-8A) fitted with a 0.5 A 80/100 mesh molecular sieve column and thermal conductivity detector operating at 40 °C with Ar as carrier gas. H\textsubscript{2} eluted at ~0.8 min. H\textsubscript{2} peak heights were converted into nanomoles of H\textsubscript{2} by comparison to standards prepared from H\textsubscript{2} diluted with Ar.

2.A.4 Miscellaneous

The reducing ability of "reducing buffer" (0.025 M Tris-HCl pH 7.4, 2 mM Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}) was confirmed by observation of a royal blue color when a drop of buffer (clear and colorless) was placed on methyl viologen-soaked filter paper (white); blue indicated the presence of excess dithionite in the buffer.

Containers of nitrogenase proteins were kept on ice during an experiment. Aliquots of nitrogenase proteins were transferred in gas-tight syringes (Fisher) that were rinsed with reducing buffer immediately before use.
The color of an Fe protein solution indicates its activity. Freshly-purified dithionite-reduced Fe protein is seaweed green; with repeated removal of aliquots from the vial containing Fe protein, the sample becomes reddish orange but retains low activity; the color indicates that decomposition of \([\text{Fe}_4\text{S}_4]\) into \([\text{Fe}_2\text{S}_2]\) clusters has occurred.

Auto-oxidation of the Fe protein occurs due to the absence of reductant, a situation which arises because the Fe protein catalyzes the oxidation of dithionite. The MoFe protein does not appear to suffer from repeated handling, for it retains its rich, dark, root-beer brown color.

Ethylene glycol (EG) was degassed, diluted to 80% with reducing buffer, and contained 20 mM dithionite before use. To quench turnover, equal volumes of EG and turnover solution were combined. The final concentration of EG (40%) was known to quench \(\text{C}_2\text{H}_4\) production by \(A.\ vinelandii\) Mo nitrogenase (Blanchard, 1995).

EPR samples that contained EG were frozen very slowly to freeze the sample homogeneously and without fractures; a sample was half-frozen in about 35 s.

Volumes of gases were transferred in Pressure-Lok glass syringes (Precision Sampling Corporation).
2.A.5 Preparation of Headspace Gas

2.A.5.a 101 kPa of CO

MgATP-regenerating solution in a vial was degassed by at least two evacuation-charge cycles as described (2.A.3.a). The vial was then charged with CO (to a $P_{CO}$ of ~110 kPa) from a CO cylinder (Scott Specialty Gases) fitted with an Oxysorb cartridge (Messer Griesheim), by three or four evacuation (~20 s)-charge (~10 s) cycles.

2.A.5.b <101 kPa of CO

An empty, stoppered vial was charged with CO by the method described above (2.A.5.a). A second empty vial (13.8 mL) was charged with Ar (2.A.3.a) after evacuation for 30 s in each cycle. A vial containing 10 kPa of CO (10% CO) was prepared by transfer of 1.38 mL of CO from the vial containing CO into the Ar-charged vial after removal of an equal volume of Ar.

For preparation of a $P_{CO}$ of <101 kPa of CO in a reaction vial, a volume of 100% or 10% CO, as appropriate, was transferred into a reaction vial containing degassed MgATP-regenerating solution, after removal of an equal volume of headspace gas.

2.A.5.c Acetylene-Ar Mixtures

For assays of nitrogenase activity under non-standard $P_{C_2H_2}$, vials containing degassed MgATP-regenerating solution under Ar (MgATP-Ar) were prepared. A separate vial was charged with $C_2H_2$ by a similar procedure to that described...
for CO (2.A.5.a), using an acetylene cylinder (Matheson Gas Products) instead of CO. A volume of 100% C₂H₂ was transferred into each MgATP-Ar vial, after removal of an equal volume of headspace gas.

2.A.5.d Exchange of Gases (Pumping)

Headspace gases were exchanged (pumped) for Ar or C₂H₂ (10% in Ar) by performance of four evacuation-charge cycles in 2 min (2.A.3.a), charging with Ar or C₂H₂, as specified.

2.A.6 Nitrogenase in CO for EPR Spectroscopy

A vial containing MgATP-regenerating solution was charged with CO, as described (2.A.5.a or b).¹

Dithionite (20 mM), MoFe protein, and Fe protein, were added, in the order given; addition of Fe protein initiated turnover. The mixture was swirled vigorously (@ 180 rpm) at room temperature (23 °C) for the specified time. At intervals, an aliquot (0.3 mL) of turnover solution was transferred into an anaerobic, quartz EPR tube (4 mm OD x 3 mm ID x 242 mm) (Heraeus Amersil) by means of a 1 mL plastic syringe (Becton-Dickson) fitted with a 12" 22 gauge stainless steel needle (Aldrich), and frozen in liq N₂; the time required to freeze one-half of the sample was ~22 s. Frozen samples were stored in liq N₂.

¹ Whether the degassed MgATP-regenerating solution was in the vial before the vial was charged with CO, or was transferred into the vial after CO, made no difference to the results.
The proportion of Fe protein to MoFe protein was that which would produce the specified electron flux. Equal volumes of component proteins, added one-by-one or in a pre-mixed protein solution, and MgATP-regenerating solution containing dithionite, were combined. Resting state MoFe protein samples were prepared in reducing buffer. Control mixtures were prepared under Ar instead of CO.

EPR spectroscopy requires that nitrogenase concentration be high. Turnover requires sufficient MgATP-regenerating solution to provide creatine phosphate and dithionite for turnover. As a compromise, turnover mixtures for EPR analysis were prepared by combination of equal volumes of nitrogenase proteins and MgATP-regenerating solution, as described (Fisher et al., 1991).

2.B EPR SPECTROSCOPY

First derivative EPR spectra were recorded on a Bruker ER300D spectrometer interfaced with a Bruker 1600 computer with ESP 200 data collection software. The spectrometer was fitted with a TE\(_{102}\) perpendicular-mode cavity resonating at X-band frequencies. Low temperatures (\(\geq 3.8\) K) were maintained with an Oxford Instruments ESR-900 helium flow cryostat positioned in the cavity. Temperatures were monitored with an FeAu/chromel thermocouple positioned directly beneath the sample, connected to an Oxford Instruments ITC-4 temperature controller.
EPR spectroscopy was performed with 9.455 GHz microwave radiation, 100 kHz modulation frequency, 0.5 mT modulation amplitude, $1 \times 10^4$ gain, and 3 mW power, at 12.0±0.2 K, unless otherwise indicated.

Cofactor signal intensity was measured as the amplitude of the $g=4.3$ or 3.65 spectral feature, corrected for EPR tube diameter; the area under the $g=4.3$ peak was used for calculation of cofactor spin concentration. Hi-CO signal intensity was measured as the peak-to-peak amplitude of the feature from $g=2.08$ to 2.04 of the hi-CO signal; either the same peak-to-peak amplitude or the area under the hi-CO signal was used in calculation of hi-CO spin concentration. Spin concentration was determined by double integration of EPR spectral features and comparison with a standard (1 mM Cu(II) in 10 mM EDTA), after appropriate corrections (Aasa & Vanngard, 1975; Hagen, 1992). Spin concentration was calculated by means of the Aasa and Vanngard expression for calculation from a single peak when conditions of EPR spectroscopy are dissimilar to that of the (Cu) standard, or, with the Hagen expression when the entire signal could be integrated (this expression was used with the Cu standard).

Measurement of amplitudes and double integration of features of first derivative EPR spectra were performed by...
means of the NIEHS-LMB-EPR software program (Laboratory of Molecular Biophysics, National Institute of Environmental Health Science).

Data were plotted with Cricket Graph software.
Azotobacter vinelandii is a nitrogen-fixing bacterium. In 1980 Bishop and co-workers reported that certain Nif- (unable to grow by nitrogen fixation) mutant strains of A. vinelandii raised in Mo-free nitrogen-free media became Nif+ (were able to fix nitrogen) when grown in the absence of Mo (Bishop et al., 1980a,b). The investigators hypothesized that these Nif+ "pseudorevertants" possessed a second, alternative nitrogen fixation system which is suppressed by Mo and is expressed when Mo concentrations are low.

This was a radical idea because it was widely believed that biological N₂ fixation required Mo and Fe; all diazotrophic organisms which had been investigated have a nitrogenase system containing those two elements. A few reports, however, had suggested that biological nitrogen fixation might be more involved. Vanadium boosted the growth of cells grown in Mo-deficient conditions. Wild-type A. vinelandii cells grown in Mo-deficient conditions showed low levels of acetylene reduction and nitrogen fixation, but acetylene was a poor substrate compared to N₂ and protons (unlike with Mo nitrogenase) (Eady & Robson, 1984).
In order to prove that the reversion of Nif\(^-\) mutants to Nif\(^+\) was due to removal of Mo repression (derepression) of an alternative N\(_2\) fixation system and was not due to low levels of Mo nitrogenase, mutant strains of \textit{A. vinelandii} were constructed which contained deletions in the structural genes for Mo nitrogenase (\textit{nifHDK}). Clearly, any N\(_2\)-fixing ability would not be due to Mo nitrogenase activity.

The deletion strains (one was called LS10) showed a Nif\(^-\) phenotype in Mo-containing media and showed N\(_2\)-fixing activity under conditions of Mo deficiency, a finding which strongly implicated the existence of an alternative N\(_2\)-fixing system at the genetic level (Bishop et al., 1986).

Initially it was difficult to obtain the alternative enzyme from LS10 cells in quantities sufficient for enzyme studies. It was possible that the presence of low concentrations of Mo in water could repress the alternative nitrogenase system; however, it is costly to try to purify water of Mo.

Tungsten substitutes for Mo in Mo nitrogenase but W-substituted Mo nitrogenase is inactive. A mutant strain of LS10, known as LS15, was developed which was W-resistant (characterized by the ability to grow by nitrogen fixation in the presence or absence of Mo or in the presence of W (tungstate), a known inhibitor of Mo nitrogenase) (Hales et al., 1986a,b). The phenotype of W resistance indicated...
that the Mo (or W-substituted) repression mechanism was inoperative, and any nitrogenase activity was due to expression of an alternative nitrogenase system.

In the original studies of the alternative nitrogenase from LS15, isolation of the component 1 protein presented difficulties. The concentration of the protein in cell extract was low; furthermore, the protein lost all activity during the gel filtration purification procedure. Hales and co-workers found that addition of sodium metavanadate to the growth media increased the activity of the protein 30-fold, and use of a shorter, wider column than usual (5.0 x 25 cm instead of 2.5 x 100 cm) for gel filtration resulted in no loss of VFe protein activity (Hales et al., 1986b).

3.A.2 V Nitrogenase Component Proteins

Like the Mo enzyme, V nitrogenase consists of two metalloproteins, component 1 (the VFe protein) and component 2 (the Fe protein). The VFe protein is an \( \alpha_2\beta_2\delta_2 \) hexamer of \( M_c \approx 220,000 \). The \( \delta \) subunit may play a role in cofactor formation (Homer et al., 1995); otherwise, its function is unknown. The metal clusters of the VFe and MoFe proteins seem to be similar, according to MCD

---

\(^1\) For comprehensive reviews on alternative nitrogenase systems, the interested reader is referred to recent reviews by Eady (1991), Hales (1990), Pau (1991), and Lowe (1996).
(Morningstar et al., 1987), Mössbauer (Ravi et al., 1994) and EXAFS (Arber et al., 1987) spectroscopy studies, except that V is present in the FeV cofactor instead of Mo. Many of the MoFe protein amino acid residues which interact with the FeMo cofactor (Kim & Rees, 1992a) are present in the V nitrogenase α subunit (Joerger et al., 1989)².

3.A.3 V Nitrogenase Enzymology

Despite similarities of structure and ability to catalyze the reduction of other small molecules besides N₂, V and Mo nitrogenases show significant differences in catalytic activities. V nitrogenase is less active than Mo nitrogenase, as measured by total electron flux under Ar (~1400 versus 2200 nmols of electrons transferred min⁻¹ (mg of protein)⁻¹. This may be partly due to a rate of electron transfer from reduced Fe protein to MoFe protein that is four times slower (~46 versus 200 sec⁻¹) in V nitrogenase compared to Mo nitrogenase (Eady, 1991). Mo nitrogenase reduces C₂H₂ to C₂H₄; in contrast, V nitrogenase reduces C₂H₂ to both C₂H₄ and C₂H₆. V nitrogenase allocates only ~50% (A. chroococcum, Dilworth et al., 1987) or ~15% (A. vinelandii, Hales et al., 1986a) of electrons to C₂H₂ reduction compared to the high percentage allocated by Mo nitrogenase (1.C.1.3). N₂ reduction is less efficient with

---

² Those amino acids in the MoFe protein that the MoFe and VFe proteins have in common are gln191, his195, cys275, ser278, gly356, gly357, arg359, and his442.
V than Mo nitrogenase; catalysis by V nitrogenase yields 3.5 moles of H\textsubscript{2} per mole of N\textsubscript{2} reduced compared to 1 mole of H\textsubscript{2}/N\textsubscript{2} by Mo nitrogenase (Dilworth et al., 1993). The K\textsubscript{m} for N\textsubscript{2} reduction by V and Mo nitrogenases are the same, however (Eady et al., 1987).

All the information so far on the VFe protein and its clusters, obtained from spectroscopic data, indicates their general similarity to MoFe protein structures, apart from their respective heteroatoms in the cofactor. Differences in activity of V and Mo nitrogenases must therefore be due to subtle structural differences between them.

CO inhibits C\textsubscript{2}H\textsubscript{4} and C\textsubscript{2}H\textsubscript{6} formation from C\textsubscript{2}H\textsubscript{2} by V nitrogenase of A. chroococcum; however, the K\textsubscript{i} of CO inhibition, with V nitrogenase, is ~50 times the value with Mo nitrogenase (Dilworth et al., 1988). There have been few reports of investigations of CO inhibition of nitrogenase turning over at different electron fluxes (Davis et al., 1979; Dilworth et al., 1988). With the aim of gaining insight into the mechanisms of nitrogenase catalysis, the kinetics of CO inhibition of C\textsubscript{2}H\textsubscript{2} reduction by A. vinelandii V nitrogenase at different electron fluxes was examined. A surprising enhancing effect of low pressures of CO on V nitrogenase C\textsubscript{2}H\textsubscript{6}-forming activity, and an unusual enhancing effect of CO on C\textsubscript{2}H\textsubscript{4} formation if enzyme flux is low, were observed and are reported here.
An account of parts of this work has been published (Cameron & Hales, 1996).

3.B MATERIALS AND METHODS

3.B.1 Bacterial Strain, Protein Purification

V-nitrogenase was isolated from LS15 cells, a strain of *Azotobacter vinelandii* containing a deletion in the structural genes (*nifHDK*) for the conventional nitrogenase (Hales et al., 1986b). LS15 cells were obtained from the Fermentation Pilot Plant of the University of Wisconsin. (Cells were grown in media containing sodium metavanadate (NaV\(_3\)O\(_5\)) in place of the sodium molybdate (Na\(_3\)MoO\(_4\)) used in growth of the UW strain, the source of Mo nitrogenase.) VFe protein and Fe protein were isolated and purified using a variation (Hales et al., 1986b) of the procedure used to purify the conventional enzyme (2.A.1); the modifications are described below. Protein concentrations were determined and enzyme activity assays were performed as described (2.A.2, 2.A.3.a), with the modifications described below.

V nitrogenase proteins were eluted from the DEAE column with a linear salt gradient (0.1-0.5 M NaCl). Considerable overlap occurred of VFe protein- and Fe protein-containing fractions, with their maxima at 0.15 and 0.17 M NaCl, respectively. Therefore, gel filtration was essential for separation and purification of the component proteins.
The Fe protein from Mo nitrogenase was used in activity assays performed to identify which fractions (after DEAE and gel filtration) contained VFe protein. V nitrogenase Fe protein-containing fractions were identified by addition of MoFe protein.

Specific activities of purified V nitrogenase proteins: 166 nmol of C\textsubscript{2}H\textsubscript{4} produced min\textsuperscript{-1} (mg of protein)\textsuperscript{-1}, VFe protein (complemented with Fe protein of V nitrogenase); 695 nmol of C\textsubscript{2}H\textsubscript{4} produced min\textsuperscript{-1} (mg of protein)\textsuperscript{-1}, Fe protein (complemented with MoFe protein).

3.B.2 Activity Assays

In experiments that monitored C\textsubscript{2}H\textsubscript{6} production, C\textsubscript{2}H\textsubscript{2} and the reduction products C\textsubscript{2}H\textsubscript{4} and C\textsubscript{2}H\textsubscript{6} were detected with a gas chromatograph (GC) (Varian, Model 3700) equipped with an alumina column and flame ionization detection. For determination of only C\textsubscript{2}H\textsubscript{2} and C\textsubscript{2}H\textsubscript{4}, the GC was fitted with a Porapak T column. H\textsubscript{2} was detected as described (2.A.3.b).

The Fe protein of V nitrogenase shows even greater O\textsubscript{2}-sensitivity than the Fe protein of Mo nitrogenase. To minimize oxidation, the V nitrogenase Fe protein was used as required and refrozen as quickly as possible.

The desired electron flux was achieved by addition of various amounts of VFe protein to a fixed amount of Fe protein. The ratios of Fe protein:VFe protein were 8:1 (high flux), 1:1 (moderate flux), or 1:5 (low-moderate...
flux); component protein ratios were calculated on the basis of $M_r$ 64 000 (V nitrogenase Fe protein) and 240 000 (VFe protein). In experiments comparing the product ratio C$_2$H$_6$/C$_2$H$_4$ as a function of electron flux, electron flux was manipulated by varying the amount of Fe protein added to a fixed amount of VFe protein.

Headspace gas mixtures that contained CO or C$_2$H$_2$ of various pressures were prepared as described (2.A.5.b,c). In experiments to determine if CO inhibition was reversible, headspace gases were exchanged, as described (2.A.5.d). Values for the $K_m$ of C$_2$H$_2$ reduction to C$_2$H$_4$ were calculated from Lineweaver-Burk plots (1/v vs. 1/[S]), and $K_i$ values of CO inhibition of C$_2$H$_4$ production were determined from Dixon plots (1/v vs. CO concentration) (Dixon 1953). The type of inhibition exerted by CO was determined by inspection of Dixon plots.

Each data point in Figures 3.2A and 3.2B is the mean of data from three experiments. The best-fit parameters for equation 6 were calculated with the function-fitting program FFIT (A Soft Answer, Australia) and are plotted as smooth lines in Figures 3.2A and 3.2B.

3.C RESULTS

3.C.1 CO and Electron Flux

Notable differences in the effect of CO on V nitrogenase-catalyzed C$_2$H$_4$ formation occurred, depending on electron flux (Figure 3.1). While CO (40 kPa) strongly
Figure 3.1  Relative rate of C$_2$H$_4$ formation in CO as a function of nitrogenase protein ratio. $R_{\text{Ethylene}}$ is the relative production ($R = \{\text{enzyme activity in the presence of CO}/\{\text{enzyme activity in the absence of CO}\}\}$ of C$_2$H$_4$ as a function of protein ratio. Solid bars, A. vinelandii V nitrogenase (40 kPa of CO); hatched bars, A. vinelandii Mo nitrogenase (P$_{\text{CO}}$, 0.8 kPa). Data are the means from three experiments.

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inhibited the activity of high flux enzyme ($R_{\text{ethylene}}$ for $C_2H_4$ formation under CO relative to that in the absence of CO) was small), CO enhanced the activity of lower flux enzyme ($R_{\text{ethylene}}$ was greater than 1). A similar investigation (Figure 3.1) showed that while Mo nitrogenase was less and less inhibited when flux was lowered, $R_{\text{ethylene}}$ never exceeded 1.

(Data obtained with different fluxes are plotted relative to $C_2H_4$ formation in the absence of CO at that flux because nitrogenase activity varies non-linearly with electron flux.)

3.C.2 Product Formation

$V$ nitrogenase-catalyzed $C_2H_4$ and $C_2H_6$ formation under CO compared to that in the absence of CO was examined as a function of $P_{CO}$, at different electron fluxes, and several surprising effects were observed.

The most startling effect of CO was that instead of inhibiting enzyme activity, a low $P_{CO}$ strongly enhanced $C_2H_6$ formation (Figure 3.2B). Furthermore, the enhancement of $C_2H_4$-forming activity was flux dependent, being greater under moderate flux than high flux. (A similar data profile was shown by $V$ nitrogenase with a 20:1 ratio of Fe protein:Fe protein.)

At higher pressures of CO ($P_{CO}$ ≥ 1.5 kPa) $C_2H_6$ formation was strongly inhibited and the inhibition was independent.
Figure 3.2 Relative rates of V nitrogenase-catalyzed C\textsubscript{2}H\textsubscript{4} and C\textsubscript{2}H\textsubscript{6} formation as a function of P\textsubscript{CO}. A, R\textsubscript{ethylene} is the relative production (R = \{enzyme activity in the presence of CO\}/\{enzyme activity in the absence of CO\}) of C\textsubscript{2}H\textsubscript{4} as a function of CO pressure. Fe protein:VFe protein, 8:1 (open squares), 1:1 (solid circles), and 1:5 (open circles). Solid lines, theoretical R values calculated from equation 6. B, R\textsubscript{ethane} is the relative production (as in Figure 3.2A) of C\textsubscript{2}H\textsubscript{6} as a function of CO pressure. Inset: R\textsubscript{ethane} in low P\textsubscript{CO} (0 to 8 kPa).
of electron flux. (A very high flux preparation (20:1 Fe protein:VFe protein) yielded the same results.) At high \( P_{CO} \) (40 kPa) the inhibition was apparently complete. At these pressures of CO \( C_2H_6 \) production was more inhibited than \( C_2H_4 \) production (Figures 3.2A,B). This is unlike the effect of CO on the activity of the \( A. \ chroococcum \) V nitrogenase, where CO equally inhibits \( C_2H_4 \) and \( C_2H_6 \) formation (Dilworth et al., 1988).

CO had dramatically different effects on V nitrogenase-catalyzed \( C_2H_4 \) formation depending on electron flux (Figure 3.2A). At low flux, CO enhanced \( C_2H_4 \) formation; maximum enhancement occurred in the presence of a low \( P_{CO} \) (~10 kPa). With enzyme turning over at moderate flux this activity was little affected by CO. Ethylene formation by high flux V nitrogenase was inhibited by all pressures of CO tested.

The absolute activities of V nitrogenase in the absence of CO (Table 3.1) show that the amount of product formed with all the fluxes tested was significant.

The rates of formation of \( C_2H_6 \) and \( C_2H_4 \) in the absence of CO were compared as a function of electron flux. The \( C_2H_6/C_2H_4 \) ratio (product ratio) increased slightly (from 0.044 to 0.051) when electron flux was increased from moderate to high flux (Fe protein:VFe protein ratios from 4 to 29). A much lower product ratio (~0.030) was observed with Fe protein:VFe protein ratios of 1 or 2. In separate
Table 3.1 Product Formation (Absolute Activities) as a Function of Protein Ratio (nmol of product formed min$^{-1}$ (mg of protein)$^{-1}$)

<table>
<thead>
<tr>
<th>Fe protein: VFe protein</th>
<th>$C_2H_4$</th>
<th>$C_2H_6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:1</td>
<td>n.d.$^*$</td>
<td>5.9</td>
</tr>
<tr>
<td>8:1</td>
<td>68.0</td>
<td>2.4</td>
</tr>
<tr>
<td>1:1</td>
<td>22.4</td>
<td>0.7</td>
</tr>
<tr>
<td>1:5</td>
<td>2.4</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d., not determined
experiments, an average product ratio of 0.036 was observed from V nitrogenase in moderate and high flux (Fe protein:VFe protein ratios of 1:1 and 8:1, respectively), values in reasonable agreement with those above. These results are similar to the product ratios observed (~0.02) with V nitrogenase of A. chroococcum (Dilworth et al., 1988).

The data show that electron flux significantly affects C2H4 and C2H6 formation under CO. The question of whether substrate binding in the absence of CO was affected by electron flux was investigated. The Km of C2H4 formation from C2H2 was found to be greater by an order of magnitude for the V compared to the Mo nitrogenase, but the Km values of high- and moderate-flux preparations of each nitrogenase were similar (Table 3.2).

On the other hand, the Ki of CO inhibition of C2H4 formation was found to increase by an order of magnitude between high flux and moderate flux forms of the V and the Mo enzyme (Table 3.2).

CO was neither a competitive nor a noncompetitive inhibitor of V nitrogenase-catalyzed C2H4 formation, and may best be described as an uncompetitive or "mixed-type" inhibitor (Cleland, 1970). Others have made the same observation in regard to the type of inhibition shown by CO of A. vinelandii Mo nitrogenase activity (Davis et al., 1979).
Table 3.2 a. Apparent $K_m$ of $C_2H_2 \rightarrow C_2H_4$ (kPa of $C_2H_2$)

<table>
<thead>
<tr>
<th>protein ratio$^a$</th>
<th>nitrogenase$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>Mo</td>
</tr>
<tr>
<td>8:1$^c$</td>
<td>4</td>
</tr>
<tr>
<td>1:1</td>
<td>2</td>
</tr>
</tbody>
</table>

b. $K_i$ of CO Inhibition of $C_2H_2 \rightarrow C_2H_4$ (kPa of CO)

<table>
<thead>
<tr>
<th>protein ratio</th>
<th>nitrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>Mo</td>
</tr>
<tr>
<td>8:1</td>
<td>3.8</td>
</tr>
<tr>
<td>1:1</td>
<td>30</td>
</tr>
</tbody>
</table>

$^a$ component 2 protein:component 1 protein

$^b$ A. vinelandii

$^c$ 8:1, high flux; 1:1 moderate flux
CO (P_{CO} \leq 20 \text{ kPa}) had no effect on V nitrogenase-catalyzed H\textsubscript{2} evolution under Ar or under 10\% C\textsubscript{2}H\textsubscript{2}; this observation is consistent with the behavior of V nitrogenase of *A. chroococcum* at P_{CO} \leq 5 \text{ kPa} (Dilworth et al., 1988).

CO inhibition was reversible (Figure 3.3); enzyme activity recovered after exchange of gases.

3.C.3 Model of CO Enhancement and Inhibition

The trends in the data, of enhancement followed by inhibition, can be modeled by a simple two site, two effect model. Enhancement is proposed to occur when CO binds to one site on the enzyme and inhibition to result from CO binding to a different site; inhibition also occurs when both sites are occupied by CO.

Defining the binding of CO in terms of the dissociation constants K\textsubscript{1} and K\textsubscript{2} for the scheme:

\[
\begin{align*}
E + I & \rightleftharpoons EI_1 & EI_1 + I & \rightleftharpoons EI_1I_2 \\
E + I & \rightleftharpoons EI_2 & EI_2 + I & \rightleftharpoons EI_2I_1
\end{align*}
\]

where subscripts 1 and 2 refer to sites 1 and 2, and \(\rightleftharpoons\) represents "is in equilibrium with", we can define the terms:
Figure 3.3  V nitrogenase-catalyzed C$_2$H$_4$ formation in CO before and after exchange of gases. Time course of C$_2$H$_4$ production by A. vinelandii V nitrogenase incubated in the presence (solid dots) or absence (open squares with dots) of CO for the first 9 min, followed by exchange of gases and incubation in C$_2$H$_2$. 

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\[
K_1 = \frac{[E][I]}{[EI_1]}
\]
\[
K_2 = \frac{[E][I]}{[EI_2]}
\]
\[
K_{12} = \frac{[EI_1][I]}{[EI_1I_2]}
\]
\[
K_{21} = \frac{[EI_2][I]}{[EI_2I_1]}
\]
\[
R_{\text{product}} = \frac{\text{enzyme activity in pres. of CO}}{\text{enzyme activity in absence of CO}}
\]
\[
= E + h_1[EI_1] + h_2[EI_2]
\]
\[
= E_0
\]

and \(E_0 = (E + EI_1 + EI_2 + EI_1I_2 + EI_2I_1)\)

\(R_{\text{product}}\) represents production of a product in the presence of CO relative to production in the absence of CO, and \(h_n\) represents the relative amount of product produced by the enzyme form \(EI_n\) compared to production in the absence of CO (for example, \(h_1\) refers to enhanced product formation as a result of having one CO bound). The factor \(h_1\) is not the same as \(EI_1/E\) because that ratio depends on the binding and dissociation of CO from E.

For this model
\[
1 + h[I]
\]
\[
R_{\text{product}} = \frac{1}{1 + [I]/K_1 + [I]^2/K_1K_2}
\]
A conceptual problem with this model, however, is that it does not allow for enhancement to occur if the first CO molecule binds to site 2.

A second model gives a better fit of both the C₂H₆ and C₂H₄ sets of data. In this model, binding of CO to one site favors product formation and the binding of CO to both sites results in inhibition. It is not necessary to distinguish to which site the first CO molecule binds, for the effect of binding to either site will be enhancement. However, if both sites are occupied by CO, inhibition occurs.

Combining the above terms, for this model, yields

\[ R = \frac{1 + h_1[I]/K_1 + h_2[I]/K_2}{1 + [I]/K_x + [I]/K_2 + [I]^2/K_{12} + [I]^2/K_{21}} \]  

Equation 2 can be simplified to

\[ R = \frac{1 + A[I]}{1 + B[I] + C[I]^2} \]  

Equation 3 fits all the data and yields the best-fit parameters shown in Table 3.3.

Note that it is not possible to determine specific values for the individual terms h₁, h₂, k₁ and k₂ because they are represented in combinations by the terms in the table. Nevertheless, these best-fit parameters support the general observations that: (i) with C₂H₆ production, enhancement by CO is dominant (A>>B) until Pₒ becomes high, and enhancement is greater with moderate rather than
high flux enzyme (3.1>2.0); and (ii) C₂H₄ formation is enhanced under CO when A>B, and inhibition is associated with B>A.

Equation 3 together with the parameters in Table 3.3 successfully simulate the data. This strengthens the proposal that V nitrogenase has two binding sites for CO.

3.D DISCUSSION

There have been few reports of V nitrogenase examined with CO and none of these studies utilized the low P_co employed in the present work.

It can be seen that both CO and electron flux modulated V nitrogenase activity. Notably, CO acted to enhance as well as to inhibit nitrogenase activity. Further investigations revealed that each product was affected differently.

The effect of CO on V nitrogenase C₂H₆-forming activity depended primarily upon the concentration of CO; the effect changed from enhancement to inhibition as P_co was increased.

With respect to C₂H₄ formation, on the other hand, it was electron flux that determined whether CO would enhance, have no effect, or inhibit activity. The concentration of CO was less significant to the outcome.

What is unusual about the data presented here, obtained with V nitrogenase of A. vinelandii, is that it shows that CO enhances product formation; C₂H₆ formation
<table>
<thead>
<tr>
<th>Product</th>
<th>Fe protein: VFe protein</th>
<th>A (kPa⁻¹)</th>
<th>B (kPa⁻¹)</th>
<th>C (kPa⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₂H₄</td>
<td>8:1</td>
<td>2.0</td>
<td>3.0</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>0.014</td>
<td>0.004</td>
<td>8.1x10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>1.94</td>
<td>0.87</td>
<td>0.012</td>
</tr>
<tr>
<td>C₂H₆</td>
<td>8:1</td>
<td>2.0</td>
<td>0.22</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>3.1</td>
<td>0.25</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Obtained by fitting expression 3 to the data shown.
was enhanced under low $P_{\text{CO}}$ and $C_2H_4$ formation was enhanced under $P_{\text{CO}} \leq 40$ kPa when the enzyme was in low flux.

3.D.1 Enhancement by an Inhibitor

The finding that CO is an enhancer of $V$ nitrogenase activity has not been reported previously for nitrogenase. CO is known to potently inhibit all wild-type Mo nitrogenase catalysis, with the exception of $H_2$ evolution (Burgess, 1985), and to inhibit the activity (except for $H_2$ evolution) of $V$ nitrogenase of *A. chroococcum*. Enhancement might be expected if CO was a substrate, but all previous reports indicate that it is not a substrate for this enzyme. One report indicated that total electron flux ($H_2$ evolution) increased under CO (101 kPa) compared to under Ar (Mortenson & Upchurch, 1980). Therefore low $P_{\text{CO}}$ somehow modulates the activity of $V$ nitrogenase.

Enhancement by an inhibitor is not unprecedented (Bell et al., 1974), but it is rare. One can speculate that CO may enhance product formation by: (i) stabilizing an intermediate, (ii) inhibiting a dissociation step or a destabilizing influence, or (iii) facilitating the reaction. Proposed mechanisms for $C_2H_6$ formation are described below.

3.D.2 $C_2H_6$ Production under CO

The data in Figure 3.2B show that a low $P_{\text{CO}}$ strongly enhances $C_2H_6$ production, a higher $P_{\text{CO}}$ strongly inhibits the
reaction, and greater enhancement occurs at moderate rather than at high flux. Clearly, the binding of CO to a single, inhibiting site would not have these effects.

The data are well fitted by a two site, two effect model based on the proposal that CO binding to one site enhances C\textsubscript{2}H\textsubscript{6} formation while CO binding to both sites inhibits the reaction. Whether enhancement or inhibition of C\textsubscript{2}H\textsubscript{6} formation occurs depends on P\textsubscript{CO}.

The bimodal action of CO (enhancement or inhibition) exhibited by enzyme at moderate and at high flux indicates that flux plays a minor role in the actions of CO on C\textsubscript{2}H\textsubscript{6} formation.

It is interesting that the enhancing effect was greater with moderate than high flux enzyme. This seems contrary to the view that the greater the electron flux, the greater the proportion of the more-reduced product. Enzyme at moderate flux turns over more slowly and produces less product than at high flux (Table 3.1), but slower turnover would not be expected to change the relative rate of product formation (in the presence versus absence of CO). If both fluxes of the enzyme showed identical rates of product formation under low P\textsubscript{CO}, then when that product formation rate is expressed relative to product formation in the absence of CO, the ratio would be expected to be greater by moderate than high flux enzyme; however, the absolute activities (rates of product formation) under low
$P_{\text{CO}}$ were greater with high flux than with moderate flux enzyme.

Could the enhanced production of $C_2H_6$ simply be due to the conversion of enzyme-bound, partially reduced $C_2H_4$ into $C_2H_6$, the $C_2H_4$ having accumulated in the enzyme because CO inhibits its release? At least three observations suggest that this is unlikely. First, the data do not show complementary changes occurring in $R_{\text{Ethylene}}$ and $R_{\text{Ethane}}$; with moderate flux enzyme no inhibition of $C_2H_4$ occurs yet $C_2H_6$ formation is enhanced when $P_{\text{CO}} \leq 8$ kPa CO. Second, with moderate flux V nitrogenase of *A. chroococcum* CO (2 kPa) more strongly inhibits $C_2H_4$ reduction to $C_2H_6$ than $C_2H_2$ reduction to $C_2H_6$ (Dilworth et al., 1988). Third, CO (9 kPa) completely inhibits $C_2H_6$ formation from $C_2H_4$ (92 kPa) by Mo nitrogenase of *K. pneumoniae* and 1 kPa of $C_2H_2$ strongly inhibits the reaction (Ashby et al., 1987).

Based on the data presented here regarding $C_2H_6$ formation in the presence of CO, an argument can be made that CO modulates enzyme activity by binding to two sites; under low $P_{\text{CO}}$, CO binds to one site and enhances $C_2H_6$ formation; at higher $P_{\text{CO}}$ CO binds to an additional site and the net effect is inhibition of the reaction.

The above proposal could be examined further with a lower flux enzyme. From the quantities of V nitrogenase component proteins utilized in these experiments, no $C_2H_6$
was detected from a low flux preparation (Fe protein:VFe protein 1:5); to confirm the observation the experiment could be repeated with greater quantities of enzyme.

Possible simple schemes for \( \text{C}_2\text{H}_4 \) formation include:

(i) stepwise formation from acetylene via two 2-electron reduction steps (\( \text{C}_2\text{H}_2 \rightarrow \text{C}_2\text{H}_4 \); and \( \text{C}_2\text{H}_4 \rightarrow \text{C}_2\text{H}_6 \)); (ii) formation by a separate pathway from that of \( \text{C}_2\text{H}_4 \) formation; or, (iii) a contribution from both a single step and a two step pathway. If CO inhibited the first step of the two step mechanism in (i) we would expect \( \text{C}_2\text{H}_4 \) and \( \text{C}_2\text{H}_6 \) to be affected similarly by CO, but they are not. Also, the bimodal influence of CO on formation of each product cannot be explained by a two site, two effect model in which the first, enhancing site is the first 2-electron reduction step and the second, inhibiting site is the second reaction. Only a two site, two effect model, unique for each product, successfully models the data. In the case of \( \text{C}_2\text{H}_6 \) formation the outcome (enhancement or inhibition) is determined by \( P_{\text{CO}} \).

The data presented here suggest that there are two binding sites for CO which have opposite effects on \( \text{C}_2\text{H}_6 \) production depending on if one or both sites are filled. Evidence exists for two routes for \( \text{C}_2\text{H}_6 \) formation from \( \text{C}_2\text{H}_2 \) in V nitrogenase. In one pathway, \( \text{C}_2\text{H}_6 \) could be formed from \( \text{C}_2\text{H}_2 \) by a mechanism in which the alkyne binds to a
metal hydride and the complex is reduced to a metal-alkene; the complex reversibly rearranges via β-elimination into a metal-alkyl intermediate; this complex is reduced, releasing the alkane (C₂H₆) from the metal (Ashby et al., 1987; Dilworth et al., 1988). Evidence for this is the lag that occurs in the production of C₂H₆ from C₂H₂.

In V nitrogenase a second pathway may exist for C₂H₆ formation that does not involve an intermediate, because there is no lag in C₂H₆ production from C₂H₄.

One reason why Mo nitrogenase does not produce C₂H₆ from C₂H₂ may be the high affinity of the enzyme for C₂H₂ (Tables 1.4, 3.2). Acetylene may displace enzyme-bound C₂H₄ before the latter is reduced, and therefore no C₂H₆ is detected (Dilworth et al., 1988).

As discussed above, it is unlikely that the metal-alkyl intermediate would be the source of the "extra" C₂H₆ resulting under CO. The data presented here suggest instead that there could be two separate paths for formation of C₂H₆, each affected oppositely by CO, or, that low Pₕ could have opposite effects on a two-stage pathway if it were separate from a C₂H₄-forming pathway.

3.D.3 C₂H₄ Formation in CO

Contrary to the expected inhibition of C₂H₄ formation by V nitrogenase incubated in CO, the data show that this activity was enhanced by CO when the enzyme was in low
flux; the conventional effect was shown by high flux enzyme. Furthermore, what determined whether $C_2H_4$ formation was enhanced or inhibited was not $P_{co}$ but flux; $P_{co}$ played a secondary role, affecting the extent of enhancement or inhibition. The data indicate that electron flux modulates the effect of CO upon enzyme activity. In this case the two site, two effect model which satisfactorily matches the data must be based on different assumptions than the model which explains CO regulation of $C_2H_4$ formation.

A two site, two effect model for CO regulation of $C_2H_4$ formation proposes that at low flux, CO binding to a site results in enhancement (i.e. CO binds to an enhancing site), and at higher flux, CO binding to another site results in inhibition (i.e. binds to an inhibiting site). At low flux an enhancing site dominates and at high flux the inhibiting site becomes dominant.

High flux may open a second site to CO, an inhibiting site. Alternatively, CO may bind to one site but have a bimodal effect, depending on flux. Instead of a two site, two effect model, one posits a two flux, two effect model whereby any nitrogenase molecule in low flux is enhanced by CO and those operating in high flux are inhibited. Flux could modulate the outcome of CO binding by, for example, determining enzyme conformation.
Flux may also affect the nature of CO binding. Flux, the rate of electron flow through the enzyme, may affect the transient oxidation-reduction states experienced by the nitrogenase metal clusters during turnover. Certain of those oxidation states may be capable of binding CO and others may not. CO binding to certain transient oxidation states may result in enhancement of metal cluster-substrate interaction. Ligand binding to a cluster Fe atom could conceivably influence the electronic capability of the metal atom to bind a substrate molecule. In a model of CO-metal cluster binding, electron donation occurs from a filled ligand (CO) $\sigma$ orbital to the metal atom, and back-donation occurs from a filled metal $d\pi$ orbital into an empty $\pi^*$ orbital of the ligand. The relative contribution of the two types of electron donation to the strength of the metal ligand bond, in metal carbonyl complexes, depends on the heteroatom and the other coordinating groups (Connor, 1975). Therefore, from an electron donation argument, binding of CO to a V nitrogenase cluster could perturb the binding of other molecules to the cluster.

The two flux, two effect model proposes that the enzyme may have an active site conformation or oxidation state of its metal clusters that is flux-dependent; the effect of CO would then depend on the enzyme's structure and electronic state. Whether nitrogenase in different
fluxes adopts different conformations or has different intermediate oxidation states remains an important question to be investigated; currently, these proposals lack spectroscopic evidence.

Whichever of the above models is adopted, the data indicate that the effect of CO on $C_2H_4$ formation is not primarily a function of $P_{CO}$ (for the range of $P_{CO}$ examined here). This suggests that, even under the lowest $P_{CO}$ utilized, all CO binding sites which affect $C_2H_4$ formation may be occupied, and the net effect of having CO bound depends on how fast the enzyme is turning over.

Evidence exists for two binding sites for CO in Mo nitrogenase. First, the majority of reports of CO inhibition of Mo nitrogenase conclude that CO is a noncompetitive inhibitor of $N_2$ or $C_2H_2$ reduction. Second, while CO is a potent inhibitor of Mo nitrogenase-catalyzed $C_2H_2$ reduction, having an effect within milliseconds and showing an inhibition constant ($K_i$) of $\sim 10^{-2}$ kPa of CO (Table 1.5), higher concentrations of CO are necessary to induce an EPR signal, and there is a $\sim 4$ s lag before the EPR signal appears (Davis et al, 1979). Third, a recent $^{13}$C ENDOR study utilizing $^{13}$CO in a pulse-chase procedure with Mo nitrogenase found evidence that one EPR signal is associated with a cluster having one CO bound, and a second EPR signal is associated with having two CO molecules bound
to a nitrogenase metal cluster (Pollock et al., 1995; see section 6).

It may be asked, are these concentrations of CO limiting? A $P_{\text{CO}}$ of 101 kPa yields a concentration of $\sim$1 mM of dissolved CO in an aqueous solution (Dean, 1985; Linke, 1958-1965). A typical enzyme activity assay utilizes VFe protein in a concentration of $\sim$1.5 x $10^{-6}$ M. Therefore, over the range of $P_{\text{CO}}$ tested (1 to 40 kPa), CO would be in excess relative to the VFe protein by a factor of 6 to 270.

3.D.4 $K_m, K_i$

The apparent $K_m$ of $\text{C}_2\text{H}_2$-$\rightarrow$C$_2$H$_4$ are similar for V nitrogenase at moderate and high flux, indicating that the enzyme affinity for this substrate is unchanged when high flux and moderate flux enzyme activities are compared. In contrast, the $K_i$ of CO inhibition of the reaction changes by an order of magnitude. This suggests that the site (and/or the nature) of CO binding is altered depending on the enzyme flux. The $K_i$ indicates that the enzyme has a greater affinity for CO when flux is high rather than moderate. The same trend was observed with Mo nitrogenase.

A similar phenomenon has been reported for Mo nitrogenase from *A. vinelandii* but another study showed no difference in $K_i$ between two different (high flux) component protein ratios (Orme-Johnson & Davis, 1977; Bergerson & Turner, 1973).
It can be seen that CO is a weak inhibitor of moderate flux V nitrogenase-catalyzed C₂H₄ production. And, with enzymes under the same flux, CO is a weaker inhibitor of V than of Mo nitrogenase.

The $K_i$ is a measure of the binding of CO to the enzyme; the data indicate that CO binds an order of magnitude more tightly to high flux enzyme than to moderate flux, and an order of magnitude more tightly to Mo than to V nitrogenase. The bonding of CO as ligand to transition metals is well known. Based on the trend in bond energies of carbonyl-metal cluster bonds (Connor, 1975) a V-CO bond is probably weaker than a Mo-CO bond. (If CO binds to an Fe in a nitrogenase metal cluster, the differences in $K_i$ between the V and the Mo enzymes would not be expected.) CO binding to V/Mo in the cofactor might disturb the ligation of homocitrate to the heterometal atom, and thereby alter activity. CO affects the activity of organic acid-substituted nitrogenases, in some cases inhibiting $H_2$ evolution, as discussed (1.C.1). The lack of an EPR signal from CO-incubated V nitrogenase (the present work) may be related to the weak binding of CO to VFe protein, indicated by the $K_i$, and possibly due to a weaker metal-carbonyl bond in the V than the Mo enzyme.

Several authors have noted that the $K_m$ of C₂H₂ reduction to C₂H₄ by Mo nitrogenase is flux-dependent, as discussed (1.C.1). Bergerson and Turner (1973) observed
that the $K_m$ was dependent on the concentration of the Fe protein (Bergerson & Turner, 1973). Therefore, it seemed likely that different fluxes of V nitrogenase would have different affinities for the substrate acetylene. To compensate for this in order to focus upon the effects of CO on enzyme activity, the studies in the present work utilized a constant concentration of Fe protein and a varying concentration of VFe protein and the data indeed show only small changes in $K_m$ with changes in electron flux (Table 3.2).

It is not always the case that higher flux leads to formation of a greater proportion of the more-reduced product, as discussed (1.C.1). In the present work, the $C_2H_6/C_2H_4$ ratio (in the absence of CO) increased when electron flux was increased. Similar product ratios were observed with V nitrogenase of *A. chroococcum* at different fluxes; the $C_2H_6/C_2H_4$ ratio increased from ~0.01 to ~0.045 when the Fe protein:VFe protein ratio was increased from 1:1 to 20:1 (Dilworth et al., 1988). The important point is that an increase in $C_2H_6/C_2H_4$ means that $C_2H_6$ production is increasing above and beyond the increase in $C_2H_4$ formation that occurs until electron flux is maximal, with an Fe protein:VFe protein ratio of ~20:1.

By expressing the data for activity under CO as relative to activity in the absence of CO, at that flux, it becomes clear that even though more $C_2H_6$ is produced by the
higher flux enzyme (as indicated by the increase in the \( C_2H_6/C_2H_4 \) ratio), a low \( P_{CO} \) enhances \( C_2H_6 \) production even further.

Another inhibitor of nitrogenase shows inhibition that is flux-dependent. Ethylene inhibits \( H_2 \) evolution by \( K. pneumoniae \) nitrogenase when the enzyme is turning over at low flux (inhibition is \(-50\%\)) or at high flux (\(-27\%\)) but almost no inhibition is detected when the Fe protein:VFe protein ratio is 1:1 (\(-2\%\)) (comparing \( H_2 \) evolution under 101 kPa of \( C_2H_4 \) to that under Ar) (Ashby et al., 1987).

The data presented here stand as challenges to two commonly held beliefs about nitrogenase; the data show that (i) an inhibitor can act to enhance, and (ii) high flux nitrogenase does not necessarily produce more of the further-reduced product than does moderate flux enzyme, given the same experimental conditions.

The fact that both sets of data (\( C_2H_6 \) and \( C_2H_4 \) formation under CO) can be satisfactorily modeled by a two-site model lends strong support to the hypothesis that two binding sites for CO exist in V nitrogenase.

The data underscore the importance of considering electron flux in any model of nitrogenase enzymology.
CHAPTER 4  LOW FLUX MO NITROGENASE IN CO

4.A  INTRODUCTION

We wished to investigate the kinetics of the formation and decay of hi-CO. The $S=3/2$ cofactor EPR signal decays rapidly following initiation of turnover at high flux (Orme-Johnson et al., 1972; Smith et al., 1973; Mortenson et al., 1974). In contrast, the cofactor signal decays slowly to an intensity of ~50% in Mo nitrogenase in low flux (Hageman & Burris, 1979; Fisher et al., 1991). Therefore, in low flux, slow development of hi-CO would be expected. CO signal development in parallel with cofactor signal decay would support the hypothesis that CO binds to the nitrogenase FeMo cofactor. In order to monitor the kinetics of the induction of hi-CO, Mo nitrogenase turning over in low flux under CO was examined.

4.B  MATERIALS AND METHODS

Procedures below are described in full in section 2.

4.B.1  Low Flux Mo Nitrogenase

A. vinelandii Mo nitrogenase proteins were purified, concentrations were determined, and activity assays were performed as described (2.A.1,2,3). Low flux Mo nitrogenase under CO was prepared for EPR spectroscopy as described (2.A.6), with the following modifications.

A pre-mixed protein solution, composed of Fe protein (0.8 μM) and MoFe protein (80 μM) under Ar, was prepared.
If ethylene glycol (EG) was to be added to the turnover solution, the pre-mixed component protein solution comprised 1.6 μM Fe protein and 160 μM MoFe protein.

4.B.2 Additional Items

Media labeled "5X ATP" comprised 12.5 mM ATP, 25 mM Mg$^{2+}$, 150 mM phosphocreatine and 0.625 mg/mL creatine phosphokinase in 38 mM Tes-KOH pH 7.4; that is, all ingredients apart from Tes were present in five times their concentrations in MgATP-regenerating solution (2.A.3.a). Samples labeled "No ATP, otherwise 5X" consisted of Mo nitrogenase in media of 25 mM Mg$^{2+}$ and 150 mM phosphocreatine in 38 mM Tes-KOH pH 7.4, and samples labeled "No ATP" were prepared with MgATP-regenerating solution that lacked ATP and creatine phosphokinase.

Mo nitrogenase samples in low P$_{CO}$ (≤10 kPa) were prepared in individual vials (4.65 mL) that contained 0.1, 0.4, 0.8, 1 kPa or 10 kPa of CO, prepared as described (2.A.5.b).

EG was used as described (2.A.4).

Exchange of gases was performed as follows. Low flux Mo nitrogenase was incubated under CO (110 kPa) for 15 min, then degassed EG (40%) was added and the mixture was incubated a further 3 min. CO was replaced by Ar, as described (2.A.5.d). The incubation was continued, and 10
and 21 min after exchange of gases, samples were prepared for EPR analysis.

EPR spectra were obtained using 100 mW power.

4.C RESULTS

4.C.1 Low Flux, High $P_{\text{co}}$

Low flux Mo nitrogenase under CO yielded the hi-CO EPR signal (hi-CO) (Figure 4.1). Onset of hi-CO occurred 4 to 5 min after initiation of turnover, and signal intensity increased in a near-linear manner with time (Figure 4.2). Identical results were obtained in media labelled "5X ATP".

The small differences in the spin concentrations of the cofactor signal from low flux Mo nitrogenase in the presence versus in the absence (data not shown) of CO may not be significant. The cofactor signal intensity diminished to ~50% after 10 to 15 min of incubation, whether or not CO was present.

4.C.2 Low Flux, Low $P_{\text{co}}$

No CO-induced EPR signals occurred following incubation of low flux Mo nitrogenase in low $P_{\text{co}}$ ($\leq$1 kPa of CO) in timed samples from one turnover mixture or in samples prepared individually. From samples incubated in 10 kPa of CO a weak inflection in the spectrum at $g$=2.04 was seen in one experiment but was not seen in a second experiment. None of the treatments above resulted in a lo-CO signal (lo-CO). By comparison, incubation of moderate
Figure 4.1 EPR spectrum of low flux Mo nitrogenase in CO. Fe protein:MoFe protein 1:100, MoFe protein concentration 40 μM, P\textsubscript{CO} 50 kPa; sample prepared after 8 min of turnover. EPR spectroscopy conditions, modulation amplitude 20 G, 100 mW, 10 K.
Figure 4.2 Time course of EPR signals of low flux Mo nitrogenase in CO. Protein ratio and concentrations as in Figure 4.1, P_CO 101 kPa. EPR spectroscopy, 15.9 mW.
flux Mo nitrogenase in 0.1 kPa of CO yielded an intense lo-CO.

4.C.3 Pulse, Pump, Chase

Incubation of low flux Mo nitrogenase in CO followed by quenching of turnover and exchange of gases (pumping) resulted in an intense lo-CO in spectra obtained 10 and 21 min after pumping. Subsequent incubation in high $P_{CO}$ of the above lo-CO-yielding nitrogenase mixture (i.e. pulse, pump, chase) resulted in hi-CO. Similar results were obtained from moderate flux enzyme (5.C.4).

4.C.4 Other

No signals in the $g=5$ to 6 region were seen in EPR spectra from low flux Mo nitrogenase in CO or after pumping.

In experiments monitoring the cofactor signal immediately following addition of EG to low flux Mo nitrogenase (in the absence of CO), inconclusive results were obtained. In one experiment, the cofactor signal immediately increased in intensity to nearly the level of resting-state enzyme. Signals observed in two other experiments showed too great a variation in intensities to allow interpretation.
4D DISCUSSION

4.D.1 Theoretical Reduction States of Low Flux Mo Nitrogenase

Hageman and Burris (1979) observed that the EPR signal from low flux *A. vinelandii* Mo nitrogenase rapidly decreases to ~50% in about the same period as the lag in H\(_2\) evolution.

Assuming that H\(_2\) is evolved from a two electron reduced form (E\(_2\)H\(_2\)) of the enzyme, and assuming that the rate of H\(_2\) evolution is fast compared to the slow rate of formation of E\(_2\)H\(_2\), then the lag period in H\(_2\) evolution should reflect the time required for formation of E\(_2\)H\(_2\).

Assuming that the bleaching of the EPR signal occurs due to reduction of the MoFe protein by one electron per active site (Munck et al., 1975), and assuming that the 50% intensity of the EPR signal in steady state represents nitrogenase in a 50:50 mixture of as-isolated (E\(_0\)) and one electron reduced (E\(_1\)H) forms, then the rate of decay of the EPR signal should correspond to the rate of electron donation to products and be reflected in the rate of evolution of H\(_2\) in steady state. Indeed, this was observed.

Using the rate constants and the assumptions of the Thorneley-Lowe model of MoFe protein reduction (1.C.1.b), Fisher and co-workers simulated both the decay in the EPR signal and H\(_2\) evolution of low flux *K. pneumoniae*.
nitrogenase (Fisher et al., 1991). The model assumes that MoFe protein is predominantly in forms $E_0$ and $E_1H$ at steady state (after ~15 min of turnover, in their experiments); before steady state the calculated concentration of $E_1H$ mirrors the observed bleaching of the cofactor EPR signal ($E_0$). The simulation predicts that after 12 min the relative protein population distribution would be: $E_0$, ~55%, $E_1H$, ~45%, and after 20 min $E_0$ and $E_1H$ would each comprise ~50% of the MoFe protein population. At no time would the species other than $E_0$ and $E_1$ comprise more than 0.02% of the total MoFe protein concentration.

Therefore, under the conditions employed in the experiments described here, steady state low flux nitrogenase includes very little $E_2H_2$. Due to the low concentration of Fe protein the rate of formation of $E_2H_2$ is low, and once formed it rapidly decays to yield $H_2$ and regenerate $E_0$. Reduction of $E_2H_2$ to $E_3H_3$ may occur at a very low rate, however nitrogenase is unable to reduce $N_2$ (Eady & Postgate, 1974).

The question of whether the rates of the electron transfer steps preceding $H_2$ evolution are the same was investigated by Fisher and co-workers (1991). They observed that the pre-steady-state rate of oxidation of the Fe protein is the same whether the Fe protein is added to the MoFe protein in a low flux preparation that had been turning over for 15 sec ($E_0$) or 12 min (55% $E_1H$), which
indicates that the rates of reduction \((k_i)\) of \(E_0\) and \(E_iH\) are the same. Fisher and co-workers interpreted this to mean either that electron transfer is not rate limiting, or, that the site receiving the electron is at the same oxidation level, in both states.

Even though nitrogenase under low flux is in a steady state mixture of ~50% \(E_0\) and ~50% \(E_1\), it is not known precisely where the electron goes when the Fe protein transfers an electron to the MoFe protein. Does the cofactor accept the electron? Does the cofactor become oxidized so that in fact two electrons are transferred to substrate? All that is known is that the system becomes EPR silent.

In the present work, low flux Mo nitrogenase was investigated using CO as a probe of turnover states of the enzyme.

Two questions are, what redox state (E state) of low flux Mo nitrogenase is revealed by the CO EPR signal? and, does CO bind only to a reduced cluster and if so, reduced by how many electrons? EPR spectroscopy allows detection of paramagnetic clusters which in this case have CO bound to them. The data obtained with low flux suggest that CO binds to higher E states than \(E_0\) and \(E_1\).

Low flux Mo nitrogenase under CO yielded hi-CO. Hi-CO showed a lag and a time-dependent increase in signal intensity. The data suggest that CO binds to \(E_2\) or \(E_3\) and
remains bound while the MoFe protein reduction cycle continues (Figure 4.3). The reasons for this interpretation are discussed below. It is assumed that (i) CO binding to the cluster does not affect the redox states of the cluster, and (ii) hi-CO arises from the cofactor that has two bound CO molecules (6.C).

4.D.2 Does CO Bind to E₀?

CO cannot be binding to E₀ because if it did, the CO signal would appear immediately and subsequently would decline; furthermore, CO does not induce an EPR signal from resting state MoFe protein (E₀).

4.D.3 Does Hi-CO Arise from CO-bound E₁H?

This is unlikely, because E₁H is assumed to be EPR-silent (Munck et al., 1975). E₀ is characterized by the $S=3/2$ signal of the paramagnetic cofactor; in this state the P clusters are diamagnetic and EPR-silent. E₁H is paramagnetic but it has an integer spin state (Munck et al., 1975). Therefore, no CO signal should arise from a CO-incubated enzyme mixture which is only in states E₀ and E₁. If this mixture could be artificially prepared (such as with Ti(citrate)) the suggestion could be tested.

Suppose that, in the presence of CO, the single electron that reduces the cofactor is somehow directed away from the cofactor (for example, by neutralizing or reducing the charged group on an amino-acid side-chain near to the cofactor), leaving the cofactor EPR-active even though the...
Figure 4.3  Model of reduction cycle of MoFe protein in CO. Theoretical reduction states (Eₙ) of the MoFe protein during turnover in CO in low electron flux conditions.
protein is reduced. If CO binds to this form of E₁H, hi-CO development would be expected to mirror the exponential decay of the cofactor signal. Instead, hi-CO develops after a lag, and linearly.

Supposing E₁H is somehow EPR-active; the data could be explained as resulting from CO binding to E₁H if CO binding occurred at a much slower rate than the rate of formation of E₁H. But there is no reason to think that the rate of CO binding is slow, since the Kᵣ for CO is very small and the supply of CO is not limiting. Furthermore, with high flux, CO signals form very quickly (t₁/₂ ~4 s; Davis et al., 1979). This suggests that it is not a slow CO binding rate which is responsible for the slow development of hi-CO under low flux, but rather the lack of suitably reduced metal clusters to which CO can bind.

Therefore hi-CO is unlikely to derive from CO-bound E₁H.

4.D.4 Does Hi-CO Arise from CO-bound E₂H₂?

Two reasons in support of this proposal are the following. By the conventional understanding, CO EPR signals arise from nitrogenase that has been reduced by multiples of two electrons (E₀, E₂, E₄,...). Therefore E₂H₂ should be detectable by EPR provided that E₂H₂ is present in high enough concentration. Second, the lag in hi-CO appears to be similar to the lag in H₂ evolution (Hageman & Burris, 1979); this suggests that the lag in hi-CO reflects
the time required to form $E_2H_2$. Furthermore, CO does not inhibit $H_2$ evolution.

These reasons are outweighed by the principal argument against the proposal, which is that the steady-state concentration of $E_2H_2$ is very low. The EPR signal of CO-bound $E_2H_2$ should be weak and unchanging in intensity at steady state. However, this does not describe the data.

Could CO bind to $E_1$ but not be detected by EPR until the enzyme is in $E_2H_2$? (So far, there is no unique spectroscopic marker of $E_2H_2$.) The question cannot be answered, because the observed hi-CO is unlikely to arise from $E_2H_2$.

It may be that a Mössbauer investigation could determine if the cofactor (in CO-treated low flux Mo nitrogenase) has been reduced by two electrons from the resting state.

4.D.5 Could CO Bind to $E_3$?

Another possibility is that CO binds to $E_3$, a "higher" redox state of the enzyme, in that the MoFe protein has received three electrons from the Fe protein without release of product.

Evidence in favor of this proposal is the lag before hi-CO appears, which suggests that the lag is the time required for formation of an EPR-detectable CO-bound species. Although the proportion of enzyme in the state $E_2H_2$ in steady state is very small, there could be a slow
but steady conversion of $E_2H_2$ into $E_3H_3$ at a rate determined by the low concentration of $E_2H_2$. The concentration of $E_3H_3$ is likely to be low since $H_2$ is evolved from $E_3H_3$ (as well as from $E_2H_2$), regenerating $E_1H$, and since $H_2$ evolution occurs rapidly relative to the slow rate of formation of $E_3H_3$. $E_3$ could bind CO.

The main reason against the proposal is that $E_3$ is expected to be EPR-silent. The hi-CO signal would have to arise from $E_2$-CO or $E_0$-CO.

Therefore, the lag before the onset of hi-CO suggests that CO binds to a higher E state than $E_0$ or $E_1$.

4.D.6 Slow Dissociation of CO

One explanation of the kinetics of hi-CO is that CO binds to $E_2$ or $E_3$ and stays bound to the cluster while the MoFe protein-redox cycle continues. In effect, this introduces a second redox cycle that occurs in parallel (Figure 4.3) with that of enzyme that does not have bound CO molecules.

The time-dependent increase in hi-CO intensity seems to suggest a steady accumulation of a CO-bound form of the enzyme, and indicates that CO dissociation is slower than the rate of CO binding. Even if only one form of the enzyme (for example, one electron reduced enzyme) binds CO, CO remains bound while the MoFe protein continues to cycle through reduction (turnover) states, and observed hi-CO arises from those turnover states which have bound CO and
are EPR-detectable. Therefore, the lag in hi-CO is due to the slow rate of formation of (an) EPR-detectable species of CO-bound enzyme.

Do changes in the cofactor signal provide direct evidence of cofactor-CO interaction? The sum of the concentrations of spins under the CO signal and those under the cofactor signal of the CO-exposed sample, compared to the concentration of spins from cofactor in the absence of CO, might be expected to indicate whether or not all the paramagnetism derives from the cofactor. However, these signals (hi-CO, cofactor) are spread over different ranges of the magnetic field, necessitating the use of two different methods in calculating their spin concentrations. Therefore, the degree of uncertainty is large when comparing concentrations of spins under the CO and cofactor signals. The question, does CO bind to the cofactor? couldn't be answered from spin quantitation of the data.

A successful model for the interaction of the inhibitor CO with nitrogenase must be able to account for these additional observations: (i) with high flux and low flux enzyme, no lo-CO occurred before the onset of hi-CO; (ii) a low $P_{CO}$ did not induce lo-CO from low flux enzyme but did from high flux; and (iii) pumping a hi-CO-yielding sample resulted in lo-CO, from low flux and high flux enzyme.
It is interesting that under high flux (Davis et al., 1979) as in low flux (the present work), no low-CO signal preceded the onset of high-CO. The lack of low-CO suggests that binding of two CO molecules (indicated by high-CO) occurs rapidly relative to the slow rate of formation of the state capable of binding CO.

A low $P_{\text{CO}}$ did not generate any CO signal from low flux enzyme. Clearly, then, it is not $P_{\text{CO}}$ which determines what signal will develop.

What determines if CO binds to a nitrogenase cluster? One factor may be accessibility of the cluster; as part of the mechanism of MgATP hydrolysis and electron transfer, conformation changes probably occur in the MoFe protein; a shifting of amino-acid residues adjacent to the cofactor could occur, thereby uncovering a potential binding site for CO on the cofactor, for example (Kim et al., 1995).

During turnover, the enzyme may adopt transient conformations and redox states that are capable of binding CO; upon quenching of turnover, bound CO molecules could be "trapped". This could explain why, following quenching and pumping, a CO nitrogenase mixture showed low-CO where, before quenching, low-CO was absent. The new signal is not due to EG. EG induced a weak signal with $g$-factors of 1.977 and 1.93 in EPR spectra of low or high flux Mo nitrogenase in the absence of CO, and EG had no effect on the high-CO or cofactor signals from low flux Mo nitrogenase.
incubated in high P_Co. EG increases the intensity of the Fe protein \( S = \frac{1}{2} \) signal (Lindahl et al., 1985).

Another factor may be the affinity of the enzyme for CO. Affinity for substrates or ligands such as CO may depend on the redox state of the cofactor (the actual charge (redox state) of the cofactor is unknown), and CO may be bound only by suitably reduced enzyme. If nitrogenase metal clusters accumulate reducing equivalents during enzyme turnover it seems likely that the more-reduced clusters would bind molecules with multiple bonds more avidly than would clusters in as-isolated enzyme. Tighter binding of CO by higher flux forms of nitrogenase was observed (3.C; Table 3.1).

However, in the Thorneley-Lowe model of \( E_n \) states, the subscript \( n \) refers to the number of Fe protein cycles that have occurred, not to the (negative) charge of nitrogenase metal clusters. The electrons are assumed to be incorporated into hydride bonds, in the absence of reducible substrate; redox changes occurring in the metal clusters during turnover are unknown.

CO binding to cofactor could alter the electron distribution of the cofactor and, as a result, alter its substrate reduction properties.

CO binding could also alter the spin density around the cofactor, and therefore EPR-detectable forms of the
enzyme would have different EPR signals compared to those produced in the absence of CO.

Bound CO may stabilize a certain turnover state.

In conclusion, the EPR data obtained with low flux Mo nitrogenase in CO suggest that (i) CO does not bind to E₀, or E₁, but rather to a higher E state (E₂ or E₃), and (ii) once bound, CO stays bound even while turnover continues. The data also establish that CO signal development is dependent not only upon P₉₀ but also upon flux.
CHAPTER 5  MODERATE FLUX MO NITROGENASE IN CO

5.A  INTRODUCTION

CO is a potent, noncompetitive inhibitor of all nitrogenase-catalyzed reactions (Figure 5.3) except H₂ evolution; the site of inhibition isn't known. When turnover commences in the absence of CO, the cofactor EPR signal disappears and no new signal occurs that would be a signature of active nitrogenase. CO induces two EPR signals (depending on PCo) from turning-over Mo nitrogenase (1.B.5.c); the origin of the signals and the nature of CO interaction with nitrogenase metal cluster(s) aren't known.

CO-induced EPR from Mo nitrogenase were investigated as a window into nitrogenase undergoing turnover. It is assumed that a CO-induced EPR signal is evidence of a nitrogenase metal cluster with bound CO. It was thought that an EPR investigation of CO-treated enzyme might reveal answers to the following questions: (i) does CO remain bound if turnover ceases? (ii) does CO remain bound in the absence of external CO? (iii) does CO remain bound after turnover has ceased and CO removed from the enzyme atmosphere? and (iv) after turnover has been quenched and headspace gases exchanged, can a second exposure to CO affect the enzyme?

To investigate the fate of bound CO, the progress of CO-induced EPR signals in timed samples from moderate flux
Mo nitrogenase under CO was examined following different treatments: (i) quenching of turnover; (ii) exchange of gases; (iii) quenching, and exchange of gases; and (iv) method (iii) followed by addition of CO. (A subsequent investigation, using method (iv) with opposite isotopes of CO in different steps, is described in a separate section {Chapter 6}.)

5.B MATERIALS & METHODS

5.B.1 Moderate Flux Nitrogenase

*A. vinelandii* Mo nitrogenase proteins were purified and concentrations were determined as described (2.A.1,2).

Mo nitrogenase activity assays were performed as described (2.A.3), with modifications (see below). Mo nitrogenase component proteins were combined in a ratio of 1:5 Fe protein:MoFe protein (moderate flux).

EG was used as described (2.A.4).

5.B.2 Time Course of Nitrogenase Activity

5.B.2.a Pre- and Post-Addition of Quenching Agent

The acetylene reduction assay was performed (2.A.3.a), except that instead of quenching turnover after 15 min of incubation, degassed EDTA (various volumes of a 0.24 M solution), EG (40% final concentration), or TCA (0.1 mL of a 30% solution) ("quenching agent") was added 9 min after initiation of turnover. At intervals before and after addition of quenching agent, aliquots (0.1 mL) of headspace gas were analyzed for C₂H₄ and C₂H₂ content. Enzyme
activity was calculated from the \( \text{C}_2\text{H}_4 / \text{C}_2\text{H}_2 \) peak height ratio corrected for loss of \( \text{C}_2\text{H}_2 \) due to removal of multiple aliquots of headspace gas. Controls were prepared with Tris (0.25 M) or TES (38 mM) instead of a quenching agent.

5.B.2.b Pre- and Post-CO

The acetylene reduction assay was performed as described (2.A.3.a), except that TCA was omitted and CO was included in the headspace gas (2.A.5.b). After 9 min of incubation headspace gases were exchanged for \( \text{C}_2\text{H}_2 \) (10\% in Ar), as described (2.A.5.d), except that the exchange-of-gases procedure was performed three times in ~1 1/2 min, then the incubation was continued. The "post-CO" time was measured from the start of the final charge with \( \text{C}_2\text{H}_2 \).

Aliquots of headspace gas, obtained before and after exchange of gases, were analyzed for \( \text{C}_2\text{H}_4 \) and \( \text{C}_2\text{H}_2 \) content and activity was determined as described (5.B.2.a). Controls were incubated in \( \text{C}_2\text{H}_2 \).

5.B.3 Time Course of EPR of Mo Nitrogenase in CO

Moderate flux Mo nitrogenase turnover mixtures under CO were prepared for EPR spectroscopy as described (2.A.6), with the modifications below. Fe protein (16 \( \mu \)M) and MoFe protein (80 \( \mu \)M) were combined (pre-mixed) under Ar. Samples for EPR spectroscopy contained 8 \( \mu \)M Fe protein and 40 \( \mu \)M MoFe protein.
5.B.3.a  Before and After Quenching

If turnover was to be quenched with EG, the pre-mixed protein solution contained 32 μM Fe protein and 160 μM MoFe protein. After at least 5 min of incubation, degassed quenching agent (5.B.2.a) was added and mixed, then the incubation was continued; aliquots of the mixture were withdrawn before and after addition of the quenching agent, for EPR analysis (2.A.6). Samples were quenched with EDTA by addition of 10.5, 62, or 250 μl of 0.24 M EDTA per mL of turnover solution (2.5, 15, or 60 mM EDTA).

5.B.3.b  Pre- and Post-CO (Pulse, Pump)

Mo nitrogenase was incubated in CO (pulse) for 5 or 10 min, then headspace gases were exchanged (pumped) for Ar or C₂H₂ (10% in Ar), as described (2.A.5.d). Aliquots of the nitrogenase mixture were removed before and at intervals after the exchange of gases, for EPR spectroscopy (2.A.6). Controls were incubated in the absence of CO. The duration (in minutes) of the post-CO incubation was measured from the time of completion of exchange of gases, i.e. from the end of the final charge.

5.B.3.c  Pulse, Pump, Chase

Mo nitrogenase was incubated under CO (pulse) for 5 min, then ethylene glycol (40%) was added and the incubation was continued for 5 min. CO was exchanged (pumped) for C₂H₂, as described (2.A.5.d). The incubation was continued for 10 min, then the C₂H₂ atmosphere was
pumped and replaced by CO (chase), and the incubation was continued for 5 min. Samples of Mo nitrogenase solution for EPR analysis were prepared during the pulse, about 10 min after the first pumping, and after 5 or 10 min of the chase.

One kind of control received TES instead of EG. Another control was incubated under C₂H₂ instead of CO during the first incubation.

5.B.4 EPR Spectroscopy

The program Rhombo was generously provided by Dr. W. R. Hagen.

5.C RESULTS

5.C.1.a Turnover-Quenching Agents

Prior to EPR studies of Mo nitrogenase in CO following quenching of activity, the quenching ability of several compounds was examined.

EDTA, EG, and TCA quenched turnover; Tris inhibited turnover, and TES proved to be the most satisfactory control.

EDTA quenched turnover (Figure 5.1); activity was quenched when EDTA was present in a 1:1 ratio with Mg²⁺ (5 mM). C₂H₄ formation continued for ~4 min after addition of EDTA. Greater amounts of EDTA did not stop the reaction any faster but may have quenched activity more effectively, in that less of an increase in C₂H₄ occurred until turnover ceased (ratios of EDTA:Mg²⁺ ranged from 1:1 to 48:1).
Figure 5.1  Time course of Mo nitrogenase activity following addition of EDTA. $\text{C}_2\text{H}_4$ formation before and after addition of EDTA (solid dots) or Tris (open squares) to Mo nitrogenase. EDTA or Tris was added 9 min after initiation of turnover.

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These effects of EDTA were also observed from Mo nitrogenase turning over in a more concentrated MgATP-regenerating solution containing 25 mM Mg$^{2+}$. Controls which received Tris or TES instead of EDTA continued to produce C$_2$H$_4$; however, Tris partially inhibited the reaction (Figure 5.1).

The effect of EDTA on solution pH was tested using solutions similar in composition to Mo nitrogenase mixtures prepared for EPR spectroscopy. Addition of 62 or 250 μl of EDTA (15 or 60 mM) to a 1 mL solution of 1:1 reducing buffer:MgATP-regenerating solution containing dithionite (20 mM) lowered the pH from 6.22 to 5.26 or 4.69, respectively. Due to concern about the unknown effects on the enzyme of pH changes caused by EDTA, EG (40%) was substituted as a quenching agent. Ethylene glycol (40%) stops C$_2$H$_4$ production (Figure 5.2, and Blanchard [1995]) and evolution of H$_2$ (data not shown).

TCA, the quenching agent utilized in the standard lab assay of nitrogenase activity, and EG, showed a delay similar to that seen with EDTA, before taking effect (Figure 5.2). The basis for the lag before turnover ceases, after addition of these quenching agents, is not known.

5.C.1.b Quench Turnover: EPR

Addition of EDTA or EG to Mo nitrogenase under CO had no effect on hi-CO, which persisted undiminished (observed
Figure 5.2 Time course of quenching of enzyme activity by addition of EG or TCA. \( \text{C}_2\text{H}_4 \) formation by Mo nitrogenase. EG (solid dots) or TCA (open circles) was added 9 min after initiation of turnover. TCA data, mean ± standard deviation of two experiments.
for ~1 h following addition of EDTA; observed for ~10 min after quenching with EG).

EDTA (in the absence of CO) had no effect on the cofactor signal nor did it induce new signals from turnover enzyme. In the presence of CO and EDTA, nitrogenase yielded hi-CO and weak $g=5.15$ and 5.78 signals (5.C.5). From Mo nitrogenase in the presence or absence of CO, EG consistently induced a weak rhombic signal with inflections at $g=2.08$, 1.976 and 1.93 (see, for example, Figure 5.6A), $g$-factors that are also characteristic of lo-CO. An EG-quenched activity assay solution had a pH of 7.6, and EPR turnover mixtures quenched by EDTA had a pH of 6.4.

When EG was combined with MgATP-regenerating solution before addition of proteins to make a (turnover) mixture, Mo nitrogenase under CO did not yield a CO signal.

In some EPR experiments, aliquots of turnover mixture were tested for activity during, or at the end of an experiment, by transfer directly into an acetylene reduction assay vial and addition of Fe protein followed by incubation in the usual manner. When aliquots of Mo nitrogenase that had been quenched with EDTA were assayed for activity, Mo nitrogenase showed activity, indicating that EDTA quenching of activity was reversible.

5.C.2.a Post-Exchange of Gases: Activity

CO inhibition was reversible; after incubation under low $P_{\text{CO}}$ and exchange of the CO-$\text{C}_2\text{H}_2$ gas mixture for fresh
C2H2, Mo nitrogenase recovered most of its uninhibited level of activity within 5 min after removal of CO (Figure 5.3a); partial recovery occurred following incubation under 101 kPa of CO (Figure 5.3b).

5.C.2.b Post-Exchange of Gases: EPR

Hi-CO diminished and, surprisingly, lo-CO appeared and intensified with time, after pumping (Figure 5.4). In the earliest spectra following exchange of gases only hi-CO was seen; the onset of lo-CO occurred about 2 min after gas exchange (~4 min after removal of CO). Sigmoidal decay of hi-CO correlated with development of lo-CO; subsequently, lo-CO decayed (Figure 5.5). The cofactor signal, meanwhile, slowly regained intensity.

Spin quantitation of the hi- and lo-CO signals of Mo nitrogenase before and after exchange of gases (Table 5.1) suggests that CO-induced signals may arise from cofactor, but the data are not conclusive. A relationship of CO and cofactor signals is also suggested by the time course of the signals (and was suggested by the low flux data {4.C}).

No serious loss of Mo nitrogenase activity was observed in samples obtained at different stages of the exchange of gases EPR experiments.

The same trends were seen in the CO-induced EPR signals after pumping, from Mo nitrogenase in a more-concentrated MgATP-regenerating solution (5X ATP). Mo nitrogenase in this medium was 72% and 68% (data from two
Figure 5.3 Time course of Mo nitrogenase activity in CO and after exchange of gases. A, \( \text{C}_2\text{H}_4 \) formation; incubation in CO (0.8 kPa in \( \text{C}_2\text{H}_2 \) (10\%)) (solid dots) for the first 9 min was followed by pumping and incubation in \( \text{C}_2\text{H}_2 \). B, \( \text{C}_2\text{H}_4 \) formation after pumping to replace CO (101 kPa, 6 min incubation) with \( \text{C}_2\text{H}_2 \). Data in B, mean ± standard deviation of two experiments.

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Figure 5.4  EPR spectra of Mo nitrogenase in CO before and after exchange of gases. Mo nitrogenase protein ratio, 1:1, 40 μM MoFe protein. A, incubation in CO (101 kPa); B, 5 min after pumping; C, 14 min after exchange of gases. EPR spectroscopy, 15.9 mW.
Figure 5.5  Time course of EPR signals of CO-incubated Mo nitrogenase after exchange of gases. Open squares, hi-CO; solid circles, lo-CO. Fe protein:MoFe protein 1:5, 75 μM MoFe protein, in a more-concentrated ATP solution (5XATP), 101 kPa of CO. EPR spectroscopy, 15.9 mW.
Table 5.1 Concentrations of Spins (μM)* of EPR Signals from CO-Treated Mo Nitrogenase in Pulse, Pump, Chase Experimentsb

<table>
<thead>
<tr>
<th>treatment</th>
<th>cofactorc</th>
<th>hi-COd</th>
<th>lo-COe</th>
<th>g=5.15f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar, r.b.</td>
<td>71</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>no CO pulse; quench; pump</td>
<td>52</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>no CO pulse; quench; pump; CO chase</td>
<td>56</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CO pulse; no quench; pump</td>
<td>24</td>
<td>1.3</td>
<td>6.6</td>
<td>n.d.</td>
</tr>
<tr>
<td>CO pulse; no quench; pump; CO chase</td>
<td>25</td>
<td>5.7</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CO pulse; pump</td>
<td>21</td>
<td>0.8</td>
<td>31</td>
<td>n.d.</td>
</tr>
<tr>
<td>CO pulse; quench; pump; CO chase</td>
<td>30</td>
<td>10.7</td>
<td>5.3</td>
<td>6.8</td>
</tr>
</tbody>
</table>

* Spin concentrations calculated as described (2.B).

b Representative data set from two experiments, shown; concentration of MoFe protein in EPR samples, 27.5 μM; pulse, pump, chase experiments performed as described (5.B.3.C), with Fe protein:MoFe protein 1:5.

c Spin concentration of the g=4.3 peak or g=3.7 signal.

d Calculated from the entire hi-CO signal or the g=2.08/2.04 peak; maximum background, 0.8 μM.

e Calculated from the g=1.93 peak; maximum background, 3.1 μM.

f Spin concentration of this signal from the S=3/2 spin system; maximum background, 1.4 μM.

g Reducing buffer

h None detected
experiments) as active as it was in the standard assay solution. Mo nitrogenase comprised of 1:1 component proteins yielded the same pattern of EPR signal changes.

Pumping on moderate flux Mo nitrogenase under low $P_{\text{CO}}$ (0.1 kPa), which showed lo-CO, yielded residual lo-CO.

5.C.3 Post-Quench, Post-Exchange of Gases: EPR

Hi-CO decayed quickly, and lo-CO appeared more quickly, showed greater intensity, and was longer-lasting, in EG-quenched samples than in samples that did not receive EG (Figure 5.6; Table 5.1).

In assays of enzyme activity under conditions mimicking those of the EPR experiments (except with 10% CO in the headspace gas), addition of EG (40%) prevented recovery of activity after exchange of gases; controls which were treated with TES or 20% EG instead of 40% EG did recover activity.

In a "quench turnover, exchange of gases" experiment, nitrogenase was assayed for activity every time an EPR sample was prepared; Mo nitrogenase regained activity, and furthermore, equal activity was observed from control (Tris instead of EDTA) and quenched (EDTA) Mo nitrogenase solutions. Therefore, MoFe protein wasn't irreversibly harmed by EDTA. Similar tests of EG-treated Mo nitrogenase confirmed that it recovered activity.
Quenching with EDTA resulted in similar trends in the post-exchange CO-induced EPR signals; however, the two signals varied in duration.

5.C.4 Post-Quench, Post-Exchange: Chase with CO

Mo nitrogenase, incubated under CO, quenched with EG, pumped, and subsequently "chased" with CO yielded hi-CO (Table 5.1).

Controls, incubated in the absence of CO, quenched, pumped, and subsequently chased with CO, did not yield a hi-CO signal. Controls that were not quenched but that were pumped, and chased with CO, showed hi-CO whether or not the enzyme was exposed to CO before gases were exchanged.

5.C.5 Other Signals

Under certain conditions, CO induced additional EPR signals from Mo nitrogenase, signals representative of another $S=3/2$ spin system. EPR signals with $g$-factors of ~5.78 and ~5.15, and a very weak, broad signal at $g=2.745$ occurred in samples containing EDTA or a more-concentrated MgATP-regenerating solution (5X ATP) (Figure 5.7). The features at $g=5.78$ and 5.15 (Figure 5.8), or 5.15 alone, also occurred in spectra of early (1 to 8 min) post-exchange samples prepared in 5X ATP. Observed values of the $g$-factors of each peak ranged from $g=5.79$ to 5.78 and $g=5.16$ to 5.13.
Figure 5.6  EPR spectra of EG-quenched Mo nitrogenase in CO before and after exchange of gases. Fe protein:MoFe protein 1:5, 36 μM MoFe protein, 40% EG. A, P<sub>CO</sub> 101 kPa; B, 6 min and C, 53 min after exchange of gases.
Figure 5.7  EPR spectrum of Mo nitrogenase in CO, showing signals from an $S=3/2$ spin system. The signals have $g$-factors of 5.79 to 5.78 and 5.15 to 5.13. Experimental conditions as in Figure 5.5 except 69 µM MoFe protein, 10 min after initiation of turnover. EPR spectroscopy, 100 mW, 10 K.
Figure 5.8  Low field signals from an $S=3/2$ spin system, as in 5.7. Fe protein:MoFe protein 1:1, $P_{CO}$ 101 kPa. A, Mo nitrogenase in a more concentrated MgATP-regenerating solution (5XATP); B, sample in standard MgATP-regenerating solution.
EPR resonances having $g$-factors of 0 to 6 arise from $S=3/2$ spin systems. An $S=3/2$ spin system has two pairs of Kramer's doublets which split in an applied magnetic field. An EPR signal occurs due to transitions between energy levels within a doublet. At any given temperature the energy levels have a Boltzman distribution of populations. As temperature is lowered, the EPR signal arising from the lower doublet increases in intensity relative to that of the upper doublet.

In zero magnetic field the two doublets are separated in energy by $\delta = 2D(1+3(E/D)^2)^{1/2}$, where $D$ is a tensor quantity, $E$ is a zero field splitting parameter, and $E/D$ is a measure of rhombicity. For the metalloenzyme system here, $D$ is large and each doublet can be treated separately as an $S_{\text{eff}}=1/2$ spin system and the $g$-factors can be computed; two methods of calculation were used. The program Rhombo calculates the theoretical values for $g_x$, $g_y$, and $g_z$ for both doublets of an $S=3/2$ spin system as a function of rhombicity. The observed $g$-factors were assigned (using a value for $g_x$ of 2.016) to a spin system having $E/D=0.222$, with $g_x=1.39$, $g_y=1.12$, and $g_z=5.78$ for one ($m_s=\pm3/2$) doublet and $g_x=2.64$, $g_y=5.15$, and $g_z=1.75$ for the other ($m_s=\pm1/2$) doublet. Manual calculation from equations (Hoffman et al., 1976) confirmed the assignments.
The theory predicts that the resonances at \( g = 5.78 \) and \( 5.15 \) arise from different doublets. Temperature dependence studies were performed between 4 and 20K (i.e., 4.0, 7.0, 10.0, 12.0, 15.0 and 20.0\( \pm 0.1 \)K) to determine which of the two doublets is the ground state doublet. The intensity of the \( g = 5.15 \) resonance increased relative to that of the \( g = 5.78 \) peak as the temperature was lowered, indicating that the \( (m_s = \pm 1/2) \) doublet is the ground state doublet and therefore \( D \) is positive. The value of \( D \), 0.43 cm\(^{-1}\), was calculated from the equation for \( \delta \), using the value of \( \delta \) (\( \delta = 0.931 \) cm\(^{-1}\)) obtained from the slope of the above plot (slope = \( \delta / k \), where \( k \) is the Boltzmann constant) and either the program-computed value or the calculated value of \( E/D \).

The predicted high field resonances were not observed; resonances with \( g \)-factors<2 are often too broad to be observed, and indeed, the resonance with a \( g \)-factor of 2.74 was broad and weak.

5.D DISCUSSION
5.D.1.a Turnover-Quenching Agents

EDTA proved to be effective as a quenching agent; the quenching abilities of EG and TCA were confirmed. An unexpected observation was the lag interval before quenching was complete, shown by all three quenching agents.
The delay (lag) cannot be the result of a solution phenomenon, which would be expected to occur quickly, such as chelation of Mg$^{2+}$ ions by EDTA, precipitation of dithionite by TCA, or dissolution of the enzyme in a less polar medium (40% EG). Since TCA probably denatures the proteins immediately, it is unlikely that the lag is due to slow dissociation of Fe protein:MoFe protein complexes and release of bound C$_2$H$_4$, nor would it be due to the enzyme gradually using up dithionite trapped within protein folds.

The most likely explanation of delayed quenching is slow diffusion to the gas phase of dissolved C$_2$H$_4$.

5.D.1.b Quench Turnover: EPR

Persistence of hi-CO from quenched nitrogenase in the presence of CO may be explained in (at least) two ways. CO may remain bound to a cluster within inactive enzyme; this might occur if quenching induces a closer folding or packing of the MoFe protein, leading to trapping of cluster-bound CO. An alternative explanation is that association and dissociation of CO molecules and a nitrogenase metal cluster (cofactor; 6.E.3) continues to occur despite the inactivity of the enzyme. This could occur if quenching resulted in increased access of the cluster to CO. (Even if, in the extreme case, quenching caused the release of metal clusters into the medium, it is unlikely that the same EPR signals would occur from protein-free clusters in CO as from enzyme-bound clusters.
in CO, and furthermore, no visible sign of denaturation of nitrogenase proteins in EG was observed.)

The persistence of hi-CO from quenched enzyme indicates that turnover is not necessary for prolongation of the signal.

The EG-induced EPR signal is of unknown origin. EG may be augmenting a previously undetected signal; EG is known to intensify the \( g=1.94 \) signal of the Fe protein (Hagen et al., 1985; Lindahl et al., 1985). The \( g=5 \) signals induced in the presence of EDTA and CO are discussed in section (5.D.5).

5.D.2.a Post-Exchange of Gases: Activity

CO inhibition of nitrogenase is reversible; this had been shown with nitrogen fixation by cell-free extracts from \( C. pasteurianum \), which is reversibly inhibited by low \( P_{CO} \) (Lockshin & Burris, 1965). Reversible inhibition following incubation under 101 kPa of CO is additional evidence that CO binds tightly (low \( K_i \)) but not permanently to (a) nitrogenase metal cluster.

Incubation under 101 kPa of CO was performed in an attempt to mimic the conditions of Mo nitrogenase in CO in preparation for EPR spectroscopy. Ideally, one would like to investigate the kinetics (enzyme activity) and the EPR spectroscopy of Mo nitrogenase under identical conditions of protein concentration, component protein ratio, headspace gas composition, and temperature of incubation,
among others. In practice, the conditions of each method of inquiry are quite different and it is a challenge to attempt to make them match. While activity assays were typically performed with sub-micromolar concentrations of MoFe protein and in high flux, EPR mixtures were prepared with $\geq 40 \, \mu M$ MoFe protein and in moderate flux (for the primary reason of diminishing the EPR signal in the $g=2$ region, attributable to the Fe protein). For acetylene reduction assays the headspace gas cannot be 100% CO as utilized in most EPR experiments.

5.D.2.b Post-Exchange of Gases: EPR

The question was posed: would the return of enzyme activity, after pumping, be accompanied by disappearance of hi-CO? To investigate the strength of binding of CO to a cluster in nitrogenase, headspace gases were exchanged; the surprising results were that instead of simply decreasing in intensity, hi-CO appeared to convert into lo-CO, with time. This suggests that the MoFe protein cluster that binds CO is not in a fixed form (conformation) independent of the number of CO moieties bound to it, but that depending on conditions such as $P_{CO}$, the cluster can adopt different forms. The EPR spectra also revealed that CO remains bound to a cluster even when the enzyme resumes activity.
The appearance of lo-CO from pumped samples that, before pumping, showed hi-CO, suggests that what determines which CO signal is produced is the concentration of CO dissolved in the medium, as suggested by others (Davis et al., 1979). An inverse relationship of the intensities of lo- and hi-CO occurs as a function of $P_{CO}$ in nitrogenase mixtures prepared individually (Davis et al., 1979); the results presented here are new in that they indicate the apparent conversion of one signal (spin system) into another, from the same sample.

That the lo- and hi-CO signals are interrelated is strongly suggested by the time course of the signals. Spin quantitation of the signals does not indicate that one signal completely converts into another; however, it is not necessary that the signals completely convert in order for them to be related. An important question is, do the two signals arise from the same metal cluster that has several (hi-CO) or few (lo-CO) CO molecules bound (one cluster, two signals hypothesis) or from two different nitrogenase metal clusters, each responsible for one signal (one cluster, one signal hypothesis)?

5.D.3 Post-Quench, Post-Exchange of Gases: EPR

EG quenching did not lock the enzyme in a hi-CO-yielding condition. Instead, a similar pattern of inversely related changes in intensity of hi- and lo-CO signals occurred, following pumping, as in the absence of
EG. Hi-CO diminished more quickly, following pumping, in EG than in the absence of EG, which suggests that EG does not stabilize the hi-CO form of a cluster. Lo-CO occurred earlier in EG-quenched samples than in turning-over mixtures, suggesting that in EG, formation of the lo-CO form of cluster is favored. The observation of lo-CO from low flux Mo nitrogenase following quenching and pumping supports that hypothesis.

EG induced an earlier, stronger, and longer-lasting lo-CO signal than occurred in the absence of EG; for those reasons, EG proved useful in subsequent studies of lo-CO-yielding nitrogenase.

EG may be stabilizing cluster-bound CO, such as by promoting the association or inhibiting the dissociation of a CO-cluster complex. Alternatively, an EG-induced conformation change in the MoFe protein may make potential CO-binding sites more accessible to dissolved CO.

5.D.4 Post-Quench, Post-Exchange: Chase with CO

An unexpected finding was that CO can bind to nitrogenase that is not undergoing turnover. CO binding to a Mo nitrogenase metal cluster may stabilize the cluster in a form that allows it to bind additional CO molecules; alternatively, CO may stabilize certain turnover states which are favorable for CO binding. Other explanations emphasizing the role of EG are described above (5.D.3). Nevertheless, in all reported instances of hi-CO including
this one, turnover is required for the initial binding of CO to a cluster; this suggests that a reduction event is necessary before CO can bind.

5.D.5 Other Signals

While the $g$-factors assigned to the resonances of the additional $S=3/2$ spin system observed here are very similar to those of the $S=3/2$ spin system of the Fe protein (Lindahl et al., 1985), the spin systems are different in that $D$ is positive here while that of the Fe protein is negative (the spin system is inverted).

The large value of the rhombicity (where $0 \leq E/D \leq 0.33$ cm$^{-1}$) suggests that the spin density of the metal cluster responsible for the signals is considerably distorted from that of a perfectly isotropic system.
CHAPTER 6 ENDOR SPECTROSCOPY OF $^{13}$CO-INHIBITED NITROGENASE AND $^{57}$Fe-LABELED HYBRID NITROGENASES

6.A INTRODUCTION

The origin of CO-induced EPR signals was not known. Was CO perturbing the environment of a nitrogenase metal cluster, or was CO binding to a cluster?

Hi- and lo-CO EPR signals were not broadened by $^{13}$CO but were broadened in nitrogenase that contained $^{57}$Fe-labeled MoFe protein (Davis et al., 1979). This finding supported the hypothesis that CO binds to a nitrogenase metal cluster. The lack of broadening of the signals from nitrogenase incubated with $^{13}$CO does not necessarily mean there is no direct interaction of CO with a metal cluster, because an interaction may be too weak to be observed.

If CO EPR signals arise as a result of direct bonding of CO to a nitrogenase metal cluster, $^{13}$C ENDOR signals might be detectable following incubation in $^{13}$CO. An EPR signal arises from a paramagnetic spin system with unpaired electrons, and an ENDOR signal may arise if there is interaction between a paramagnetic spin system and a nucleus with spin (Hoffman, 1991).

Preliminary EPR investigations of nitrogenase incubated in $^{13}$CO revealed evidence of slight broadening of the CO signals.

Small $^{13}$C ENDOR splittings of both CO EPR signals were detected from nitrogenase samples prepared by another
laboratory (see Figure 6.1) (Hoffman & Orme-Johnson, unpublished results). This provided evidence that CO binds directly, albeit weakly, to a nitrogenase metal cluster.

CO-induced EPR signals from Mo-nitrogenase are interchangeable (5.C). It was proposed to employ a pulse-pump-chase procedure, in combination with ENDOR spectroscopy, to inquire into the relationship of lo- and hi-CO. By generating lo-CO from nitrogenase incubated with $^{13}$CO and chasing with $^{12}$CO, the question, is the second CO adding to the same metal cluster that has one bound CO, or to a different cluster? could be addressed.

Hyperfine interactions that would be too weak to cause broadening of the EPR signal are more likely to be revealed in the ENDOR spectrum because of the much greater resolution and sensitivity of the ENDOR technique (Hoffman, 1991).

An account of this work has appeared (Pollock et al. 1995).

6.B MATERIALS & METHODS

6.B.1 Nitrogenase Samples

A. vinelandii Mo nitrogenase component protein purification, protein concentration assay, and activity assays were performed as described (2.A.1,2,3.a). Mo nitrogenase proteins were combined in the ratio of 1:5 Fe protein:MoFe protein (moderate flux).
6.B.2 Apparatus

Isotopically enriched gas ([¹³C]CO {¹³CO}) was obtained from Cambridge Isotope Laboratories.

The assembly for connecting a flask of ¹³CO to a reaction vial, and instructions for how to charge a vial with ¹³CO, are described in the Appendix.

6.B.3 Experiments: "Pulse, Pump" and "Pulse, Pump, Chase"

Mo nitrogenase turnover mixtures were prepared for EPR spectroscopy, as described (2.A.6), with the modifications described here. Turnover solutions were prepared in serum vials (4.65 mL) and contained 8 μM Fe protein and 40 μM MoFe protein, or, in experiments with a very brief "chase", 15 μM Fe protein and 75 μM MoFe protein.

Samples were transferred by means of a gas-tight 100 μL syringe (Fisher) fitted with a 3" 22 gauge SS needle into Ar- or N₂-charged ENDOR (Q-band EPR) tubes made of Suprasil Quartz (2 mm OD x 1 mm ID x ~63 mm) (Heraeus Amersil) and double-stoppered with serum stoppers (Aldrich). Samples were frozen and stored in liq N₂.

In some experiments an EPR sample was prepared, as described (2.A.6), at the same time as an ENDOR sample. (When both an ENDOR and an EPR sample were to be made, the ENDOR sample was made first.)

The "pulse, pump" and "pulse, pump, chase" experiments were performed as described (5.B.3.b,c), with the following modifications. Typically, the duration of each incubation
was: first incubation, 5 min; after adding EG, 5 min; after the first pumping, 5 or 10 min; after the second pumping, 5 min. Turnover mixtures that were small in volume (when no EPR samples were to be made) were incubated for 1 min in the first incubation and for 1 min after pumping, after addition of EG and mixing by vortexing for 10 s; next, the sample was pumped and chased with either $^{12}$CO or $^{13}$CO for either 1 min or for as short a time as possible (see below), as specified.

"Pulse, chase" experiments using opposite isotopes of CO made use of $^{12}$CO as the pulse and $^{13}$CO as the chase, and vice-versa.

6.B.4 Development of a Method for a Short Chase

Three methods were tested to make the "chase" as short as possible. A Mo nitrogenase turnover mixture under CO was prepared for EPR spectroscopy, as described (2.A.6). Turnover was quenched with EG, the atmosphere was pumped, and the incubation was continued for about 10 min.

Then, (i) the atmosphere was pumped and charged with CO, and the solution was incubated and, at intervals, transferred into tubes and frozen; (ii) the solution was transferred into a CO-charged vial (101 kPa of CO), swirled, and at intervals, transferred into tubes and frozen; or (iii) the solution was transferred into a CO-charged EPR tube and was slowly pumped up and down in the
tube by means of the transfer syringe (three times), then the tube was frozen.

Method (i) was rejected because the time of initial contact of the solution with CO could not be accurately determined.

Method (ii) yielded the hi-CO signal, even when the solution was injected into and immediately withdrawn from the CO vial (in total, 10 s exposure to CO).

Method (iii) resulted in spectra that showed both hi- and lo-CO signals of inversely varying amplitudes depending on how long the solution was present in the tube before being frozen; solutions that were present in the tube for <30 sec showed the lo-CO signal only. However, this method was rejected because of concern about inhomogeneous exposure of the solution to CO.

Therefore, method (ii) was adopted in all experiments: a reaction solution was transferred into a CO-charged vial and immediately transferred into an ENDOR or EPR tube and frozen.

6.B.5 EPR and ENDOR Samples

In several experiments, the ENDOR sample was examined by X-band EPR before being shipped for ENDOR analysis. The frozen ENDOR tube was placed inside a N$_2$(g)-charged EPR tube which was immediately stoppered and frozen in liq N$_2$. N$_2$(g) was used instead of Ar because with an Ar-charged EPR tube, Ar liquifies when the tube is immersed into liq N$_2$. 

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and vaporizes when the frozen tube warms in room air. When an Ar-charged EPR tube is used to hold an ENDOR tube, liquid Ar accumulates in the EPR tube when the double tube is frozen; when the EPR tube is transferred from liq N₂ into the cryostat, the liquid Ar vaporizes and causes the ENDOR tube to be ejected out of the EPR tube!

When in place in the cryostat, the EPR tube containing the ENDOR tube was continuously flushed with He(g) to exclude oxygen from the position of the sample in the cavity.

6.B.6 ENDOR Spectroscopy

CW EPR and ENDOR spectra were recorded at Q-band frequencies on a modified Varian E-109 spectrometer equipped with a liquid helium immersion dewar. Spectra were recorded at 2K under rapid-passage conditions. ENDOR signals were observed as an intensity change in the 100 kHz field-modulated dispersion mode EPR signal and were accumulated in the computer. ENDOR spectroscopy was performed by Mr. Hong-In Lee in the laboratory of Dr. B. M. Hoffman, Department of Chemistry, Northwestern University.

6.C RESULTS

ENDOR spectroscopy involves measuring the NMR spectrum of a nucleus associated with an electron spin center. A change in the populations of nuclear energy levels is detected as a change in the EPR signal intensity (Hoffman, 1991).
Figure 6.1.A1 shows the $^{13}$C ENDOR spectrum of a sample prepared under $^{13}$CO and showing lo-CO. The spectrum was taken at the low field inflection ($g=2.09$) of the EPR signal. The ENDOR spectrum is centered at the $^{13}$C Larmor frequency (black inverted triangle) and is split by $A$, the hyperfine coupling parameter.

The spectrum shows one doublet centered at $v^{(13)}C$ and split by $A^{(13)}C \sim 3$ MHz; this indicates that one $^{13}$CO is coupled to the electron spin system. The weak feature in the center of the spectrum, also present in the $^{13}$C ENDOR spectrum of a sample prepared under $^{12}$CO (Figure 6.1.A2), is attributed to ENDOR of natural abundance $^{13}$C in the protein.

The $^{13}$C ENDOR spectrum of hi-CO prepared under $^{13}$CO is shown in Figure 6.1.B2; the spectrum was taken at the $g=2.17$ inflection of the hi-CO EPR signal. The doublet centered at $v^{(13)}C$, with a hyperfine coupling of $A \sim 6$ MHz, indicates that a single $^{13}$CO interacts with the paramagnetic center. The spectrum also shows a central peak that is much more intense than the central peak present in the $^{13}$C ENDOR spectrum of a sample prepared under $^{12}$CO (Figure 6.1.B6). The peak (in Figure 6.1.B2) was resolved into two peaks split by $A \sim 1$ MHz (Figure 6.1.B1), by the pulsed ENDOR technique (Hoffman et al., 1993). This indicates that a second $^{13}$CO is bound (but more weakly coupled) with the paramagnetic center. The
Figure 6.1. $^{13}$C ENDOR spectra of Mo nitrogenase following $[^{12/13}\text{C}]\text{CO}$ treatment. Explanations in text (6.C).
very weak coupling suggests that either CO is bound to a diamagnetic metal ion such as low spin Fe$^{2+}$ or to a metal center with spin density of almost zero.

There are (at least) two explanations for these data: (i) the higher $P_{CO}$ that induces hi-CO simply adds a second CO molecule to the same cluster which has one bound CO; or (ii) two different clusters are each binding one CO molecule, in hi-CO samples.

A pulse-chase approach, employing opposite-isotope CO as either the "pulse" or "chase", presented a way to discern between these possibilities.

Active Mo-nitrogenase was exposed to $^{12}$CO or $^{13}$CO during either the "pulse" or "chase" steps. Samples were examined by $^{13}$C ENDOR spectroscopy.

Figure 6.1.B3 is the $^{13}$C ENDOR spectrum of the $^{12}$CO pulse, $^{13}$CO chase sample. This spectrum is almost identical to that of the hi-CO sample incubated in $^{13}$CO (Figure 6.1.B2). The sample prepared with the opposite combination of isotopes, $^{13}$CO pulse, $^{12}$CO chase, shows no $^{13}$C ENDOR peaks (Figure 6.1.B4). There are two explanations for these data: (i) lo-CO represents CO bound to one metal cluster and hi-CO indicates CO binding to the other cluster; or (ii) one cluster is responsible for both signals, but exchange of bound CO moieties occurs and $^{13}$CO that bound during the "pulse" is replaced by $^{12}$CO during the "chase" incubation.
A way to distinguish between these two possibilities is to "chase" with \(^{12}\text{CO}\) for a very short time before freezing the sample. Figure 6.1.B5 shows the \(^{13}\text{C ENDOR}\) spectrum of such a sample. The spectrum shows the same doublet (with hyperfine coupling \(\Delta \sim 6\ \text{MHz}\)) as that of the \(^{13}\text{CO hi-CO sample}\) (Figure 6.1.B2), indicating the presence of a single, bound \(^{13}\text{CO}\). The spectrum lacks the central peak (observed in Figure 6.1.B2) that indicates a second, weakly-coupled \text{CO} molecule. Even though hi-CO has to have been induced by the additional \(^{12}\text{CO}\), the \(^{13}\text{C ENDOR signal}\) is clear and can only have come from the \(^{13}\text{CO}\) in the samples that had been bound to the lo-CO state. This means that the \(^{13}\text{CO}\) molecule of the lo-CO state has become the \(^{13}\text{CO}\) of the hi-CO state, and the two EPR signals must arise from the same cluster.

The absence of the central peak (compare Figures 6.1.B5 and 6.1.B2) strengthens the interpretation that the "chase" adds a second CO molecule (not seen by \(^{13}\text{C ENDOR}\) when the chase is \(^{12}\text{CO}\)) to the cluster which has a single bound CO (and which results in the conversion of lo- into hi-CO).

Furthermore, spectra B5 and B4 in Figure 6.1 outline a time-scale for the rate of exchange of bound versus free CO; in B5 the bound \(^{13}\text{CO}\) is present in lowered intensity after 30 s exposure to \(^{12}\text{CO}\) but is not observed after a 5 min incubation (B4).
6.D DISCUSSION

ENDOR spectroscopy is a sensitive technique useful for investigating interactions of substrates or inhibitors with a paramagnetic metal center in an enzyme. Hyperfine interactions that would be undetectable by EPR (because of being too small to show as a broadening of the linewidth of an EPR signal) can be revealed in the ENDOR spectrum (DeRose & Hoffman, 1995).

$^{13}$C ENDOR signals from $^{13}$CO-nitrogenase provide direct evidence that CO binds to a nitrogenase metal cluster and that a cluster with bound CO is the center responsible for CO-induced EPR signals.

The experiments presented here investigated the hypothesis that lo- and hi-CO EPR signals arise from one metal cluster that has a variable number of bound CO molecules (one cluster, two signals hypothesis). Alternatively, the CO signals might each arise from distinct metal clusters (the two cluster, two signal hypothesis).

Lo-CO was generated from nitrogenase in $^{13}$CO. When $^{12}$CO was added to this form of the enzyme the resulting hi-CO did not show $^{13}$C ENDOR if the incubation in $^{12}$CO (the chase) lasted 5 min; however, when the chase was brief the hi-CO showed $^{13}$C ENDOR. Significantly, the $^{13}$C ENDOR spectrum resembled that of lo-CO from $^{13}$CO-treated enzyme, providing evidence that lo- and hi-CO arise from the same
metal cluster, the difference being in the number of CO molecules bound to the cluster. A cluster with one bound CO molecule yields lo-CO, and hi-CO arises from a cluster with two bound CO molecules.

It is unlikely that rapid transfer of CO (movement from one cluster onto another) accounts for the results of the 30 s chase, for the following reasons: (i) CO is known to be a tight-binding inhibitor (Morrison, 1969), and (ii) when CO is replaced by substrate as the headspace gas, CO inhibition is reversed, and hi-CO decays, after a lag of a few minutes (5.B.3.b).

The results cannot be explained as resulting from rapid jumping of CO, because the $^{13}$C ENDOR spectrum for the 30 s chase does not show the central peak, indicative of a second bound $^{13}$CO, present in the $^{13}$C ENDOR spectrum of the hi-CO sample prepared under $^{13}$CO. Therefore, it seems that if CO moves from one cluster to another, it does so at a slow rate and little exchange occurs during the 30 s of the $^{12}$CO chase.

ENDOR has revealed what EPR could not: evidence of direct, albeit weak, interaction of CO with a MoFe protein metal cluster.

The results presented here establish that lo- and hi-CO arise from the same metal cluster, one that has either one (producing lo-CO) or two (hi-CO) bound CO molecules.
It is not known if CO binding to a MoFe protein metal cluster is related to CO inhibition of enzyme activity.

Evidence exists for two binding sites for CO (1.C.2.d) (Davis et al., 1979): (i) inhibition occurs within 90 ms, whereas EPR is detected after 4 s; (ii) inhibition occurs with very low $P_{CO}$, whereas $lo$-CO is detected from Mo nitrogenase in $P_{CO} \geq 0.1$ kPa of CO; (iii) with increasing $P_{CO}$, $lo$-CO increases to a maximum then declines rapidly at the same time as $hi$-CO appears (i.e. the reverse of the results of pumping); and (iv) $V$ nitrogenase data suggest that two binding sites exist for CO (3.C.3).

The hyperfine interactions observed in these samples are weaker than those that have been reported for two hydrogenases. Incubation of oxidized Hydrogenase I from C. pasteurianum W5 with CO converts the rhombic EPR signal ($g=2.09$, 2.04, 2.001) into an axial signal ($g=2.07$, 2.01); $^{13}$CO slightly broadens the EPR signal. The $^{13}$C ENDOR spectrum shows a much larger hyperfine coupling ($A \sim 21$ MHz) compared to that of $^{13}$CO-nitrogenase (Telser et al., 1986).

CO has an even more dramatic effect on the EPR spectrum of Hydrogenase II (uptake) from C. pasteurianum W5, oxidized form. The rhombic EPR signal ($g_{av}>2$) arising from a [3Fe-xS] cluster is converted into a different rhombic signal and the signal shows pronounced splitting (Telser et al., 1987).
These enzymes and nitrogenase differ in sensitivity to CO. High $P_{CO}$ irreversibly inhibits both hydrogenases, and low $P_{CO}$ irreversibly inhibits Hydrogenase II; in contrast, CO inhibition of nitrogenase is reversible at high and low $P_{CO}$.

Taken together, information from $^{13}$C ENDOR spectra and enzyme kinetics studies suggests a model whereby CO interaction with a metal cluster influences the degree of CO inhibition of these enzymes; however, there is no direct evidence for this mechanism.

6.E $^{57}$FE ENDOR SPECTROSCOPY OF CO-INHIBITED MO NITROGENASE

6.E.1 Introduction

$^{13}$C ENDOR of Mo nitrogenase treated with $^{13}$CO revealed that one or two CO molecules bind to a MoFe protein metal cluster and this binding results in lo- or hi-CO, respectively (6.C). The question remained: to which metal cluster does CO bind? The question of which metal cluster (P cluster or FeMo cofactor) binds CO was addressed by preparing MoFe protein with selectively $^{57}$Fe-labeled clusters. An account of this work has been published (Christie et al., 1996).

In the following study, the proteins, prepared in another lab and treated there with CO, showed weak CO EPR signals and no $^{57}$Fe ENDOR signals. It was thought that more intense CO signals were needed before a conclusion about the presence or absence of $^{57}$Fe ENDOR could be drawn.
6. E. 2 Materials & Methods

The following hybrid protein was prepared by P. Christie in the laboratory of Dr. W. H. Orme-Johnson at M.I.T.

Apoprotein was purified from a \textit{nifB}^{-} strain of \textit{A. vinelandii} (UW45), a strain which synthesizes component 1 protein that lacks the FeMo cofactor. To this protein was added cofactor extracted from the MoFe protein of \textit{57Fe}-grown wild-type cells. The resulting hybrid protein, containing unlabeled P clusters and \textit{57Fe}-labeled cofactor, was called "M57,P56".

Apoprotein containing \textit{57Fe}-labeled P clusters was prepared by growth of UW45 cells in \textit{57Fe}-containing media.

Four forms of MoFe protein were prepared, which incorporated: natural abundance Fe in both clusters (P56,M56); \textit{57Fe} in both clusters (P57,M57) (globally-enriched); \textit{57Fe}-labeled P cluster and natural abundance cofactor (P57,M56); or natural abundance P cluster and \textit{57Fe}-labeled cofactor (P56,M57).

In initial experiments, no \textit{57Fe} interaction with the CO-bound paramagnetic center was detected. In our lab a second experiment was attempted.

M57,P56 nitrogenase was diluted in non-reducing buffer (0.025 M \textit{Tris-HCl} (pH 7.4), 0.2 M NaCl) and re-concentrated in a Minicon A25 concentrator (Amicon). This step was performed in an Ar-filled glove box. A turnover mixture
under CO was prepared using M57,P56, as described (6.B), except that the mixture was incubated was for ~1.5 min and the ratio of Fe protein:MoFe protein was 2.

Nitrogenase containing each form of MoFe protein was examined by $^{57}$Fe ENDOR spectroscopy of the hi-CO signal. If CO binds to an $^{57}$Fe-containing cluster, CO-induced EPR would be expected to show hyperfine interaction with $^{57}$Fe nuclei. Each magnetically-distinct $^{57}$Fe would be revealed in the ENDOR spectrum as a doublet centered at A/2 and split by $\pm v_{Fe}$ with frequencies of $v_{\pm}=A/2\pm v_{Fe}$.

6.E.3 Results

Figure 6.2.B1 shows the $^{57}$Fe ENDOR spectrum of hi-CO from M57,P56 under CO. Three, possibly four, overlapping doublets can be observed. Each pair of peaks is centered at A/2 and is split by 2$v^{(57)Fe}$. Comparison with spectra obtained from non-hybrid, fully-labeled nitrogenase indicates that the splitting of each doublet is ~3 MHz, therefore $v^{(57)Fe}$ ~1.5 MHz. The hyperfine coupling (~22-33 MHz) of the 3 or 4 iron atoms to the CO-bound cluster indicates that CO is bound to the $^{57}$Fe-labeled cluster.

Globally-enriched protein (Figure 6.2.A1) shows strong $^{57}$Fe ENDOR signals, which are clear evidence of CO interaction with two types of Fe and tentative evidence of interaction with two additional Fe atoms. Natural abundance Fe-containing protein does not show ENDOR signals (Figure 6.2.A2). Of the hybrids, only nitrogenase with the
Figure 6.2  $^{57}\text{Fe}$ ENDOR spectra of hybrid forms of Mo nitrogenase in CO. For explanations see text (6.E.3).
M57,P56 form of MoFe protein shows $^{57}$Fe ENDOR (Figures 6.2.B1 and 2).

6.E.4 Discussion

$^{57}$Fe ENDOR spectroscopy of CO-inhibited Mo nitrogenase hybrids containing metal clusters selectively enriched with $^{57}$Fe revealed that CO binds to the FeMo cofactor and not to the P cluster. This finding is significant, for it strengthens the suggestion that the site of CO inhibition and probably substrate reduction is the cofactor.

Christie and co-workers (1996) speculated that CO binds to a reduced state of the cofactor during turnover but hi-CO arises from CO-bound cofactor that has returned to its resting state reduction level and now has an alternate conformation compared to the protein in the absence of CO.

Without a (spectroscopic) technique capable of detecting differences in the conformation of the protein micro-environment surrounding the cofactor, this hypothesis is difficult to test. EXAFS spectroscopic investigations, currently underway, may provide relevant information as to whether cofactor with CO bound and cofactor lacking CO have significantly different structures.

An alternative hypothesis is also reasonable. As described (4.D), CO may bind to enzyme in $E_2$ or $E_3$, and stay bound as the enzyme cycles through its redox states; what is detected by EPR may be $E_0$-CO or $E_2$-CO.
The finding that CO binds to the cofactor is a significant step on the path towards elucidation of the mechanism of nitrogen fixation by nitrogenase.
CHAPTER 7 CURRENT & FUTURE INVESTIGATIONS

For CO- or substrate-induced signals to appear under what are, normally, EPR-silent conditions (turnover) suggests that in the presence of CO (or substrate) EPR-active intermediate states accumulate and become detectable (Yates & Lowe, 1976; Eady, 1980). CO or the substrate may stabilize an intermediate turnover state which is transient in the absence of CO or substrate (5.D). It is also possible that CO or substrate may bind to an EPR-silent state and remain bound as the protein relaxes to the next lower reduction state, which then becomes EPR-detectable (discussed in 4.D).

The CO pulse-chase method in combination with ENDOR spectroscopy has the potential to reveal interactions of substrate or inhibitor molecules with nitrogenase metal clusters, using the CO signal as a marker of metal cluster involvement in a turnover state (section 6). Mo-nitrogenase, incubated in the presence of CO and isotopically-labeled substrates or inhibitors other than CO and examined by ENDOR spectroscopy, may reveal direct evidence of substrate- or inhibitor-metal cluster interactions.

EPR signals unique to nitrogenase under various substrates (in the absence of CO) (Lowe et al., 1978) have been insufficiently explored by researchers and merit
investigation with isotopically-labeled substrates and ENDOR spectroscopy.

7.A C_2H_2 AND C_2H_4

Weak EPR signals (2-4% of unpaired-electron spins per mole of MoFe protein) can be detected from nitrogenase incubated in C_2H_2, C_2H_4, or Ar (Lowe et al., 1978). Like those induced by CO, the signals are only observed from turnover samples.

7.B CN^-

Cyanide ion (CN^-), like CO, is a potent inhibitor of nitrogenase. Studies with CN^- are complicated by the possibility of nitrogenase-catalyzed reduction of HCN. By dissolving the sodium salt of cyanide ion in buffer of a controlled pH, a supply of the ion can be assured.

CN^- inhibits H_2 evolution by nitrogenase; CO does not; however, low concentrations of CO relieve the cyanide inhibition (Li et al., 1982). The EPR signal from isolated FeMo cofactor changes form and g-factors on binding CN^- (Richards, 1985, cited in Smith et al., 1985). These data suggest that CN^- may interact directly with a nitrogenase metal cluster. Therefore, CN^- is a candidate for an ENDOR study with nitrogenase.

7.C N_2

Isotopically enriched gas ([^{15}N]N_2 {^{15}N_2}) was obtained from Cambridge Isotope Laboratories.
Initial investigations utilizing a CO pulse, $^{15}$N$_2$ chase did not reveal $^{15}$N ENDOR resonances. This result, although negative, supports an earlier report that one of the EPR signals observed under C$_2$H$_4$ is abolished by C$_2$H$_2$, HN$_3$ or HCN but not by N$_2$ (Lowe et al., 1978). It has been suggested, based on nitrogenase enzymology, that N$_2$ binds at a different site than that of other nitrogenase substrates or inhibitors, which may explain these observations.

7.D C$_2$H$_2$ AND $^{13}$C ENDOR SPECTROSCOPY

Regarding the question, does C$_2$H$_2$ interact with cofactor? we proposed to use $^{13}$C ENDOR spectroscopy (in collaboration with Dr. B. M. Hoffman and Mr. H-I. Lee) to look for evidence of interaction between $^{13}$C]C$_2$H$_2$ and lo- or hi-CO-yielding Mo nitrogenase.

For hi-CO samples, moderate flux Mo nitrogenase was incubated (1.0 min at 200 rpm, 23°C) in a gas mixture consisting of either 80% $^{12}$C$_2$H$_2$ or 80% $^{13}$C$_2$H$_2$ and 20% CO, and samples for ENDOR spectroscopy were prepared. The concentration of MoFe protein in ENDOR samples was 225 µM.

For lo-CO samples, Mo nitrogenase was incubated as described above, quenched with EG, pumped, and chased for 10 min with same-isotope $^{12/13}$C$_2$H$_2$ (the chase atmosphere was prepared by transfer of the gas into the vial using a lockable syringe).
No $^{13}$C ENDOR signals were detected from the hi-CO sample. Difficulties were encountered in generating lo-CO; improvements are needed in the method so that it can be performed with small volumes of nitrogenase mixtures contained in small vials.

7.E \textbf{C}_2\text{H}_2 \text{ AND EPR SPECTROSCOPY}

Further to the question, does \text{C}_2\text{H}_2 interact with cofactor? EPR investigations were undertaken.

Resting state MoFe protein under 100\% \text{C}_2\text{H}_2 was examined by EPR spectroscopy; controls were prepared under \text{Ar} (EPR tubes were charged with the same gas as that of the incubation atmosphere).

Preliminary results indicated that the $g=4.3$ resonance shifted in position (from 4.30 to 4.28), but was not broadened, in the presence of \text{C}_2\text{H}_2.

Moderate flux Mo nitrogenase turning over in a gas mixture consisting of either 80\% $^{12}$\text{C}_2\text{H}_2 or 80\% $^{13}$\text{C}_2\text{H}_2 and 20\% \text{CO yielded a cofactor signal that was not shifted in its } g=4.3 \text{ resonance and that showed a suggestion of broadening of the } g=3.67 \text{ resonance; hi-CO also appeared to be slightly broadened but the signal was weak.}

7.F \textbf{ADDITION OF CO AFTER EG QUENCH}

Moderate flux Mo nitrogenase, incubated in the absence of \text{CO}, quenched with EG, incubated again in the absence of \text{CO}, then chased with \text{CO}, did not yield \text{CO} EPR signals, as expected for a system in the absence of turnover. It was
of interest to determine the time course of the inability of the cluster to bind CO.

Moderate flux Mo nitrogenase was incubated in the absence of CO for 30 s, quenched with EG (controls received MgATP-regenerating solution instead of EG), incubated again for variable times, quickly pumped, then chased with CO for a fixed interval (40 s) before EPR samples were prepared. The experiment was performed twice.

CO binding occurred when CO was added at 10, 20 and 30 s after addition of EG and did not occur 1 min after the quench, in the first experiment, and CO binding was detected when CO was added within 2 min after EG but was not observed from the 5 min sample, in the second experiment. These results may be interpreted as indicating that EG-quenching of turnover occurs slowly, allowing CO to bind to a nitrogenase cluster (cofactor) within the period before turnover ceases, an interpretation in agreement with the observed lag in EG-quenching of activity (5.C.1.a).

7.G COFACTOR SIGNAL AFTER EG QUENCH

The return of the cofactor signal is biphasic, following addition of EG, with high flux Mo nitrogenase (Blanchard, 1995). If the first phase corresponds to the relaxation of proteins in "higher" E states to E₁, and the second phase corresponds to E₁→E₀, then EG treatment of a turnover mixture comprised of only E₁ and E₀ should show
only one relaxation phase ($E_1 \rightarrow E_0$) in response to addition of EG.

Low flux Mo nitrogenase in steady state (anaerobic incubation in the absence of CO, 12 to 20 min at 180 rpm, 23°C) was quenched by addition of EG, and timed samples following the quench were examined by EPR spectroscopy.

The results were inconclusive. Following addition of EG (40%), the cofactor signal slowly regained intensity but showed wide variation in intensity between samples, in one experiment. Addition of 27% EG, in other experiments, may have been insufficient to quench turnover. These results are preliminary, and improved techniques for rapid preparation of samples for EPR spectroscopy should result in less variation between samples.

7.H MO NITROGENASE IN SUBSTRATE GASES

Mo nitrogenase was incubated in various substrate gases ($C_2H_2$, $C_2H_4$, $N_2$) and examined by EPR spectroscopy. Substrate-induced EPR signals might provide evidence for substrate binding to transient turnover states of the enzyme. Preliminary investigations did not reveal unique substrate-specific spectroscopic signals.

7.I FTIR

CO binds to Mo nitrogenase cofactor (see 6.E.3). The nature of CO binding is not known; does it bind to an Fe or a Mo atom, or bind in some other way? FTIR spectroscopy
can detect metal-carbonyl bonding, and may be useful for determining the metal to which CO may bind.

Establishment of the FTIR instrument conditions necessary for use with nitrogenase is being undertaken in collaboration with Ms. Brenda Weiss in the laboratory of Dr. S. Cramer, Department of Physics, University of California at Berkeley. Mo nitrogenase under CO, prepared here, has been used in development of experimental techniques for use with the FTIR instrument.

7.J ALF-ADP-INHIBITED TRANSITION STATE COMPLEX

Two very recent, independent studies of an ALF-ADP-inhibited nitrogenase (Duyvis et al., 1996; Renner & Howard, 1996) show that the Fe protein in the Fe protein-MoFe protein complex is apparently frozen in a transition state conformation (by means of the Al fluoride-ADP combination). The x-ray crystal structure of the ALF-ADP inhibited transition state complex of the Fe protein-MoFe protein complex is currently being determined (D. C. Rees & co-workers, Caltech). Experiments in the presence of CO would reveal if CO could bind to Mo nitrogenase in a transition state.
CHAPTER 8 SUMMARY

The details of the mechanism of nitrogenase-catalyzed reduction of N₂ and C₂H₂, among other substrates, are little understood. In the present work, the inhibitor and spectroscopic probe CO was used to investigate properties of nitrogenases undergoing turnover.

The responses of V nitrogenase to low P<sub>co</sub> suggest that the enzyme has two binding sites, opposite in effect, for CO, and that C₂H₄ and C₂H₆ formation probably occur by separate mechanisms. The mechanism for C₂H₄ formation is more sensitive to the rate of electron flux than to P<sub>co</sub>, while the C₂H₆-forming process is more influenced by P<sub>co</sub> than by the electron flux rate.

Examination of low flux Mo nitrogenase allowed the onset and development of hi-CO to be observed. The inhibitor binding characteristics (revealed as the hi-CO EPR signal) of Mo nitrogenase in low flux indicate that CO binds to a mechanistically late-stage form of the MoFe protein, and the rate of CO dissociation is much lower than its rate of association. Two or more reduction events may be necessary for the formation of an EPR-active form of the enzyme capable of binding CO.

The inversely correlated intensities of lo-CO and hi-CO from pumped Mo nitrogenase in moderate flux suggest that these signals arise from the same metal cluster; however,
the data can also be explained on the basis of one metal cluster yielding one signal and the other signal arising from the other metal cluster. A $^{13}$C ENDOR investigation of a pulse, pump, chase experiment utilizing different C isotopes of CO allowed discernment that the one cluster, two signal hypothesis is correct; both CO-induced EPR signals arise from the same metal cluster.

$^{57}$Fe ENDOR was used to observe CO interaction with the nitrogenase cofactor, and additional ENDOR investigations have been proposed to further investigate interactions between CO-bound cluster and isotopically-labeled substrates.

Results from this study suggest that (i) only a suitably ($\geq$two-electron) reduced form of the enzyme may have the ability to bind CO; (ii) nitrogenase metal clusters with CO already bound may be better able to bind additional CO molecules than clusters lacking CO; and, (iii) the mechanisms of product formation may be uniquely affected by inhibitor concentration and by electron flux.

CO has proven to be a useful means to probe nitrogenase during turnover. It is hoped that the findings presented here will serve to stimulate further inquiry into the nature of nitrogenase catalysis.
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APPENDIX: ASSEMBLY FOR CONNECTING A FLASK OF $^{13}$CO TO A REACTION VIAL

1. Flask Fittings

A 1 L break-seal flask of $^{13}$CO is fused to a glass tube terminating in a stopcock (A) and fitted with a side-arm containing a piece of steel rod capable of being moved by a magnet. One end of a cannula is pushed through the rubber stopper plugging the exit tube from the stopcock; the other end of the cannula is inserted through the rubber septum of a reaction vial fitted to a Schlenk line.

2. The First Time the $^{13}$CO Flask is Used

2.a. (Stopcock A is open.) By repeated evacuation-Ar-charge cycles, finishing with an evacuation, make the entire assembly from the Schlenk line to the seal of the $^{13}$CO flask, anaerobic.

2.b. Close stopcock A. Use a magnet to move the piece of steel rod from the side arm into the main tube. Let the piece of steel drop onto the seal and break the seal.

3. To Fill a Reaction Vial with $^{13}$CO

3.a. Make sure stopcock A is closed and the chamber between it and the $^{13}$CO flask is charged with $^{13}$CO (steps 1 and 2 above).

3.b. Repeat the evacuation-Ar-charge cycle at least 3 times, finishing with an evacuation.

3.c. Close stopcock at Schlenk line (stopcock B). Open stopcock A (which fills the reaction vial with $^{13}$CO). Wait
(count to 12) before closing stopcock A. If the vial contains a solution, gently shake the vial while charging it with $^{13}$CO.

3.d. Withdraw cannula from the reaction vial stopper. Detach the reaction vial from the Schlenk line, and continue the incubation.
VITA

Linda is one of three children of Ian and Jean Cameron, of Vancouver, B.C., Canada. Linda attended Handsworth Secondary School in North Vancouver and the American International School in Kuala Lumpur, Malaysia. After high school, she lived with her family in Belo Horizonte, Brazil for two years. Linda obtained a B. Sc. in Biochemistry from the University of British Columbia (U. B. C.), Vancouver in 1978. She was a lab technologist in a hospital chemistry lab, then after a period of travel she studied Pathology (M. Sc., 1988) at U. B. C. She carried out research in the Philippines towards her M. Sc. degree.

Linda shares her parents love of music, reading, and walks by the seashore.
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Date of Examination:

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