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Iron–Sulfur Clusters in the Molybdenum–Iron Protein Component of Nitrogenase. Electron Paramagnetic Resonance of the Carbon Monoxide Inhibited State†

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ABSTRACT: Carbon monoxide inhibits reduction of dinitrogen (N_2) by purified nitrogenase from *Azotobacter vinelandii* and *Clostridium pasteurianum* in a noncompetitive manner (K_{ii} and K_{is} = 1.4×10^{-4} and 4.5×10^{-4} and 7×10^{-4} atm and 14×10^{-4} atm for the two enzymes, respectively). The onset of inhibition is within the turnover time of the enzyme, and CO does not affect the electron flux to the H_2-evolving site. The kinetics of CO inhibition of N_2 reduction are simple, but CO inhibition of acetylene reduction is complicated by substrate inhibition effects. When low-temperature (~13 K) electron paramagnetic resonance (EPR) spectra of CO-inhibited nitrogenase are examined, it is found that low concentrations of CO ([CO] = [enzyme]) induce the appearance of a signal with *g* values near 2.1, 1.98, and 1.92 with $t_{1/2} \approx$ 4 s, while higher concentrations of CO lead to the appearance of a signal with *g* values near 2.17, 2.1, and 2.05 with a similar time course. The MoFe proteins from *Rhizobium japonicum* and *Rhodospirillum rubrum*, reduced with *Azotobacter* Fe protein in the presence of CO, give similar results. Under conditions which promote the accumulation of H_2 in the absence of CO, an additional EPR signal with *g* values near 2.1, 2.0, and 1.98 is observed. The use of *Azotobacter* nitrogenase components enriched selectively with ^{57}Fe or ^{95}Mo, as well as the use of ^{13}CO, permitted the assignment of the center(s) responsible for the induced signals. Only ^{57}Fe, when present in the MoFe protein, yielded broadened EPR signals. It is suggested that the MoFe protein of nitrogenase contains one or more iron–sulfur clusters of the type found in the simple ferredoxins. It is further proposed that the CO-induced signals arise from states of the MoFe protein in which CO inhibits electron flow to the N_2-reducing site so that the iron–sulfur cluster achieves steady-state net charges of −1 (high CO complex) and −3 (low CO complex) in analogy to the normal paramagnetic states of high-potential iron–sulfur proteins and ferredoxins, respectively. The "no-CO" signal may be either an additional center or the N_2-reducing site with H_2 bound competitively.

Of the iron–sulfur enzymes (Orme-Johnson, 1973), surely nitrogenase is among the least understood with regard to structural features of the prosthetic groups. Enzymatic activity—the MgATP-dependent reduction of N_2, H^+, or acetylene—requires both an Fe protein and a MoFe protein as well as MgATP and a low-potential reductant (Burris & Orme-Johnson, 1974).

The Fe protein binds MgATP and appears to be the proximal reductant of the MoFe protein. The Fe protein in most organisms appears to contain four iron and four labile sulfur atoms per molecule of about 6×10^4 molecular weight. The electronic (Ljones, 1973) and EPR[1] (Orme-Johnson et al., 1972; Eady et al., 1972; Mortenson et al., 1973) spectroscopic properties suggest that the protein possesses a ferredoxin-like iron–sulfur cluster. However, the key property, *MgATP-dependent* electron transfer or "reductive dephosphorylation", remains unexplained.

The structures present in the MoFe protein are less clearly defined. The metal composition is still uncertain [see Eady et al. (1972), Zumft & Mortenson (1975), and Orme-Johnson & Davis (1977) for discussions of the data]. The limits of recently reported compositions seem to be 1 to 2 Mo atoms and 15 to 33 iron atoms per molecule of 2×10^5 to 2.5×10^5 molecular weight. In addition to possible differences between organisms, it has been suggested that partially metal-depleted (or deficient) protein species may accompany the MoFe protein during purification (Zumft & Mortenson, 1973). For example,

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[1] Abbreviations used: EPR, electron paramagnetic resonance; Cp1, MoFe protein from *C. pasteurianum*; Cp2, Fe protein from *C. pasteurianum*; Av1 and Av2 similarly from *A. vinelandii*; nomenclature of Eady et al. (1972).

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in our recently reported Mössbauer study of ^{57}Fe-enriched MoFe protein from *Azotobacter vinelandii* (Münck et al., 1975), we found that the recrystallized protein, homogeneous by gel electrophoresis, contained 2 g at Mo and 24 g at Fe per 3×10^5 g of protein. We based our discussion of the Mössbauer spectra on the assumption that functioning molecules contain two Mo atoms, though the reported molecular weight of the protein is 2.2×10^5 (Kleiner & Chen, 1974) to 2.5×10^5 (Swisher et al., 1975). More recent evaluations suggest that 33 ± 2 Fe and 2 Mo is the correct composition (Zimmerman et al., 1978). Under such circumstances it is especially important to determine whether there is indeed more than one population of proteins in the preparation, whether there are distinguishable prosthetic groups, and whether all of the spectrally detectable species are involved in catalysis. With the MoFe protein of nitrogenase, this program has only begun (Orme-Johnson et al., 1978).

The MoFe proteins isolated to date exhibit EPR spectra with g values near 4.3, 3.7, and 2.0 [this work and also Zumft & Mortenson (1975)]. These signals have been assigned to an iron–sulfur center in the spin 3/2 state which is reduced to an EPR-silent form in the steady state present during nitrogen reduction. For the MoFe protein of *A. vinelandii* (Münck et al., 1975), we estimated that the zero-field splitting was $\Delta k \approx 15$ K and that ca. one spin per Mo was present. The Mössbauer spectra of both *A. vinelandii* (Münck et al., 1975; Zimmerman et al., 1978) and *Klebsiella pneumoniae* (Smith & Lang, 1974) MoFe proteins contain a component, associated with the EPR signal and comprising roughly 40% of the Fe present, which becomes EPR-silent (integer spin) on passing to the reduced steady-state form of the MoFe protein. We deduced from these data that one cluster per Mo atom, containing six iron atoms with a net spin state of 3/2, is responsible for these phenomena. We have also recently detected Fe_4S_4 cores in the protein, in addition to the $S = 3/2$ centers (Orme-Johnson et al., 1978). In this communication we report the results of EPR measurements on nitrogenase functioning in the presence of the potent inhibitor CO, in which we observed additional spectroscopic states identifiable with specific oxidation states of the simpler ferredoxins and high-potential iron–sulfur proteins. These states originate from the MoFe protein component of nitrogenase.

Materials and Methods

Nitrogenase components were prepared by Dr. V. K. Shah from *A. vinelandii* using the method of Shah & Brill (1973). Those from *Clostridium pasteurianum* were prepared by the method of Tso et al. (1972) or our own unpublished procedure (Henzl et al., unpublished experiments). MoFe proteins from *Rhodospirillum rubrum* and *Rhizobium japonicum* were generously provided by Dr. Paul Ludden and Dr. H. J. Evans. Morpholinepropanesulfonic acid (Mops) was the product of Sigma Chemical Co., and tris(hydroxymethyl)aminomethane (Tris) was the enzyme research grade of General Biochemicals Corp. Natural-abundance CO was from Matheson Gas Products and was stored over 10% pyrogallol and 10% sodium carbonate in water in order to remove O_2. The ^{13}CO (90% enrichment) was obtained from Merck of Canada, Ltd. The ^{57}Fe (91% enrichment) was obtained from Union Carbide Corp. as Fe_2O_3 and was converted to $FeCl_3$ before incorporation in the growth medium for *A. vinelandii*, as described earlier (Münck et al., 1975). The ^{95}Mo was converted from metal (Union Carbide) to sodium molybdate by alkaline fusion. Sodium dithionite was obtained from British Drug Houses through the Gallard Schlessinger Co., and other chemicals were reagent-grade commercial products.

The apparatus utilized for anaerobic manipulations of the nitrogenase components has been described before (Orme-Johnson et al., 1972; Orme-Johnson & Beinert, 1969). For several experiments in which a large excess of sodium dithionite was present in the enzyme solution, quartz EPR tubes (5-mm o.d.) capped with serum vial stoppers were employed. The air initially in the tubes was replaced with N_2 or Ar ($O_2 <$ 1 ppm) by repeated cycles of evacuation and flushing via a 22-gauge needle inserted through the stopper. Enzyme components and reactants were subsequently introduced via Hamilton syringes. The samples were frozen by plunging the EPR tubes into a rapidly stirred isopentane bath held at 130 K. EPR spectroscopy was performed either with a modified Varian V-4500 instrument (Palmer, 1967) in the laboratory of H. Beinert or with a Varian E-9 spectrometer. Both spectrometers are fitted with apparatus for low-temperature operation at 9.2 GHz. This technique and the evaluation of spectra by double integration and comparison to standards were as described (Tsai et al., 1970; Palmer, 1967).

The measurement of nitrogenase activity was carried out by three methods: by the MgATP-dependent rate of dithionite oxidation (Ljones & Burris, 1972) as measured spectrophotometrically at 350 nm where the molar absorbancy change per 2 equiv of electrons transferred is 1300 (Davis et al., 1975), by the gas chromatographic determination of the reduction of acetylene to ethylene (Shah et al., 1972), and by the measurement of ammonia production after microdiffusion using the indophenol method (Burris, 1974). The first procedure was carried out in 1-cm path length silica cuvettes capped with serum stoppers, while the latter two assays were run in volume-matched sets of serum vials (5 or 15 mL of nominal volume). The reactants were introduced via gas-tight syringes after evacuation and flushing of the vials.

Enzyme activities are expressed as International Enzyme Units (units = micromoles per minute) or, for specific activities, as nanomoles per minute per milligram of protein. Concentrations of substrates and inhibitors used in this study are expressed in atmospheres. For calculations of substrate or inhibitor concentrations in the aqueous phase, we have used the tables in Lange's Handbook of Chemistry. Our own determination of the partitioning of ethylene and acetylene between aqueous and gas phases under conditions representative of typical enzyme assays agreed with the published values to within the error limits of our gas chromatographic technique. As will become clear under Results, inhibition of nitrogenase by CO occurs under two different limiting conditions. For steady-state kinetic assays, the total amount of CO in the system is large relative to the amount of enzyme, whereas for most EPR experiments the amount of CO accessible to the enzyme is not in large excess. In describing both kinds of experiments, we express concentrations in terms of partial pressures (atmospheres) of gas which are in equilibrium with the aqueous phase in the absence of enzyme. EPR signals elicited from the functioning enzyme in the presence of CO are classified as high pCO or low pCO signals on the basis of their EPR characteristics and not on the basis of absolute concentrations of CO in a particular system.

Results

Steady-State Kinetics of CO-Inhibited Nitrogenase. It has been known since the reports of Lind and Wilson (Lind & Wilson, 1941; Wilson & Lind, 1943) that carbon monoxide is a potent and specific inhibitor of nitrogen fixation in vivo. Lockshin & Burris (1965) reported that CO is a competitive inhibitor of nitrogen reduction in crude extracts from *C. pasteurianum*, whereas Hwang et al. (1973) found that when

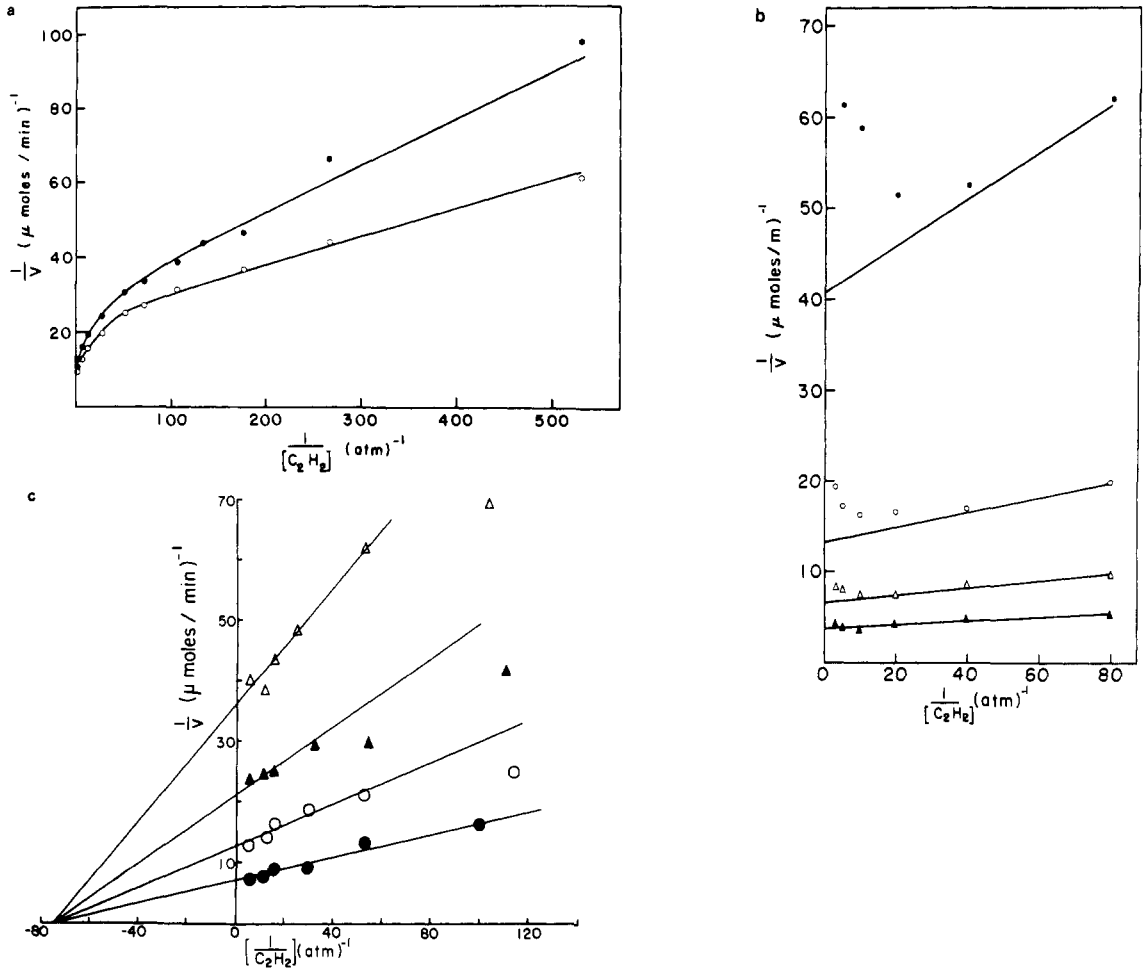

FIGURE 1: (a) Acetylene reduction by purified nitrogenase components from *C. pasteurianum*. Assays were carried out as described by Shah et al. (1972) using a buffer of 100 mM Tris–Mops at a final pH of 6.9 at 30 °C. The ordinate is the reciprocal of micromoles per minute and the abscissa is in reciprocal atmospheres of acetylene. Enzyme components were purified by the method of Henzl et al. (unpublished experiments). The MoFe protein (Cp1) had a specific activity greater than 1000 nmol/(min mg) while the specific activity of the Fe protein (Cp2) was 1500 nmol/(min mg) in a standard assay at pH 6.9 with 0.5 atm of acetylene. Solid circles are for a relative excess of Cp2; open circles are for a relative excess of Cp1; lines are a least-squares computer fit to the experimental data. For "excess" Cp2 there was 0.097 unit of Cp1 and 0.224 unit of Cp2 per mL, while for "excess" Cp1 there was 0.194 unit of Cp1 and 0.112 unit of Cp2 per mL. (b) Substrate inhibition of *A. vinelandii* acetylene reduction with varying component ratio. Assays were carried out by using the method of Shah et al. (1972) with 0.025 M Tris-HCl buffer, pH 7.4. Av1 was a crystallized fraction (Shah & Brill, 1973), while Av2 was a fraction from the second DEAE chromatography as described by Shah & Brill (1973). Abscissa and ordinate are as described in (a). The concentration of Av1 was kept constant at 530 μg/mL (0.75 unit/mL) with Av2 added at the indicated amounts: (▲) 0.025; (△) 0.062; (●) 0.125; (○) 0.250 unit. The lines were visually fitted to an apparent K_m of 0.0125 atm. By use of this apparent K_m, the inhibition constant fitted manually as described by Cleland (1970) was 0.3 atm (omitted for clarity). (c) Apparent K_m for *A. vinelandii* nitrogenase with excess Av2. Assays carried out by the method of Shah et al. (1971) using a buffer of 100 mM K^+–Mops, pH 7.2, at 30 °C. Av1 had an activity of 15.5 units/mL with a specific activity under standard assay conditions of >1500 nmol/(min mg). Av2 had an activity of 710 units/mL with a specific activity of 1000 nmol/(min mg). Reaction mixtures contained 5 μL of Av1 and and varying amounts of Av2 from 2.5 to 25 μL. Abscissa and ordinate are as described in (a). Solid circles (●) are activities observed with 25 μL of Av2 (component ratio of 0.075 unit of Av1/0.25 unit of Av2); open circles (○), 10 μL of Av2; solid triangles (▲), 5 μL of Av2; open triangles (△), 2.5 μL of Av2. The lines are fitted to an apparent K_m of 0.0125 atm.

nitrogen-fixing particles from *A. vinelandii* were employed, CO inhibited the reduction of nitrogen and acetylene in a noncompetitive fashion. Bergerson & Turner (1973) reported competitive inhibition of soybean bacteroid nitrogenase.

(*a*) *Electron Utilization.* Carbon monoxide is not an inhibitor of H^+ reduction (MgATP dependent) catalyzed by nitrogenase (Hwang et al., 1973), whereas CO does inhibit H^+ reduction by *C. pasteurianum* hydrogenase (Erbes et al., 1975) in a competitive manner. A series of manometric experiments conducted in collaboration with Dr. D. Erbes verified that CO, at either 0.01 or 0.001 atm (with argon making up the balance of the gas phase), does not affect the rate of hydrogen evolution from purified *C. pasteurianum* nitrogenase within the accuracy of the measurements (+10%). We also found that, when hydrogen evolution was allowed to proceed until the MgATP was exhausted, the amount of dithionite used per MgATP consumed (approximately 0.2 mol/mol) was unaffected by the presence of CO. This verified earlier findings of Hwang et al. (1973). Thus, CO does not alter the steady-state rate of electron utilization by nitrogenase.

(*b*) *Acetylene Reduction.* Before examining the effect of CO on acetylene reduction by purified nitrogenase components, we did a set of control experiments with acetylene as the variable substrate. It has previously been reported (Shah et al., 1975) that there is substrate inhibition by acetylene in assays of *A. vinelandii* nitrogenase. The results obtained with *C. pasteurianum* are in striking contrast to this as shown in Figure 1. The results in Figure 1a can be analyzed in terms of a 2:1 function (Cleland, 1970). One simply assumes that there are two forms of an enzyme present, which have different K_m values for the reducible substrate, and generates a least-squares fit to determine the function of the variable

substrate concentration. The lines in the figure are computer generated; data points used to generate the lines are shown as solid or open circles for two different component ratios. The low K_m derived from these fittings is 0.003 ± 0.002 atm while the high K_m is 0.23 ± 0.1 atm. Although these are very different discrete K_m values, they would not be recognized as such if acetylene concentration was varied less than 10-fold (Bergerson & Turner, 1973) about the previously reported K_m of 0.01 atm (Schöllhorn & Burris, 1967).

In parts b and c of Figure 1, we show the effect of varying the component ratio and acetylene concentration on *A. vinelandii* nitrogenase. In these parts of the figure, it can be seen that there is substrate inhibition in *A. vinelandii* which varies as a function of the component ratio. Over the range where Av1 is the limiting component (Figure 1c), there is little observable substrate inhibition, but there is some indication that there may be a very low K_m form of the enzyme (data points for low substrate concentration lie below the lines drawn on the reciprocal plot). Additional experiments (L. C. Davis and Y. L. Wang, unpublished experiments) using short reaction times and very low substrate concentrations indicate that a very low K_m form (~0.001 atm) is present in *A. vinelandii*. In Figure 1b, where Av2 is the limiting component, there appears to be one K_m near 0.0125 atm as found in Figure 1c and a K_i of about 0.3 atm. The lines on this part of the figure were constructed by using the manual method described by Cleland (1970) in which a line passing from the vertical intercept of the asymptote to the minimum point of the $1/v$ activity curve has a slope twice that of the asymptotic line. The position of the K_i is that at which the separation of the activity curve and the asymptote is equal to the vertical intercept. Obviously, precise determination of the K_i requires more data than we have shown in this figure. The point to be made is that estimation of CO inhibition of acetylene reduction becomes difficult when there are multiple forms of the enzyme produced in steady-state conditions of assay.

A complication in dealing with gaseous substrates and inhibitors is that the gas–liquid partitioning must be taken into account. In addition, CO is in the class of tight-binding inhibitors as discussed by Morrison (1969). Concentrations of CO in the aqueous phase which yield 50% inhibition of the nitrogenase enzyme assay are of the order of 1 μM or less while the enzyme is typically present at a level of 0.1–2.5 μM (cf. Figure 1). Thus, when levels of CO much below the apparent K_i are used, the enzyme may bind a significant fraction of the CO in the aqueous phase and perturb the gas–liquid equilibrium. This means that, generally, the potency of CO as an inhibitor is underestimated, and the inhibition curves may not be a simple function of time, substrate concentration, inhibitor concentration, or enzyme concentration. Morrison has clearly shown that the rate equations in such cases must contain both linear and squared terms in v, making interpretation of inhibition patterns difficult in the absence of a known mechanism for the enzyme action.

Figure 2 shows the results of a set of assays used to estimate the inhibition of *A. vinelandii* nitrogenase by CO. We have drawn a set of lines indicating the apparent K_m of 0.0125 atm for acetylene as was used in Figure 1, assuming that CO does not alter the K_m of the enzyme for the reducible substrate acetylene. The alternative extreme assumption, that CO has no effect on V_{max} but only on K_m (Bergerson & Turner, 1973), does not appear very likely for the data shown. We also did assays under conditions such that the substrate inhibition by high concentrations of acetylene is readily observable and found that addition of CO did not prevent the substrate inhibition effect. From Figure 2a we cannot really say whether CO affects both V_{max} and K_m or only V_{max}.

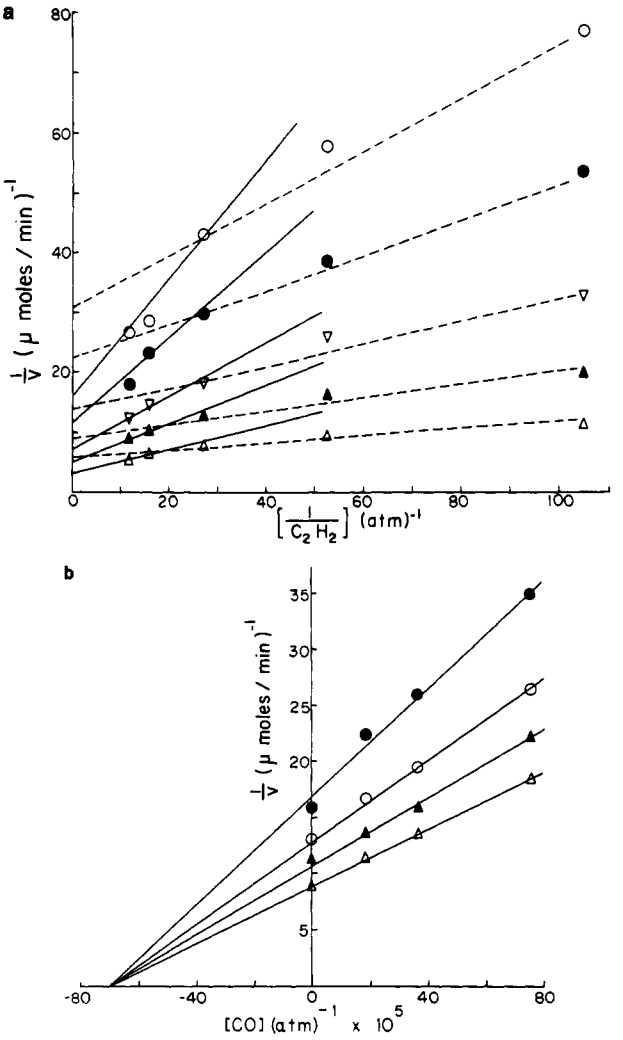

FIGURE 2: (a) CO inhibition of acetylene reduction by *A. vinelandii* nitrogenase. Assays were carried out as described in Figure 1c using a mixture of 10 μL of Av1 and 25 μL of Av2 per assay. Abscissa and ordinate are as described in Figure 1a. Levels of acetylene were as indicated on the graph. The partial pressure of CO was varied from 0 to 0.00185 atm: open triangle (△), no CO; solid triangle (▲), 0.00037 atm of CO; inverted triangle (▽), 0.00074 atm of CO; solid circle (●), 0.0012 atm of CO; open circle (○), 0.00185 atm of CO. Reactions were run for 2 min at 30 °C. The dashed lines are fitted to a K_m of 0.0125 atm, assuming linear noncompetitive inhibition by CO, while solid lines are fitted to a K_m of 0.06 atm. (b) CO inhibition of acetylene reduction by *C. pasteurianum* nitrogenase. Nitrogenase components were combined in a ratio of 1:1 in units of activity, so that the final concentration of enzyme was 0.15 unit/mL in each component (see Figure 1a and Materials and Methods). Assays were initiated by adding 100 μL of enzyme mixture to 900 μL of buffer containing ATP mixture previously equilibrated with acetylene and CO at 30 °C. After 60 s the reaction was terminated by addition of 0.1 mL of 30% Cl_3AcOH. Production of ethylene was determined by gas chromatography. Partial pressures of CO are as indicated in the figure. Levels of acetylene were varied as follows: solid circles, 0.01 atm; open circles, 0.025 atm; solid triangles, 0.05 atm; open triangles, 0.1 atm. Lines are visually fitted to a "pure noncompetitive" type of inhibition for a K_i of 0.0007 atm (Dixon & Webb, 1964).

An alternative replot of the data of Figure 2a, using the method $1/v$ vs. [I], as described by Dixon & Webb (1964) indicates that inhibition of acetylene reduction by CO is a nonlinear function of the CO concentration (~$[CO]^{1.33}$). The inhibition is clearly not competitive on this type of plot (Bergerson & Turner, 1973) but is of the "mixed type" (noncompetitive in Cleland's terminology). Replots of 1/

Table I: Inhibition Constants for CO Inhibition of Nitrogen Reduction

source of nitrogenase	inhibn type	$K_{ii} \times 10^{-4}$ (atm)	$K_{is} \times 10^{-4}$ (atm)	ref
C. pasteurianum crude extract	competitive			Lockshin & Burris (1965)
C. pasteurianum[a] purified	linear noncompetitive	7 ± 1	14 ± 2	this work
A. vinelandii	linear noncompetitive	1.14 ± 0.24	4.7 ± 0.6	Hwang et al. (1973)
A. vinelandii[b] purified	linear noncompetitive	1.4 ± 0.6	4.5 ± 1	this work

[a] Nitrogenase components purified by the method of Henzl, Davis, and Orme-Johnson (unpublished experiments) were used for this experiment. Cp1 had an activity of 16 units/mL and Cp2 had an activity of 25 units/mL when assayed with 0.5 atm of C_2H_2 in the same assay mixture at pH 6.9 in 100 mM K^+-Mops. Specific activities were >2000 nmol/(min mg) for each component. Ten microliters of Cp1 and Cp2 was used for each assay. N_2 was varied over the range 0.025–1 atm and CO was varied from 0 to 0.002 atm. Ar was used to make up the assays to 1 atm. Reactions were initiated by addition of an enzyme mixture and terminated after 5 min by addition of 1 mL of saturated K_2CO_3 (Burris, 1974). [b] Nitrogenase components purified by the method of Shah & Brill (1973) were used for this experiment. Ten microliters of Av1 and twenty-five microliters Av2 were used for each assay. This mixture showed an extrapolated V_{max} for C_2H_2 reduction of 230 nmol/(min assay) (see Figure 1c). N_2 concentration was varied from 0.1 to 1 atm with CO over the range 0–0.003 atm. Ar was used as inert diluent for CO and to bring assay to a final pressure of 1 atm, before enzyme addition. Reactions were initiated by addition of enzyme mixture and terminated by addition of 1 mL of saturated K_2CO_3.

Table II: Time Course of CO Inhibition of Acetylene Reduction

CO concn (aq phase)[a]:	19 nmol/mL		81 nmol/mL		222 nmol/mL	
acetylene redn:	nmol of C_2H_4[b]	mol/mol of Mo[c]	nmol of C_2H_4	mol/mol of Mo	nmol of C_2H_4	mol/mol of Mo
time of assay (5)						
15	18.5	3.6	7.2	1.4	5	0.4
30	33.3	6.6	10.3	2.0	5	0.4
60	45.8	14.4	14.4	2.8	5.5[d]	0.4

[a] CO concentration was calculated by assuming that 1 atm of CO gives 860 nmol/mL in the aqueous phase. [b] Total amount of ethylene produced per assay in the time indicated. Assays were carried out as described under Materials and Methods and the legend to Figure 1. Reactions were initiated by addition of small aliquots of enzyme and were terminated by addition of 3 M Cl_3AcOH. [c] Analytical determination of molybdenum by the method of Clark & Axley (1955). Maximal activity of the enzyme in the absence of CO was 2.75 units/nmol of Mo which corresponds to 220-ms turnover time per Mo, or 110 ms/mol of enzyme containing two Mo's. [d] Assays using half or twice the amount of enzyme showed 2.5 and 9.3 nmol of ethylene produced, respectively. This series was carried out by using the same enzyme concentration as in the first two experiments but a different total reaction volume.

v(slope) or $1/v$(intercept) from Figure 2a vs. [I] provide an estimate of the inhibition constant for the lines drawn in Figure 2a. These plots both yield 0.0004 atm as the K_i for CO inhibition of acetylene reduction.

Inhibition of acetylene reduction by CO for nitrogenase of *C. pasteurianum* (Figure 2b) appeared to be linear noncompetitive over the range of substrate, enzyme, and inhibitor concentrations used, even though the substrate saturation curves yielded nonlinear reciprocal plots as shown in Figure 1a. By determining the effect of CO at either very high or very low acetylene concentrations, we were also able to examine whether there was a different relative inhibition of the high and low K_m forms of the enzyme.

The results in Figure 2b are consistent with slope and intercept replots (of $1/v$ vs. $1/[S]$ plots) for the same data derived by using only the two highest concentrations of acetylene. We obtained the same pattern in 10-min assays using half as much enzyme. In another experiment the potency of inhibition by CO was tested by using N_2, Ar, or H_2 as the diluent for 0.18 atm of acetylene. In this experiment there was no difference in CO inhibition as a function of diluent gas; the apparent K_i was 0.00085 atm of CO. In another experiment the apparent K_i obtained from plots of $1/v$ vs. [I] at 0.0023 atm of acetylene was 0.0006 atm of CO when either a mixture of 0.25 unit of Cp1 plus 0.22 unit of Cp2 or a mixture of 0.25 unit of Cp1 plus 0.66 unit of Cp2 was used. In summary, we can say that the K_i for CO as an inhibitor of acetylene reduction lies between 10^{-4} and 10^{-3} atm and that the inhibition appears to be noncompetitive.

(*c*) *Nitrogen Reduction.* When N_2 was used as reducible substrate, the kinetics for CO inhibition appeared to be more straightforward. Table I lists the inhibition constants derived from our experiments with soluble enzymes from *A. vinelandii* and *C. pasteurianum*, as well as some earlier values from the literature. The soluble enzymes exhibited linear noncompetitive inhibition by CO, from which we can conclude that to produce inhibition only one CO per active site needs to be bound and that CO may bind to a form of the enzyme produced prior to that which reduces N_2 (Cleland, 1970).

Pre-Steady-State Inhibition by CO. As shown in the above experiments, the apparent inhibition constant for CO is below 0.001 atm, which results in a concentration in the aqueous phase approximately equimolar with enzyme concentrations. Under some conditions, as in the EPR experiments to be described below, the total amount of CO in the system is less than the amount of enzyme present. To avoid having to estimate the rate of gas–liquid equilibration in a nonequilibrium system, we did the following experiments.

The aqueous phase of several assay vials containing the ATP mixture was equilibrated with acetylene and CO in varying concentrations. Small aliquots of enzyme were added to initiate the reaction, and assays were terminated after short times by addition of 3 M Cl_3AcOH (Table II). A sixfold molar excess of CO over enzyme in the aqueous phase produced 95% inhibition within 15 s. Further inhibition occurred within an apparent first-order process with a $t_{1/2}$ of about 30 s. When the concentration of CO was increased, the reduction of acetylene decreased; with an 80-fold molar excess of CO over enzyme, there was no detectable enzyme activity after the first 15 s. The amount of acetylene reduced in this time can be calculated to be less than 1 mol/2 mol of molybdenum (Table II). The amount of ethylene produced at the highest

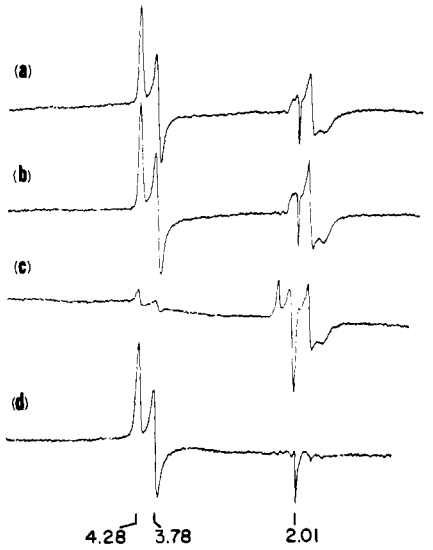

FIGURE 3: Effect of a high concentration of CO on the EPR and turnover of nitrogenase from *C. pasteurianum*. Reactions were carried out at ambient temperatures (22 °C) by using a buffer of 60 mM K^+–Mops, pH 7.4, and final concentrations of the following: ATP, 2.5 mM; Mg^{2+}, 5 mM; creatine-*P*, 25 mM; creatine kinase, 0.2 mg/mL; CO was present at 0.3 atm in N_2. Reactions were initiated by addition of Cp2 and were quenched by plunging the EPR tube into cold isopentane 45 s after initiation of reaction (except part d). EPR spectroscopy carried out as indicated under Materials and Methods at 13 K, with 3-mW incident power, modulation amplitude of 10 G, sweep rate of 100 G/min, field center of 2600 G, and frequency of 9.2 GHz. The ordinate is an arbitrary linear function of the first derivative of microwave power absorption; the abscissa is a magnetic field, increasing to the right. The values of *g* corresponding to certain field values are marked along the abscissa. Enzyme components were purified by the method of Tso et al. (1972) and had specific activities greater than 1000 nmol/(min mg) under optimum assay conditions. Final concentrations of proteins: Cp1, 5.5 mg/mL; Cp2, 2.2 mg/mL. The samples were (a) CO present, MgATP absent; (b) both CO and MgATP present; (c) both CO and MgATP present for 45 seconds; (d) same as (c) after 20-min incubation.

CO concentration tested was proportional to the enzyme concentration and was consistent with inhibition of enzyme activity occurring within a single turnover, on the average.

The results in Table II are consistent with the inhibition constant previously derived under steady-state conditions if one used the results of 1-min assays (cf. Figure 2b). When we attempted to determine the inhibition by CO using 1.25 μM enzyme and 0.5 atm of acetylene, 50% inhibition of enzyme activity was obtained with 0.003 atm of CO (which gives 2.6 μM CO in the liquid phase). From this we can estimate a binding constant of 5×10^5 M^{-1}, assuming that only one molecule of CO binds to each enzyme active site that it inhibits. The results in Table II suggest that the actual binding constant must be greater than this, since the inhibition is progressive and somewhat greater than the calculated amount for this binding constant at 1 min. With low concentrations of enzyme (0.2 nmol) and a large total excess of CO (200 nmol) in a typical assay vial with a 5-mL gas phase and a 1-mL aqueous phase at 50% inhibition, the steady-state kinetic assay would not detect a second weaker binding constant. The inhibition is so potent that instrumental limitations prevent assaying the enzyme activity at much higher CO levels, except under the nonequilibrium conditions such as we used for the experiments shown in Table II.

CO Binding Site. Carbon monoxide has no effect on MgATP-dependent H_2 evolution whereas N_2 does inhibit H_2 evolution in a manner such that the sum of electron fluxes is roughly constant (Hwang et al., 1973). The N_2 inhibition of H_2 evolution is consistent with either a competition between

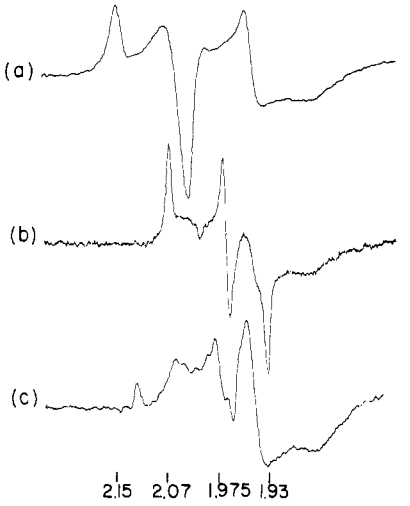

FIGURE 4: Signals elicited during turnover of nitrogenase with and without CO. Reactions were carried out as indicated in Figure 3, varying only the partial pressure of CO. For (a), 0.3 atm of CO; for (b), 0.00125 atm of CO, for (c), no CO in N_2 gas phase. EPR spectroscopy was as indicated under Materials and Methods with a sweep rate of 400 G/min and a field center of 3400 G at 9.2-GHz frequency. For (a) the temperature of observation was 13 K with a power of 3 mW; for (b) the temperature was 23 K and the power was 30 mW; for (c) the temperature was 13 K and the power was 30 mW. These were nonsaturating powers in each case at the temperature of observation. The abscissa and ordinate are as described in Figure 3. The *g* values of some prominent features are indicated below the spectra. The large, broad signal in the $g = 1.94$ region is that of Cp2 (see Figure 3).

N_2 and H^+ for a common pool of electrons or a second type of interaction whereby N_2 interrupts electron flow to the H^+-reducing site. We tested this second possibility in a dithionite oxidation assay with either 0.9 atm of N_2 plus 0.1 atm of CO or 0.9 atm of Ar plus 0.1 atm of CO in the gas phase. If N_2 interrupted electron flow to the H^+ site, there should be inhibition of the net electron flux in the presence of N_2 plus CO. To within the experimental accuracy of the method, there was no difference between dithionite oxidation rates with or without N_2. It appears that CO interrupts electron flow to the site at which N_2 is reduced, while the site which interacts with H^+ (independent of N_2) is unaffected. The site to which CO binds could be the N_2 binding site if CO binds to the enzyme in a different redox state than that which binds N_2. This would produce noncompetitive kinetics if the change of redox state were not a readily reversible process.

EPR of the CO-Inhibited Steady State. We have found that CO in reaction mixtures containing nitrogenase and a MgATP generator elicits new EPR signals in a reversible manner. As shown in Figure 3a,b, CO did not affect nitrogenase in the absence of MgATP. When the MgATP source was added and the enzyme frozen and examined after 45 s, the $g = 3.7$ center of the MoFe component was reduced, the same as in the absence of CO (Orme-Johnson et al., 1972), but, in addition, a new axial signal with *g* values near 2.15 and 2.05 appeared (Figure 3c). When the system was allowed to incubate for 20 min, by which time the electron source was exhausted, the new signal was gone, as were the signals near $g = 2$ attributed to the Fe protein. The MoFe protein signal at $g = 4.3$, 3.7, and 2.01 was restored. The latter two events are exactly as those observed in comparable samples without CO (Orme-Johnson et al., 1972). This suggests that the N_2 reduction site and the $g = 3.7$ EPR active center are separate entities. The axial signal is produced in the presence of concentrations of CO far in excess of the K_i. When the CO concentration was lower and comparable to the enzyme

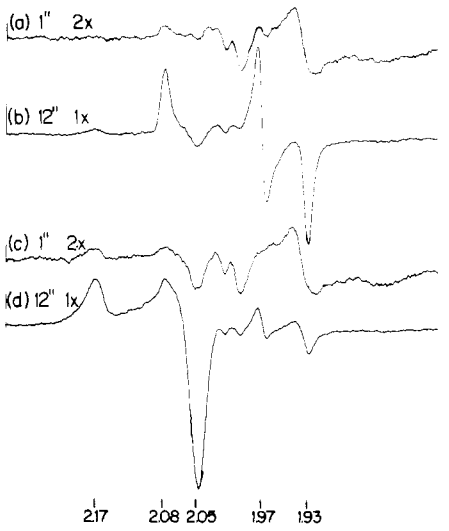

FIGURE 5: Time dependence of elicitation of high and low pCO signals in *A. vinelandii*. Reactions were initiated by mixing equal volumes of an ATP mixture and an enzyme mixture in a rapid-mixing chamber. After the indicated time at 30 °C, reactions were terminated by injection into cold isopentane. The ATP mixture was prepared to give the same final concentrations as described in Figure 3 except that creatine kinase was at 5 mg/mL final concentration. For (a) and (b) the ATP mixture was equilibrated with 0.08 atm of CO, while for (c) and (d) it was equilibrated with 0.8 atm of CO. The enzyme mixture contained 12 mg/mL Av1 and 12 mg/mL Av2 (equal moles of active protein) and approximately 45 μM Mo/mL. The conditions of EPR spectroscopy and the abscissa and ordinate are as described in Figure 3.

concentration, at short times after addition of the MgATP an entirely different signal was elicited in the presence of CO. As can be seen in Figure 4, a rhombic signal appeared with g values near 2.07, 1.97, and 1.92.

We also observed the development of another new set of signals in the controls (+ N_2, − CO) which were not previously observed,[2] namely, a signal with g values near 2.13 and 1.98. This was more strongly developed in the presence of N_2 than of Ar or in vacuo under which most of our earlier long-term (seconds range) reaction mixtures were prepared. It was also easier to elicit in Av1 than in Cp1.

The CO signals develop rather slowly. Figure 5 shows spectra from samples freeze-quenched (Bray, 1961) at 1 and 12 s after mixing MgATP with the *A. vinelandii* enzyme at 30 °C. For the *C. pasteurianum* enzyme the results were entirely similar; in both cases at 30 °C the signals of either type developed with apparent first-order kinetics with a $t_{1/2}$ of approximately 4 s when the ratio of components was 3 Fe protein/1 MoFe protein. Two conclusions may be drawn from these findings. First, the development of the EPR-active state is at least 2 orders of magnitude slower than the onset of CO inhibition of N_2 reduction; the latter is complete in about 90 ms. Second, the process leading to the formation of the signal elicited at high CO concentrations must possess a rate comparable to or slightly faster than the rate of formation of the first signal. Note that in Figure 5d there is a small amount of the low pCO signal remaining at 12 s. The finding of no appreciable low pCO signal at 1 s in the presence of 0.4 atm of CO (Figure 5c) is only possible if, as seems most likely, the low pCO EPR-active state is formed from an EPR-silent CO

[2] After completion of this manuscript, we learned from Dr. G. Yates that he and his colleagues have observed similar resonances from *A. chroococcum* nitrogenase, under similar circumstances (Lowe et al., 1978). They find a variety of resonances that they also attribute to FeS centers in the MoFe protein.

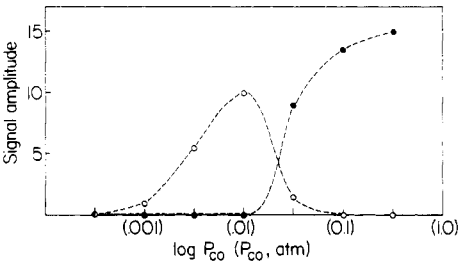

FIGURE 6: Variation of signal height with pCO. The ATP mixture and enzyme used were the same as described for Figure 5 but one-third as much enzyme was used (final concentration 2.4 mg/mL Av1). CO partial pressures were varied, as indicated, by dilution with N_2. Samples were incubated at 30 °C for 45 s after initiation of the reaction by addition of Av2. EPR spectroscopy was carried out as indicated under Materials and Methods and Figure 3. The signal amplitudes are corrected to constant amplifier gain and are proportional to the concentration of paramagnetic species. Filled circles, signal at g_z = 2.165 (high potential iron protein type centers); closed circles, signals at g = 2.084 (reduced ferredoxin-type center).

Table III: Size of CO Signal as a Function of Temperature and Component Ratio in *C. pasteurianum* and *A. vinelandii*[a]

	ratio of Av1/Av2					
	10:1		3:1		1:1	
T (°C)	g = 2	g = 4	g = 2	g = 4	g = 2	g = 4
30	49	75	80	90	81	>95
15	26	50	62	90	75	>95
10	16	38	43	75	70	>95
5	8	25	16	25	56	75
0	–		–		0	
	ratio of Cp1/Cp2					
	10:1		4:1		1:1	
T (°C)	g = 2	g = 4	g = 2	g = 4	g = 2	g = 4
30	12.5	93	40	96	40	98
25	6	93	20	96	36	98
20	4	93	11	96	27	98
15	0	93	3	96	12	98
0	0	3	0	30	–	–

[a] Values for the g = 2 region are normalized signal sizes at a non-saturating microwave power at 13 K. Values for the g = 4 region are percent decrease in signal relative to a control in buffer rather than an ATP mixture. Samples were prepared as described under Materials and Methods, incubated at the specified temperature for 30 s and quenched by plunging the EPR tube into stirred isopentane at 130 K. The ratios of components are mole ratios estimated from the quantities of proteins added.

complex (the inhibited enzyme) by a slow shift in the oxidation state of one of the prosthetic groups, taking place during many turnovers. It is also clear that there is at least one CO binding site or state other than the inhibitory one. Raising the pCO from 0.001 to 0.1 atm had no effect on the electron flux as measured by H_2 evolution, which means that neither site is directly on the electron path to the H^+-reduction site, and it is plausible that both CO binding sites are on the electron path from the g = 3.7 EPR center to the N_2-reducing site.

The effect of variation of pCO on the type of EPR signal observed in the steady state reinforces the concept of two binding sites. Samples of the MoFe protein plus ATP generator were equilibrated with different partial pressures of CO at 30 °C, and the reaction was initiated by the addition of Fe protein. After 20 s of further incubation, the mixtures were frozen and examined by EPR, with the results depicted in Figure 6. The low pCO signal increased with pCO to a maximum and declined rapidly as the high pCO signal appeared. These experiments were performed with MoFe protein

Table IV: Nuclear Hyperfine Effects[a] on EPR Signals Elicited under CO

	high pCO		low pCO			no CO
isotope tested	g = 2.17	g = 2.05	g = 2.08	g = 1.97	g = 1.98	g = 1.98
Av1 natural	34	32	17	14	14	25
Av1 ^{95}Mo	34	33	18	14	14	
Av1 ^{57}Fe	*40*	*44*	*30*	*30*	*29*	*48*
Av2 ^{57}Fe	34	32	17	14	14	
^{13}CO	33	32	17	14	14	

[a] Line width at half-height (gauss). Samples were prepared as indicated under Materials and Methods; spectra were obtained under standard conditions, matching relative intensities to equalize peak height and then determining the width at half-height for the several maxima and minima (see Figure 5).

concentrations (12 μM) far in excess of the K_i values ($\approx$1 μM) for CO. The maximal low pCO signal appeared when [CO] $\simeq$ [MoFe protein]. Thus, it appears that CO in excess of tightly bound first CO molecule on the MoFe protein reacts with the initial CO–protein complex to form a second complex with a distinct EPR signal which we have called the high pCO signal.

We also studied the development of the high pCO signals as a function of incubation temperature and component ratio, keeping the incubation time constant. The results indicate the relative rates of complex formation (Table III). The enzymes from *C. pasteurianum* and *A. vinelandii* differ markedly in that the rate of formation of the EPR-active species in *C. pasteurianum* nitrogenase decreases very quickly in the region of 15 °C, whereas the *A. vinelandii* enzyme is much less temperature sensitive. This difference is more pronounced at low concentrations of the Fe protein. Evidently, the appearance of the signals is affected by the rate of electron transfer from the Fe protein to the MoFe protein, relative to efflux at the H^+ site, and the rate of electron influx may become the rate-limiting step in the normal operation of the enzyme at low temperatures.

Although the slow appearance of the signals is initially first order, neither signal reaches the intensity expected for one spin per molecule of MoFe or Fe protein, even under the most favorable conditions (higher temperature, higher Fe/MoFe protein ratios). This suggests that the observed signals arise from steady-state forms of the enzyme that are not quantitatively produced under these conditions, presumably because the EPR-active center is still connected to an electron egress site (the H^+-reduction site). We can estimate the spins per molecule that could be formed if the first-order process went to completion. Under the conditions of Figure 5, per Av1 molecule (limiting component) at 30 °C, the hypothetical maximal signals from the high and low pCO forms would correspond to $\sim$0.49 and 0.40 spin/molecule, respectively. The maximal amount of either of these signals that we have seen so far, under a variety of conditions, is about 0.5 spin/molecule. Since these signals occur in steady states of the catalytic cycle, it is perhaps not surprising that the absolute amounts seen are low.

Origin of the EPR of the CO-Inhibited State. The availability of Av1 and Av2 from *A. vinelandii* grown on medium enriched in either ^{57}Fe or ^{95}Mo (Münck et al., 1975) allowed us to determine which of the two protein components of nitrogenase contains the metal centers responsible for the CO-induced EPR signals. We carried out a series of otherwise identical incubations of nitrogenase in the presence of CO and the MgATP generator, varying only the isotopic state of the Fe and MoFe proteins (or of the CO). If the center responsible for the elicited EPR signal contained a given element, and if the Fermi contact interaction of the nuclear spin with the electron spin were comparable to or larger than the line width of the EPR signal in the absence of the nuclear spin, then replacement of a spin = 0 nucleus (^{56}Fe, ^{100}Mo, ^{12}C) with nuclei of nonzero spin (^{57}Fe, ^{95}Mo, ^{13}C) would lead to observable broadening of the EPR (Beinert & Orme-Johnson, 1968). The results of evaluations of the widths of individual spectral features from samples prepared in this way are given in Table IV. It can be seen that only Fe of the MoFe component produces broadening in the signals elicited by high or low CO concentrations. It should be noted that Erbes et al. (1975) discovered a small (about 2 G) broadening due to ^{13}C in the CO complexes of hydrogenase. Failure to observe broadening in such experiments does not exclude participation of the element in question; the nuclear hyperfine interaction may be too small to observe. It can, however, be said with confidence that the MoFe protein contains one or more *iron* complexes that become paramagnetic in the presence of CO when electrons are being supplied from the Fe protein. This conclusion is supported by experiments in which Av2 was used as the electron donor to MoFe proteins of *Rho. rubrum* and *Rhi. japonicum* in the presence of MgATP. As can be seen in Table V, the g values of CO-induced signals from these species vary with the source of the *MoFe protein* used.

Discussion

The two EPR signals induced in nitrogenase in the presence of MgATP and CO resemble signals characteristic of low molecular weight iron–sulfur proteins (Orme-Johnson & Sands, 1974). The low pCO signal, for which the average g value is less than 2.0, can be reasonably assigned to an iron–sulfur cluster in the same oxidation state as a reduced ferredoxin. The high pCO signal has a g average above 2, and thus it resembles the signal from the oxidized form of the high-potential iron–sulfur proteins. On the basis of the finding of Holm & Ibers (1977) that the iron–sulfur clusters $Fe_4S_4(RS)_4$ (R = alkyl or acyl) can exist in net charge states of −1, −2, and −3, Carter et al. (1972) proposed that in fact the high-potential iron–sulfur protein in the oxidized form is

Table V: Transient EPR Signals of Nitrogenase of Various Organisms[a]

	g values								
organism	high pCO			low pCO			no CO		
A. vinelandii	2.17	2.08	2.05	2.08	1.98	1.93	2.09	2.01	1.98
C. pasteurianum	2.15	2.07	2.04	2.07	1.975	1.92	2.13	1.99	1.98
Rho. rubrum	2.17	2.09	2.05	2.08	1.975	1.93	not done		
Rhi. japonicum	2.18	2.11	2.06	not done			2.14	2.01	1.98

[a] Apparent g value of maximum, minimum, or crossover point (see Figure 5). High pCO values were determined at about 0.1 atm of CO, low pCO values were determined with about 0.01 atm of CO, and no CO signals were determined under an atmosphere of N_2. Component II of *A. vinelandii* was used for elicitation of the CO signals of *Azotobacter, Rhodospirillum,* and *Rhizobium.* The added Av2 served to reduce the MoFe protein from these organisms, in the presence of MgATP.

in the −1 net charge state, that the reduced form of this protein and the oxidized form of bacterial ferredoxins are in the −2 state (which is EPR silent), and that reduced ferredoxin is in the −3 state. The EPR signals characteristic of the oxidized high-potential iron–sulfur proteins and the reduced ferredoxins are believed to be due to the −1 and −3 net charge state, respectively. Cammack (1973) gave great support to this theory by showing that a high-potential iron–sulfur protein could be reduced to a state with EPR like that of a ferredoxin. A reasonable hypothesis is that there exist iron–sulfur centers in the MoFe protein of nitrogenase of the same type represented in the low molecular weight proteins and in Holm's analogues and that these may be induced to exhibit EPR from −1 and −3 net oxidation levels when certain electron flow pathways in the enzyme are interrupted. Our demonstration of Fe_4S_4 cores in Av1 and Cp1 by cluster transfer methods strengthens this hypothesis (Orme-Johnson et al., 1978). We do not know if these signals arise from the same iron–sulfur cluster, but the reciprocal behavior of the two signals as a function of pCO as well as their quantitation suggests this possibility. It may be that the signals arise from the same iron–sulfur cluster responsible for the $g = 3.7$ signal which is present in the MoFe protein as isolated; Mössbauer investigations to this point are in progress.

The signals elicited in the absence of CO are less well understood.[2] The same signal is observed in the presence of N_2, N_3^-, Ar, or H_2, but the relative intensities vary somewhat from one condition to another. It was discovered that a somewhat larger signal was produced under N_2 than under Ar. Azide elicited a still smaller signal, although the $g = 4.3$ region indicated that the enzyme was at least as deep into the reduced steady state with N_3^- as with N_2 or Ar. N_2O produced no detectable signals although the enzyme went into steady state. Both NO and CN^- potently inhibited the enzyme so that it did not go into the reduced steady state; no new signals were elicited. The one factor common to all conditions in which the signal appeared was the presence of H_2 accumulated during the turnover of the enzyme prior to freezing. Thus, it may be a center specific for N_2 reduction which becomes oxidized in the presence of H_2 (g average is very near 2), or it may be a center to which H_2 is directly bound, by analogy with the situation in hydrogenase (Erbes et al., 1975). These alternatives are currently being examined by using isotopic substitutions.

The appearance of an EPR spectrum (the high pCO signal), from what is likely a rather *oxidized* iron–sulfur cluster in the *reduced* steady state of the MoFe protein, is at first sight puzzling. Equally surprising is the disappearance of this *oxidized* state when the enzyme becomes *reoxidized* upon exhaustion of the *reductant* (cf. Figure 3). Assuming that our assignment of these signals to the oxidation states of a familiar iron–sulfur cluster is correct, one should notice that *even* oxidation states of these clusters should either be diamagnetic in the ground state or be even-spin cases in which event they would be EPR silent. One plausible explanation of the behavior of the CO-induced signals would then be that when no CO is present the center is mostly in an even-electron state (though a small amount may be present in a −3 state; see Table IV), whereas, when the first CO is bound and N_2 reduction is inhibited, the center is reduced on the average toward the −3 state. When the second CO is bound, the average oxidation level rises toward −1, and, when the electron supply vanishes, the center reaches an even higher oxidation level, possibly the 0 state (formally equivalent to having all iron atoms in a $Fe_4S_4(RS)_4$ cluster be in the *ferric* state). These ideas are depicted in the following chart for the origin of the CO-reduced signals.

equivalent low M_T cluster	state of nitrogenase
$[Fe_4S_4(RS)_4]^{-1}$	high pCO steady state
$[Fe_4S_4(RS)_4]^{-2}$	normal steady state
$[Fe_4S_4(RS)_4]^{-3}$	low pCO steady state

This diagram refers to the hypothetical center responsible for the CO-induced effects and not necessarily to the center giving EPR at $g = 3.65$ in the MoFe protein as isolated. The cluster must be connected to the H_2-evolving site which furnishes the oxidizing force, while the Fe protein provides the electron influx which is modified by the presence of CO. In any event, the hypothesis that the MoFe protein of nitrogenase contains recognizable iron–sulfur clusters appears to be strengthened by these observations.

Acknowledgments

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Ca^{2+} Regulation of Sarcoplasmic Reticular Protein Phosphatase Activity†

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ABSTRACT: Isolated rabbit skeletal muscle sarcoplasmic reticulum contains Ca^{2+}-dependent protein kinase and protein phosphatase activities which may regulate the Ca^{2+} transport ATPase [Hörl, W. H., & Heilmeyer, L. M. G., Jr. (1978) *Biochemistry 17*, 766–772; Hörl, W. H., Jennissen, H. P., & Heilmeyer, L. M. G., Jr. (1978) *Biochemistry 17*, 759–766]. Addition of ATP–Mg^{2+} to the concentrated suspension of sarcoplasmic reticulum in the presence of 10 μM Ca^{2+} inhibits the endogenous protein phosphatase activity to 74–90%. This inhibition can be reversed by reducing the free Ca^{2+} concentration with ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N'N'*-tetraacetic acid (EGTA) or by high dilution of the membrane suspension. Kinetically, 10 μM Ca^{2+} increases the K_m' for troponin about 10-fold without any change in V_{max}. Dilution of the membranes in the presence of Ca^{2+} (10 μM) leads to an approximately sevenfold decrease in the K_m' for phosphorylase *a* and an approximately fourfold increase in V_{max}; in the absence of Ca^{2+} (10 nM) no change in K_m' is observed and the V_{max} increases approximately two- to threefold. Membranes without the ATP–Mg^{2+} step during preparation do not show the Ca^{2+}-induced protein phosphatase inhibition; however, after preincubation with the catalytic subunit of the cyclic AMP dependent protein kinase and ATP–Mg^{2+}, the Ca^{2+}-dependent inhibition of the endogenous protein phosphatase reappears. Centrifugation experiments show that the association of the protein phosphatase to the membranes is Ca^{2+} and protein concentration dependent. In the presence of 10 μM Ca^{2+} and at a protein concentration higher than 5 mg/mL, approximately half the amount of phosphatase activity remains in the supernatant in comparison to 10 nM Ca^{2+}.

A Ca^{2+}-dependent protein kinase which is similar but probably not identical with phosphorylase kinase[1] and a protein phosphatase are associated with isolated rabbit skeletal muscle sarcoplasmic reticulum. These two enzymes may regulate the Ca^{2+} transport ATPase activity (Hörl et al., 1978; Hörl &

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[1] Enzymes: phosphorylase kinase (ATP:phosphorylase *b* phosphotransferase, EC 2.7.1.38); phosphorylase *b* or 1,4-γ-glucan:orthophosphate γ-glycosyltransferase (EC 2.4.1.1); phosphorylase phosphatase or phosphorylase phosphohydrolase (EC 3.1.3.17); ATPase or ATP phosphohydrolase (EC 3.6.1.3).

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