

Kinetic and Spectroscopic Analysis of the Inactivating Effects of Nitric Oxide on the Individual Components of *Azotobacter vinelandii* Nitrogenase[†]

Michael R. Hyman,[†] Lance C. Seefeldt,[§] T. Vance Morgan,[§] Daniel J. Arp,[†] and Leonard E. Mortenson^{*,§}

Laboratory for Nitrogen Fixation Research, 4100 Cordley Hall, Oregon State University, Corvallis, Oregon 97330-2902, and Center for Metalloenzyme Studies, Department of Biochemistry, University of Georgia, Athens, Georgia 30602

Received September 6, 1991; Revised Manuscript Received December 19, 1991

ABSTRACT: The effects of nitric oxide (NO) on the individual components of *Azotobacter vinelandii* nitrogenase have been examined by kinetic and spectroscopic methods. Incubation of the Fe protein (Av2) for 1 h with stoichiometries of 4- and 8-fold molar excesses of NO to Av2 dimer resulted in a complete loss of activity of Av2 in C₂H₂-reduction assays. The kinetics of inactivation indicated that the minimum stoichiometry of NO to Av2 required to fully inactivate Av2 lies between 1 and 2. The rate of inactivation of Av2 activity by NO was stimulated up to 2-fold by the presence of MgATP and MgADP but was unaffected by the presence of sodium dithionite. Unexpectedly, complete inactivation of Av2 by low ratios of NO to Av2 also resulted in a complete loss of its ability to bind MgATP and MgADP. UV-visible spectroscopy indicated that the effect of NO on Av2 involves oxidation of the [4Fe-4S] center. EPR spectroscopy revealed that the loss of activity during inactivation of Av2 by NO correlated with the loss of the $S = 1/2$ and $S = 3/2$ signals. Appearance of the classical and intense iron-nitrosyl signal ($g = 2.03$) was only observed when Av2 was incubated with large molar excesses of NO and the appearance of this signal did not correlate with the loss of Av2 activity. The effects of NO on the MoFe protein (Av1) were more complex than for Av2. A time-dependent inactivation of Av1 activity (C₂H₂ reduction) was observed which required considerably higher concentrations of NO than those required to inactivate Av2 (up to 10 kPa). In addition, the effects of NO on Av1 were significantly affected by the presence of sodium dithionite. In fact, kinetic evidence suggests that an Av1-catalyzed, NO-dependent consumption of dithionite occurs before Av1 is inactivated by NO. A correlation between UV-visible and EPR spectral features and the extent of NO inactivation has been established. The inactivation of either nitrogenase component by NO did not lead to aggregation or dissolution into their constitutive subunits. However, NO inactivation did cause changes in both proteins since neither NO-treated protein inhibited C₂H₂-reducing activity in assays containing equimolar concentrations of untreated protein. The effects of NO on both nitrogenase components are interpreted in terms of the known reactivity of NO with Fe-S centers.

Nitrogenase from *Azotobacter vinelandii* is composed of two protein components. The Fe protein (Av2) is a homodimeric protein (M_r 64 000) which contains one [4Fe-4S] ferredoxin-like center which bridges the two subunits. The Fe protein catalyzes the transfer of an electron from reduced carriers to the second nitrogenase component, the molybdenum-iron (MoFe) protein. This electron transfer is coupled with hydrolysis of MgATP. The MoFe protein (Av1, M_r 250 000) is a complex protein ($\alpha_2\beta_2$) which contains approximately 30 Fe and 2 Mo per tetramer. The metals in the MoFe protein are found in two sets of two chemically distinct clusters (Mortenson & Thorneley, 1979). One of these, the M cluster (6-8 Fe, 1 Mo, 8 S⁻, 1 homocitrate), can be separated intact from the protein in the form of a FeMo cofactor and is regarded as the site where substrate is reduced (Orme-Johnson, 1985). The majority of the remaining Fe in the tetramer is found in two Fe-S clusters, each of which comprises either two linked [4Fe-4S] clusters or two new clusters of 8 Fe each (Bolin et al., 1990). The establishment of the role of the metal clusters in both components of nitrogenase is fundamental to our understanding of the enzymology of this complex metalloprotein.

In addition to catalyzing the physiologically relevant reduction of N₂ to ammonia, nitrogenase is also capable of reducing numerous alternative substrates (Dilworth, 1966; Schollhorn & Burris, 1967). Studies of the reactivity of nitrogenase toward such substrates and their interactions as substrates and/or inhibitors of N₂ fixation have played an important role in elucidating the mechanism of this enzyme (Burgess, 1985). One series of compounds, the oxidized forms of nitrogen (NO₃⁻, NO, NO₂⁻, and N₂O) have received considerable attention as inhibitors of nitrogenase by virtue of their important individual roles in the biological nitrogen cycle. Recent studies have shown that N₂O is actively reduced to ammonia via N₂ as an intermediate (Jensen & Burris, 1986) whereas NO₃⁻ is entirely unreactive (Vaughn & Burgess, 1989). On the other hand, studies with NO₂⁻, recognized as an inhibitor of nitrogenase, are not clear-cut. Trinchant and Rigaud (1980) and Meyer (1981) reported that the inhibitory effects of NO₂⁻ are reversible while Michalski and Nicholas (1987) reported that they are irreversible. This matter was partially resolved by the recent demonstration that NO₂⁻ is a substrate for nitrogenase (Vaughn & Burgess, 1989). However, this result, and the observations of several others (Meyer, 1981; Trinchant & Rigaud, 1982), indicated that the effects of nitrite are complicated by a time-dependent inactivation of nitrogenase. It was suggested that NO is responsible for this inactivation and that inhibitory concentrations of NO can be generated nonenzymatically from the reduction of nitrite by dithionite, the common reductant used in nitrogenase

[†]This work was supported by National Institute of Health Grant R01-GM40067 to L.E.M. and Department of Energy Grant DEFG 690ER20013 to D.J.A.

* To whom correspondence should be addressed.

[†]Oregon State University.

[§]University of Georgia.

assays (Meyer, 1981). Inhibition studies with pure NO also produced equivocal results. Lockshin and Burris (1965) described NO as a potent, but fully reversible competitive inhibitor of N₂ fixation. However, the effects of NO deviated significantly from the behavior expected for a strictly competitive inhibitor. In contrast, Trinchant and Rigaud (1982) described NO as a noncompetitive inhibitor of acetylene reduction by nitrogenase. Still other studies with a variety of approaches have demonstrated that the Fe protein is very susceptible to irreversible inactivation by NO (Meyer, 1981; Michalski & Nicholas, 1987; Liang & Burris, 1989), while the MoFe protein is largely insensitive.

NO is known to react with Fe-S centers present in both proteins and model compounds to produce iron-nitrosyl derivatives which exhibit distinctive EPR signatures (Butler et al., 1988). The aim of the research described in this paper has been to investigate the reactivity of NO with both components of nitrogenase, to examine the effects of NO treatment in terms of changes in the physical, kinetic, and spectroscopic properties of the nitrogenase components, and to use NO as a probe of the catalytic mechanism.

MATERIALS AND METHODS

Materials. Adenosine 5'-triphosphate (ATP), creatine phosphokinase, creatine phosphate, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). Ar (99.99%), C₂H₂ (99.6%), C₂H₄ (99.9%), and nitric oxide (NO) (99.9%) were obtained from Airco (Murray Hill, NJ). Higher *N*-oxides were removed from NO by passage of the gas through a Dreschel bottle containing an aqueous solution of 5 N KOH. Sodium dithionite was obtained from Sigma Chemical Co. (St. Louis, MO). [8-¹⁴C]-ATP and -ADP (tetrasodium salt) were purchased from Amersham (Arlington Heights, IL) and had specific activities of 58 and 56 mCi·mmol⁻¹, respectively.

Nitrogenase Purification and Assays. *A. vinelandii* nitrogenase components were purified as described previously (Burgess et al., 1980). The specific activities of the proteins were approximately 1700 nmol of product·min⁻¹·(mg of Av2)⁻¹ and 1500 nmol of product·min⁻¹·(mg of Av1)⁻¹, where the substrates were H⁺ or acetylene and the products were H₂ or ethylene. Unless otherwise stated, all assays were performed at 30 °C in 9.5-mL glass vials containing the specified gas phase sealed with butyl rubber stoppers and aluminum crimp bands (Wheaton Scientific, Milleville, NJ). The concentrations of the components in the assays for nitrogenase activity were as described by Burgess et al. (1980). The vials containing the reaction mixture were degassed and reequilibrated with the appropriate gas mixture (Ar or Ar with 10 kPa of C₂H₂). Five minutes before the start of the reaction, sodium dithionite was added, the appropriate quantity of either Av1 or Av2 was added, and the vials were allowed to preincubate in a water bath shaker for at least 2 min. Finally, the reactions were initiated by the addition of the complementary nitrogenase component, either Av1 or Av2 (see figure legends for exact protein concentrations). The assay vials were incubated at 30 °C for 5 min in a water bath shaker, and the reaction was quenched with 250 μL of 2.5 M H₂SO₄.

Gas Chromatography. H₂ and NO were determined by gas chromatography using a Varian apparatus fitted with a Teflon-lined stainless steel column (6 feet × 1/8 inch) packed with molecular sieve 13X and a thermal conductivity detector. Helium (He) was used as carrier gas. The column was operated at 35 °C, and the detector was operated at 135 °C. C₂H₄ was determined using a Varian chromatograph appa-

ratus fitted with a stainless steel column containing Porapak R and a flame ionization detector. Argon was used as carrier gas. The column was operated at 40 °C, and the detector was operated at 100 °C.

Incubation Conditions. For experiments involving incubation of less than 100 μL of the nitrogenase components with NO, glass serum vials (9.5 mL) containing an open-topped inner vial (1 mL volume) were used. The purpose of the inner vial was to retain the small sample volume while at the same time providing a large surface area for gas diffusion into the liquid phase. For larger volumes, glass serum vials (9.5 mL) were used without the inner vial. In both cases, the larger vials were stoppered with butyl rubber stoppers and aluminum crimp seals. Just prior to use, the vials were made anaerobic by several cycles of evacuation and refilling with Ar using a vacuum manifold. Two minutes before the start of the reaction, the appropriate volumes of NO (either undiluted NO or NO in the form of a known stock solution of NO diluted with Ar) were added to the vials as an overpressure using Ar-flushed gas-tight syringes since NO reacts rapidly with O₂. The reactions were initiated by the addition of the appropriate volume of each nitrogenase component, and the vials were incubated at 25 °C on a rotary shaker (200 rpm).

Spectroscopy. UV-visible spectra of nitrogenase components were obtained using a Cary 2200 spectrophotometer. All protein samples (300 μL) were added to stoppered, Ar-flushed quartz cuvettes (2 mm path length). EPR spectra of nitrogenase components were obtained using a Bruker ER200D spectrometer interfaced to a Bruker ER1600 computer. Helium temperatures were obtained with an attached Oxford ESR9 cryostat. All EPR conditions are listed in the figure legends. Samples of NO-treated proteins were evacuated and reequilibrated with argon five times within 1 min to remove free NO. The latter treatments did not alter the EPR spectra and were required to limit further reactions between NO and nitrogenase components and to minimize discrepancies between the level of inactivation measured by activity assays and the associated EPR spectrum. The samples were loaded into Ar-flushed EPR tubes and immediately frozen by immersion into a liquid N₂-hexane slurry so that not more than 2 min passed from the time the NO was removed to the time the sample was frozen.

Fe Determination in Proteins. The content of Fe in proteins was estimated colorimetrically by its reaction with *o*-phenanthroline using a modification of the procedure described by Lovenberg et al. (1963). Protein (400–800 μg) was heated to 80 °C for 10 min in a volume of 1.5 mL of 0.1 M HCl. After removal of precipitated protein by centrifugation, 1 mL of the resulting supernatant solution was transferred to a clean glass tube and the following were added: 3.0 mL of water, 0.2 mL of 10% (v/v) hydroxylamine, and 1 mL of 0.1% (w/v) *o*-phenanthroline. The ingredients were mixed and allowed to stand at room temperature for 30 min, and the absorbance was read at 512 nm. A standard curve was prepared from a stock solution of FeSO₄.

MgATP and MgADP Binding Assay. Measurement of MgATP and MgADP binding to the NO-inactivated Av2 was achieved by a modification of the equilibrium binding assay described by Bui and Mortenson (1968). A total of 5.2 mg of Fe protein (untreated or NO inactivated for 1 h with a 4 to 1 molar ratio of NO) was loaded onto a 3-mL Sephadex G-25 column preequilibrated with 50 mM Tris, pH 8, containing 50 mM NaCl, 10 mM MgCl₂, and 2 mM dithionite. For ADP binding experiments, the latter buffer also contained 89.2 μM ADP and 1.8 μM [¹⁴C]ADP (56 mCi·mmol⁻¹). For

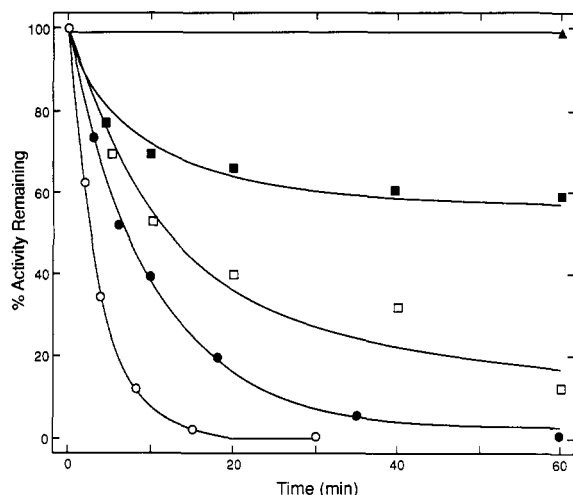


FIGURE 1: The time course of NO inactivation of Av2. Five anaerobic, Ar-filled (101 kPa) incubation vials (9.5 mL) were prepared as described in Materials and Methods. Nitric oxide of known concentration (NO diluted in Ar) was added to vials using Ar-flushed gas-tight syringes. Treatments were initiated by the addition of Av2 (50 μ L at 39 mg of protein·mL⁻¹) to the vials. At the indicated times, samples (5 μ L) were removed and added to a C₂H₂-reduction assay mixture containing untreated Av1 (15 μ L at 35 mg of protein·mL⁻¹). C₂H₂-reducing activity was determined as described in Materials and Methods. The molar ratios of NO to Av2 in the incubation vials (gas phase plus solution phase) were (▲) 0; (■) 1; (□) 2; (●) 4; and (○) 8.

ATP-binding experiments, the above buffer contained 90 μ M ATP and 2 μ M [¹⁴C]ATP (58 mCi·mmol⁻¹). For a non-nucleotide-binding control, the experiment was repeated using 5 mg of carbonic anhydrase instead of Fe protein. The column was developed with the same buffer, and 300- μ L fractions were collected. A 100- μ L sample was removed from each fraction into 10 mL of scintillation fluid, and the radioactivity was measured using a Beckman liquid scintillation counter (LS-3801). The concentration of nucleotide in each fraction was calculated by subtracting the background ¹⁴C counts on the column from the total ¹⁴C counts in each fraction. The protein concentration in each fraction was determined on a sample of each column fraction by the biuret method (Gornall et al., 1949).

RESULTS

Effect of the NO to Av2 Ratio on the Rate and Extent of Inactivation of Av2. Several previous studies have suggested a time-dependent loss of activity when Fe proteins from a variety of nitrogenase systems are exposed to low concentrations of NO, but no stoichiometries were reported (Meyer, 1981; Liang & Burris, 1989). It was therefore important to determine the minimal stoichiometry of NO to Av2 dimer required to fully inactivate this protein since this could provide an indication of the mechanism of inactivation. Samples of Av2 (50 μ L containing 27 nmol of dimer) were incubated for up to 1 h in the presence of 0-, 1-, 2-, 4-, and 8-fold molar ratios of NO to Av2. For residual activity measurements during the incubation, samples of NO-treated Av2 were added to C₂H₂-reduction assay mixtures containing untreated Av1. The rate of loss of Av2 activity increased with increasing ratios of NO to Av2 dimer (Figure 1). A complete loss of activity occurred within 1 h with 8- and 4-fold molar excesses of NO whereas an incomplete loss of activity was observed over the same time with lower stoichiometries of NO. When Av2 was incubated in the presence of equimolar concentrations of NO, approximately 35% of the initial activity was lost over the first 20 min of incubation. This was followed by a further loss of

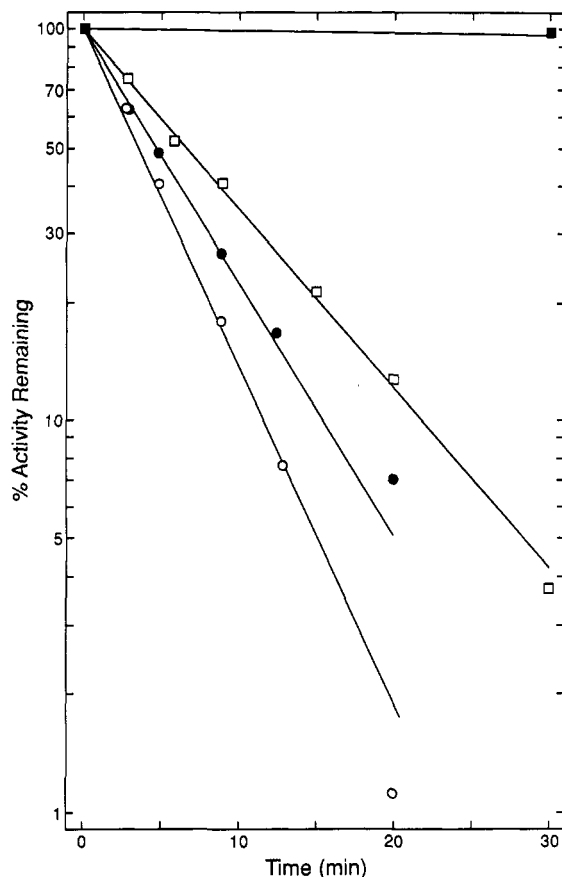


FIGURE 2: Effects of MgATP and MgADP on the rate of NO inactivation of Av2. Anaerobic, Ar-filled incubation vials were prepared as described in Materials and Methods, and NO was added as described in Figure 1. The reactions were initiated by the addition of Av2 (50 μ L at 39 mg of protein·mL⁻¹). At the indicated times, samples (5 μ L) were removed from the reaction vials and added to C₂H₂-reduction assay mixtures containing untreated Av1 (15 μ L at 35 mg of protein·mL⁻¹). The log of remaining C₂H₂-reducing activity is plotted versus time of incubation of Av2 with NO: (■) no NO; (□) a 4-fold molar excess of NO; (○) a 4-fold molar excess of NO plus 5 mM MgATP; (●) a 4-fold molar excess of NO plus 5 mM MgADP.

less than 10% of initial activity over the following 40 min of incubation. A qualitatively similar response was observed for Av2 incubated with a 2-fold molar excess of NO although the later time points showed a greater decrease in activity between the 20- and 60-min time points. When the data of Figure 1 were plotted as the log of the percent activity remaining versus time, the loss of activity of Av2 incubated with either an 8- or 4-fold molar excess of NO was a pseudo-first-order process with half-lives of 2.7 and 6.9 min, respectively. In contrast, the loss of activity of Av2 incubated with either a 1- or a 2-fold molar excess of NO deviated significantly from a first-order process (data not shown).

Effect of MgATP and MgADP on the Rate of Inactivation of Av2 by NO and Effects of NO on MgATP and MgADP Binding to Av2. The pseudo-first-order loss of activity of Av2 incubated with a 4-fold molar excess of NO was used to investigate and quantify the effect of MgATP and MgADP on the rate of loss of Av2 activity by NO. A plot of the log of the percent activity remaining versus time for Av2 incubated with a 4-fold molar excess of NO in the absence of either MgATP or MgADP was linear and the estimated half-life of activity was 6.7 min (Figure 2). In the presence of a 4-fold molar excess of NO and either 5 mM MgATP or MgADP, the loss of activity remained a pseudo-first-order process but the rate of inactivation increased. The half-lives of activity decreased from 6.7 min to 3.5 min and 4.5 min for Av2 in-

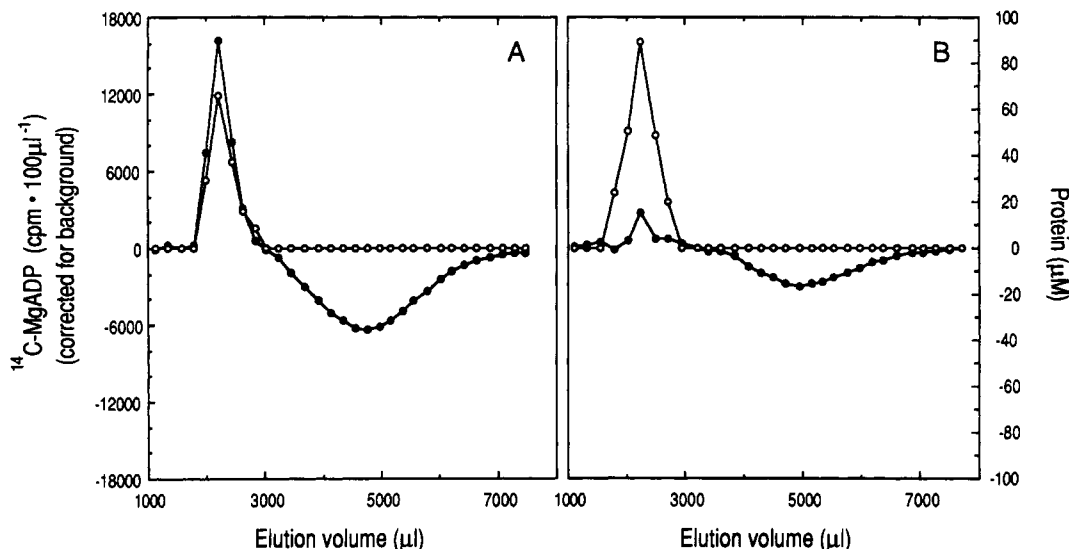


FIGURE 3: [^{14}C]MgADP binding to NO-inactivated Av2. A total of 5.2 mg of untreated (panel A) or NO-inactivated (panel B) Av2 (treated with a 4 to 1 molar ratio of NO to protein for 1 h) was loaded onto a 3-mL Sephadex G-25 column equilibrated with buffer (see Materials and Methods) containing 89 μM ADP and 1.8 μM [^{14}C]ADP. The proteins were eluted from the column with the same buffer, and 300- μL fractions were collected. A 100- μL aliquot from each fraction was counted in a scintillation counter, and the net counts were calculated by subtracting the background counts (20280 cpm·100 μL^{-1}) from the counts in each fraction. The net counts (solid circles) for the 100- μL aliquot of each fraction is plotted versus the elution volume. The protein concentration in each fraction also is plotted versus the elution volume (O).

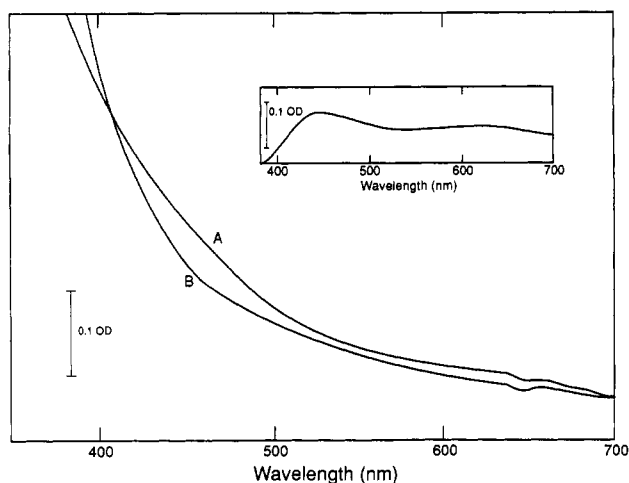


FIGURE 4: UV-visible absorption spectrum of NO-inactivated Av2. Three hundred microliters of Av2 (39 $\text{mg}\cdot\text{mL}^{-1}$) was treated under anaerobic conditions for 1 h with NO such that the molar ratio was 4 NO to 1 Av2 and Av2 was 99% inactivated. The absorption spectrum was recorded in a 3-mm-pathlength cell containing a gas phase of argon (A). A spectrum of untreated enzyme was also recorded (B). The difference spectrum, NO-treated Av2 minus control Av2, is shown in the inset.

cubated with MgATP and MgADP, respectively. No loss of activity was observed over the time course of the incubation for Av2 incubated in the absence of NO. Additional experiments demonstrated that the effect of ATP and ADP on the rate of inactivation of Av2 by NO depended on the presence of Mg^{2+} . The addition of 5 mM sodium dithionite to Av2 during the incubation with NO had no effect on the rate of inactivation (data not shown). Although the presence of either MgATP or MgADP stimulated the rate of inactivation of Av2 by NO, suggesting the nucleotides were bound to Av2, Av2 first inactivated by NO in the absence of either nucleotide could no longer bind MgATP (not shown) or MgADP (Figure 3). The NO-treated Av2 binds less than 8% of the MgADP of untreated Av2. This trace of binding is equivalent to what we found for the non-nucleotide-binding control protein, carbonic anhydrase.

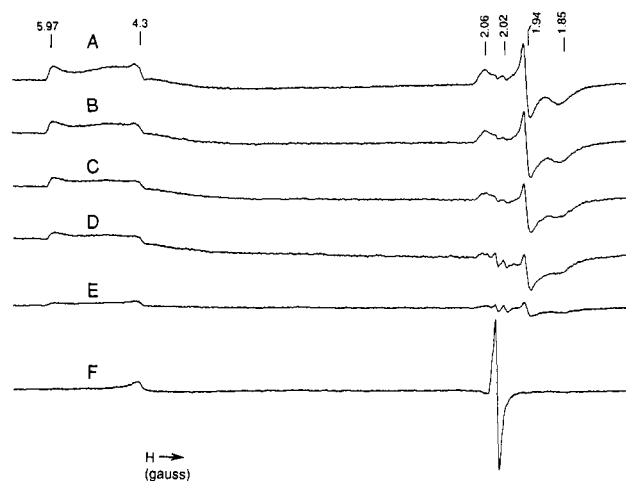


FIGURE 5: The EPR spectra of NO-treated Av2. Six samples of Av2 (300 μL at 39 mg of protein· mL^{-1}) were incubated in Ar-filled vials containing molar ratios of NO to Av2 dimer between 0 and 16. After 1 h of incubation, samples (5 μL) were removed and residual Av2 activity was determined as described for Figure 1. The remaining protein from each treatment was analyzed by EPR spectroscopy as described in Materials and Methods. EPR spectra were recorded for samples treated at each of the following NO to Av2 ratios: A, 0; B, 0.5; C, 1; D, 2; E, 4; F, 16. The spectra were recorded at 3.8 K, with a microwave power of 15 mW, a modulation amplitude of 1 mT, and a frequency of 9.44 GHz.

Spectroscopic Changes Associated with Inactivation of Av2 by NO. In addition to its effects on the activity and nucleotide-binding properties of Av2, NO interaction also resulted in significant alteration of the UV-visible and EPR spectra of the treated protein. The UV-visible spectrum of fully NO-inactivated Av2 showed increased absorbance in the region of 400–500 nm (Figure 4) compared to untreated Av2. The difference in absorbance had a maximum at approximately 450 nm, as seen in Figure 4 (inset). Absorbance in this region is characteristic of absorbance by Fe-S centers in proteins.

An examination of the effects of NO on the [4Fe-4S] center of Av2 as a function of the extent of inactivation was determined by EPR spectroscopy. The progressive loss of activity of Av2 in the presence of NO was accompanied by a con-

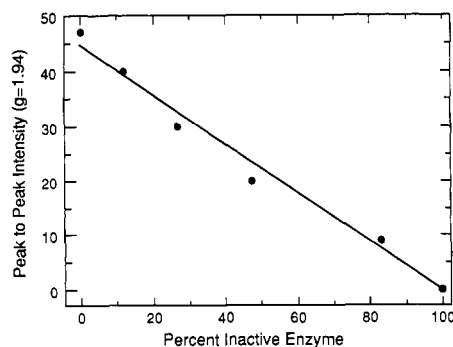


FIGURE 6: Correlation between EPR signal intensity and extent of inactivation of Av2 by NO. From the data presented in Figure 5, the peak intensity of the $g = 1.94$ EPR signal (taken as the distance between the maximum and minimum around the $g = 1.94$ center) of the reduced [4Fe-4S] center of Av2 treated with increasing concentrations of NO was plotted against the percent of NO-inactivated Av2, as determined by the loss of C_2H_2 -reduction activity when it was assayed together with native Av1.

comitant decrease in both the spin = $3/2$ ($g = 5.97$) signal and the spin = $1/2$ ($g = 1.94$) signal (Figure 5). When the intensity of the $g = 1.94$ signal was plotted as a function of the percent of enzyme inactivated by NO treatment, the plot was nearly linear (Figure 6). This indicates that the loss of this 1.94 signal correlates closely with the loss of activity during NO inactivation. The spin = $3/2$ signal ($g = 5.97$) decrease was comparable to the $g = 1.94$ signal. An additional intense feature in the EPR spectra ($g = 2.03$) was observed when Av2 was completely inactivated after incubation with a 16-fold molar excess of NO. Since little of this $g = 2.03$ signal was seen in samples of Av2 in which activity had been extensively inactivated by lower stoichiometries of NO (Figure 5), one can conclude that the loss of Av2 activity and the development of the $g = 2.03$ signal, typical of a paramagnetic iron-nitrosyl complex, are not related events. This suggests that there are at least two reactions of NO with Av2. The first correlates with the loss of activity, is produced by nearly stoichiometric concentrations of NO, and results in a decrease in both the $g = 5.97$ and $g = 1.94$ signals. The second requires a much larger molar excess of NO and does not correlate with the loss of activity but instead correlates with the formation of the species responsible for the $g = 2.03$ feature.

Effects of Preincubation with NO on the Activities of Av1. The consensus from previous studies of the reactivity of nitrogenase components during treatment with NO is that the Fe protein is considerably more sensitive to inactivation than the MoFe protein (Kelly & Lang, 1970; Meyer, 1981; Liang & Burris, 1989). As a result, the effects of NO on the MoFe protein component have received little attention.

Av1 was preincubated with 10 kPa of NO (180 μ M NO in solution), followed by assay of a sample of the treated Av1 for its ability to catalyze C_2H_2 reduction. Significant losses of activity (50%) were observed after preincubation for 20 min. However, because high concentrations of NO were used in these experiments, we had to make sure that part of the loss of activity was not the result of inactivation of Av2 by the NO transferred with the Av1 sample to the assay vial. Experiments in which (a) a sample from an NO-treated buffer solution without Av1 was added to assay vials containing untreated nitrogenase components and (b) the NO present in the Av1-treated sample to be assayed was removed by either evacuation or by complexation with a solution of $Fe^{2+}/EDTA$ (Hyman & Arp, 1991) prior to addition to the assay vial revealed that the amount of NO transferred to C_2H_2 -reduction assays at a maximum could result in only a 6% inhibition of ethylene

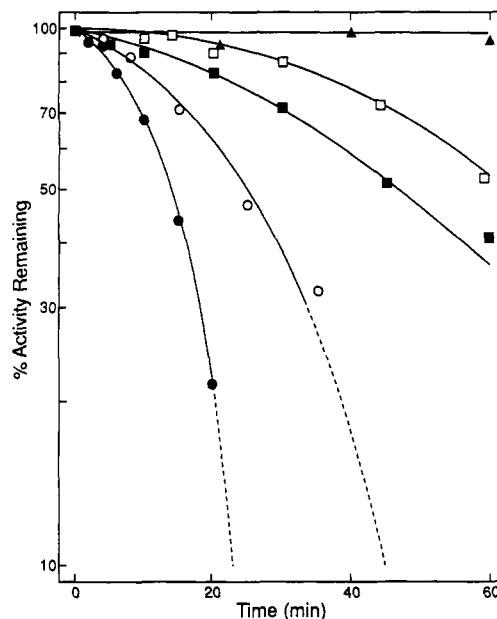


FIGURE 7: Inactivation of Av1 during incubation with NO. Five anaerobic, Ar-filled (101 kPa) incubation vials (9.5 mL) were prepared as described in Materials and Methods. NO was added to the vials as an overpressure using Ar-flushed gas-tight syringes. NO treatment was initiated by the addition of Av1 (120 μ L at 35 mg of protein·mL⁻¹). At the indicated treatment times, samples (15 μ L) were removed and added to a C_2H_2 -reduction assay mixture containing untreated Av2 (5 μ L at 39 mg of protein·mL⁻¹) and C_2H_2 -reducing activity was determined as described in Materials and Methods. The log of the percent Av1 activity remaining is plotted versus time of incubation of Av1 with NO. The gas phase of the treatment vials contained (▲) no NO; (□) 1% NO; (■) 2.5% NO; (○) 5% NO; and (●) 8% NO (v/v). The dashed lines represent extrapolated results for activity measurements below accurate detection limits.

formation over the 5-min assay period. As all reported experiments utilized samples of Av1 preincubated with gas-phase concentrations of NO of 8 kPa or less, the effect of NO on Av2 in activity assays was considered insignificant.

Unlike the loss of Av2 activity, the loss of Av1 activity did not appear to arise from a pseudo-first-order process (Figure 7). When incubated with 1 and 2.5 kPa of NO (18 and 45 μ M NO in solution, respectively), less than 15% of the initial Av1 activity was lost during the first 20 min. Subsequently, a progressive and more rapid loss of activity was observed which appeared to slowly develop into an exponential process. Similar features were also apparent in the plots for the loss of activity by incubation with higher gas-phase concentrations of NO. This suggested that the kinetics of inactivation after the first 20 min at lower NO concentrations were qualitatively similar to those at higher NO concentrations after a shorter initial period. The most pronounced effect was observed for Av1 incubated with 8 kPa of NO. The plot of log of the percent Av1 activity remaining versus time was nonlinear with an initial slow phase (5 min) followed by a progressively increasing rate of inactivation which resulted in a complete (>99%) loss of activity within 30 min (Figure 7). Qualitatively similar effects were observed for Av1 incubated with 5 kPa of NO except that in this case the entire process was more gradual and resulted in complete inactivation (>99%) of Av1 activity only after incubation with NO for 1 h.

Two further aspects of the inactivation of Av1 by NO were investigated. First, it was observed that the kinetics of inactivation of both C_2H_2 -reducing and proton-reducing activities of Av1 were the same during preincubation of the enzyme in the presence of 5 kPa of NO. Second, in contrast to the effects of NO on Av2, it was found that the presence or absence of

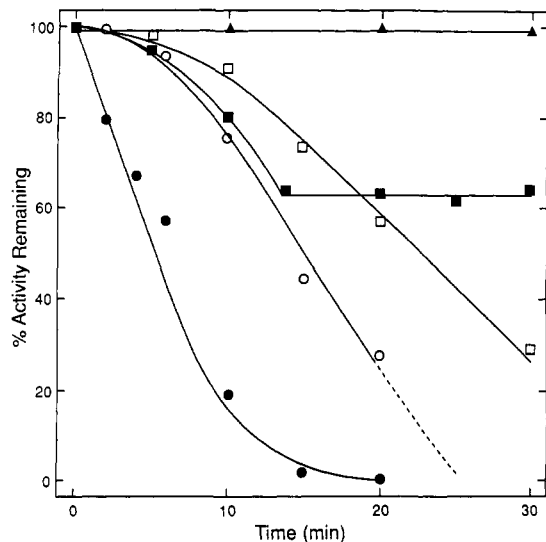


FIGURE 8: Effect of sodium dithionite on the rate of NO inactivation of Av1. Five anaerobic, Ar-filled (101 kPa) treatment vials (9.5 mL) were prepared as described in Materials and Methods. Purified NO (800 μ L) was added to vials as an overpressure using Ar-preflushed syringes. The treatments were initiated by the addition of Av1 (120 μ L at 35 mg of protein·mL⁻¹). At the indicated times, samples (15 μ L) were removed and added to a C₂H₂-reduction assay mixture containing untreated Av2 (5 μ L at 39 mg of protein·mL⁻¹). The C₂H₂-reducing activity was estimated as described in Materials and Methods. The percent Av1 activity remaining is plotted against the time of treatment. Treatment conditions: (▲) no NO; (○) no additional sodium dithionite ("as isolated"); (□) "as isolated" Av1 plus 5 mM sodium dithionite; (●) "as isolated" Av1 after removal of dithionite by passage through a Sephadex G-25 desalting column; (■) "as isolated" Av1 except that at $t = 15$ min the NO was removed by evacuation and refilling of the incubation vial with Ar (101 kPa) and at $t = 25$ min sodium dithionite was added to give a final concentration of 5 mM.

sodium dithionite had a significant effect on the kinetics of NO inactivation of Av1. This effect of dithionite is further examined in the experiment depicted in Figure 8. The rate of loss of C₂H₂-reducing activity for the "as purified" Av1 containing 1 mM dithionite when treated with 8 kPa of NO followed the kinetic profile previously shown in Figure 7. This involved a slow phase in the loss of activity followed by a more rapid pseudo-first-order decrease in activity which subsequently led to complete inactivation within 30 min. However, when the sodium dithionite present in the "as purified" Av1 was increased to 5 mM, the lag in inactivation was extended without affecting the rapid rate of inactivation which occurred after the lag phase. A second finding was that the effects of NO on the "as isolated" enzyme could be halted by the removal of NO from the preincubation vessel but that subsequent addition of sodium dithionite (5 mM final concentration) to this partially inactivated but NO-free sample did not lead to recovery of lost activity. Finally, it was found that the removal of the sodium dithionite present in the "as isolated" enzyme by passage of the enzyme through a Sephadex G-25 column prior to exposure to NO totally eliminated the slow phase in the loss of activity of Av1 (Figure 8). The pseudo-first-order rate of inactivation of the dithionite-free enzyme sample was slightly faster than the maximal rate of inactivation observed for all Av1 samples containing dithionite.

Spectroscopic Changes Associated with Inactivation of Av1 by NO. As was observed with samples of NO-inactivated Av2, the effects of NO inactivation on Av1 produced significant effects on both the UV-visible and the EPR spectra of Av1. The color of Av1 changed from dark brown to yellow during inactivation by NO, and to examine this change, a series of

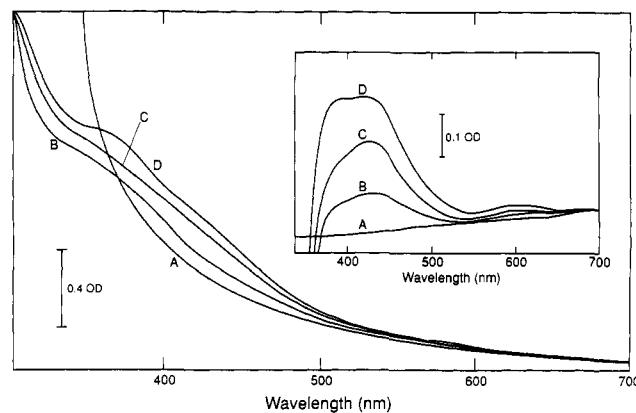


FIGURE 9: Effect of NO on the UV-visible spectrum of Av1. Samples (320 μ L) of Av1 (35 mg of protein·mL⁻¹) were incubated in 9.5-mL anaerobic Ar-filled (101 kPa) treatment vials containing 500 μ L of purified NO added as an overpressure. The protein samples contained approximately 1 mM dithionite when the experiment was initiated. After the specified periods of treatment, samples (15 μ L) were removed by syringe and added to C₂H₂-reduction assay mixtures containing untreated Av2 (5 μ L at 39 mg of protein·mL⁻¹). C₂H₂-reducing activity was determined as described in Materials and Methods. The remaining protein in the treatment vials was then transferred to an anaerobic, Ar-filled stoppered cuvette (3-mm pathlength), and the spectral changes associated with NO inactivation were recorded. The times of incubation with NO and percent activity remaining: (A) untreated control, 100%; (B) 20 min, 71%; (C) 45 min, 53%; (D) 90 min, 7%. The inset shows the difference spectra obtained by subtracting untreated AV1 from NO-treated Av1.

spectra of Av1 were recorded at varying levels of inactivation during incubation of the enzyme in the presence of 5 kPa of NO. The spectra (Figure 9) show several features including an increase in absorption in the region of 400–450 nm which correlated with increasing levels of inactivation of Av1. The absorption below 400 nm also underwent significant changes including a rapid decrease in the intense absorption associated with sodium dithionite and the progressive appearance of an additional feature at wavelengths just below 400 nm. As expected, the difference spectra obtained from the above showed the presence of two spectral features which appeared to develop at different rates during the inactivation process. The first feature to appear during the inactivation process occurred at higher wavelengths and was centered at 428 nm. The second feature, which appeared when Av1 was more inactivated, was centered at 387 nm.

The effects of NO on the EPR spectrum of Av1 were more complex than those observed with Av2. Spectra were obtained from samples of Av1 after exposure to 5 kPa of NO for varying periods of time and show (Figure 10) that the progressive loss of activity of Av1 correlates with the loss of signal intensity at $g = 3.6$ and with the complete change of the normal $g = 2.01$ signal to an axial $g = 2.03$ signal with secondary features at $g = 1.97$ and $g = 1.94$. From these spectra, a close correlation between the extent of loss of activity and the loss of signal intensity in the $g = 3.6$ region was established (Figure 11). No change in the EPR signals occurred when the NO-treated samples of Av1 were passed through anaerobic columns of G-25 before being syringed into EPR tubes, establishing that the EPR-active species are still associated with the protein.

Effects of NO on the Subunit Composition, Metal Content, and Complex Formation of Av1 and Av2. An important consideration in the characterization of the inactivation of either nitrogenase component by NO is the potential effect this inactivator might have on the fundamental physical and structural aspects of the proteins. Using anaerobic gel permeation chromatography, we have established that the com-

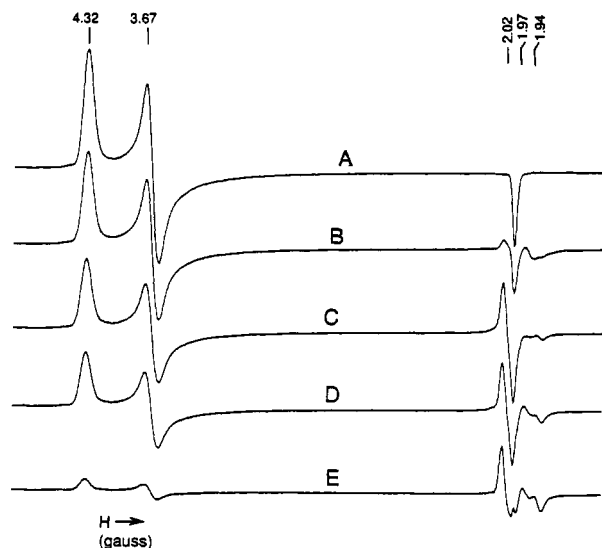


FIGURE 10: Effect of NO on the EPR spectrum of Av1. Five samples of Av1 ($300 \mu\text{L}$ at $35 \text{ mg}\cdot\text{mL}^{-1}$) were treated in Ar-filled vials with 5% (v/v) NO. The protein samples contained approximately 1 mM dithionite at the beginning of the experiment. At the indicated times, $15\text{-}\mu\text{L}$ aliquots were removed and assayed for activity and then each treatment vial was evacuated and filled with Ar to remove NO. The samples, now under Ar, were transferred to EPR tubes and frozen, and the EPR spectra were taken. Times of NO treatment: A, 0 min; B, 15 min; C, 30 min; D, 45 min; E, 90 min. EPR conditions were 3.8 K, 1 mW microwave power, a modulation amplitude of 1 mT, and a microwave frequency of 9.44 GHz.

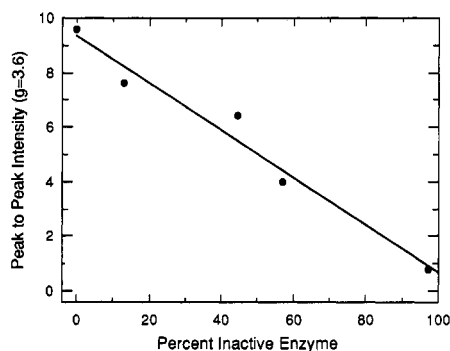


FIGURE 11: Correlation between EPR signal intensity and extent of NO inactivation of Av1. The peak to peak intensities at $g = 3.67$ for the spectra of Figure 10 were plotted against the percent of inactive Av1 determined as in the legend of Figure 10.

plete inactivation of either Av1 or Av2 by NO is not accompanied by either aggregation or polymerization of the individual proteins, nor does it lead to dissolution of the proteins into their component subunits (data not shown). Column separation techniques also established that the altered chromophores observed with the inactivated proteins coelute with the intact, inactivated enzymes and do not, therefore, represent dissociated prosthetic groups. The Fe content of fully NO-inactivated Av2 was the same as that of an untreated sample, confirming that the metal remains in Av2 after reaction with NO (data not shown).

NO-inactivated Av2 did not compete with untreated Av2 for the binding to Av1 required in normal acetylene reduction assays. Only 0.4% inhibition was detected when equimolar amounts of NO-treated Av2 and untreated Av2 were added to the assay. We found similar results for NO-inactivated Av1. In this case, addition of equimolar amounts of NO-inactivated Av1 and untreated Av1 added to untreated Av2 in the normal assay resulted in 6.7% inhibition when compared to an assay without NO-inactivated Av1 added.

DISCUSSION

NO Interaction with Av2. The results presented in this paper confirm and expand upon previous descriptions of NO as a potent inactivator of the Fe protein component of nitrogenase [e.g., Meyer (1981) and Liang and Burris (1989)]. We provide both kinetic and spectroscopic evidence that allows us to better define the site and mode of action of NO on the Fe protein. In addition, we will discuss the interactions between the binding sites of MgATP and MgADP and the [4Fe-4S] center of the Fe protein revealed by NO inactivation.

First, we discuss our kinetic results and how they fit with previous findings on NO interaction with Fe protein. A study by Meyer (1981) examined the time-dependent inactivation of Av2 activity in the presence of between 10- and 380-fold molar excesses of NO. In all cases, the losses of activity were pseudo-first-order processes. It was argued from these results that a minimum of one NO per active dimer was required for inactivation. In the present study, we have attempted to further define the minimum stoichiometry required for inactivation by examining inhibition with considerably lower concentrations of NO. For example, complete and pseudo-first-order losses of activity were observed when Av2 was incubated with 4- and 8-fold molar excesses of NO. At lower stoichiometries (1- and 2-fold excess), we observed a deviation from first-order kinetics and less than complete inhibition within 1 h of incubation. Obviously, the rate of reaction will decrease and the deviation from apparent first-order kinetics will increase as the relative concentrations of the reactants decrease to a point where the reaction will exhibit second-order kinetics. Several experimental factors prevented a more unequivocal estimation of the minimum stoichiometry. The most significant of these factors include (1) the instability of Av2 during long-term incubation, (2) the limiting rate of diffusion of NO from the gas to liquid phases, and (3) nonrelevant reactions of NO (e.g., absorption onto or reaction with stoppers, or the reaction of NO with dithionite or O_2) which result in a decrease in the total available concentration of NO for reaction with Av2. Our present empirical estimate of the minimum stoichiometry required for complete inactivation lies between 1 and 2 NO molecules per Av2 dimer (Figure 1).

The EPR spectra for samples of NO-treated Av2 demonstrate that the effects of NO on the [4Fe-4S] center fall into two categories. The first is a loss of the $g = 1.94$, $S = 1/2$ signal and the accompanying $S = 3/2$ signal near $g = 6$. The decrease in these signals correlates with the extent of NO-dependent inactivation of Av2 activity and suggests that the reduced form of the [4Fe-4S] center of Av2 is converted to an EPR-silent NO-complexed state. Potentially, the formation of an EPR-silent NO complex of the iron-sulfur cluster could arise from NO-binding either in the form of a nitroxyl (NO^-) or a nitrosyl (NO^+) ligand. While our data do not allow us to unambiguously distinguish between these two forms of ligation, the increases in the UV-visible absorption of the protein which occurred at wavelengths typically associated with oxidation of the Fe-S center are consistent with the metal-oxidizing effects of a nitroxyl-based ligation.

The second effect of NO on Av2, which only occurs at high NO concentrations, is the appearance of a $g = 2.03$ EPR signal which develops only after the loss of the $g = 1.94$ signal. From this it can be assumed that the paramagnetic species which is responsible for this signal is formed subsequent to the species which leads to the loss of Av2 activity. The fact that the signal persists even after size-exclusion separation on Sephadex G-25 shows that the modified center remains complexed to the protein.

The fact that model Fe-S centers which contain a single NO bound to Fe are EPR-silent (Butler et al., 1988) allows us to infer that the species created on reaction with Av2 is similar. The loss of the $g = 1.94$ signal cannot be attributed to oxidation of reduced Av2 alone because addition of dithionite does not restore activity or the $g = 1.94$ signal.

Since the minimum stoichiometry of NO required to fully inactivate Av2 is probably 1 or 2 per Av2 dimer, we suggest that the concomitant loss of Av2 activity and the $g = 1.94$ and 5.97 signals is a result of the formation of either one or two EPR-silent NO-Fe species. In contrast to the EPR-silent state of single NO-Fe complexes, the distinctive $g = 2.03$ EPR signal is characteristic of a dinitrosyl-iron complex (Butler et al., 1988). Such dinitrosyl complexes are easily formed from model Fe-S centers after reaction with NO or nitrite and are represented by complexes such as $(\text{Fe}(\text{NO})_2\text{SR}_2)^-$ (Butler et al., 1988). Within a protein-bound [4Fe-4S] center, such a complex could be formed by the displacement of two sulfur-containing ligands (either two sulfides or a cysteine thiol and a single sulfide) from a single Fe. Clearly, further experimentation is needed to define the true minimum stoichiometry of NO binding to Av2 and the chemical composition of the center after reaction with stoichiometric and excess NO. For example, one could postulate that because the Fe protein is a homodimer with two cysteine ligands from each monomer complexing to the [4Fe-4S] center, the NO could react with two Fe atoms each associated with one of the identical monomers.

Another interesting finding of this work is the relationship between nucleotide binding and NO inactivation of Av2. We found that nucleotides bound to the Fe protein influenced the rate of NO inactivation of Av2 and that NO inactivation of Av2 influences the binding of nucleotides to Av2. The rate of NO inactivation of Av2 accelerated in the presence of MgATP and MgADP, with MgATP giving the greatest acceleration. This suggests that the binding of these nucleotides increases the reactivity of the [4Fe-4S] center to NO. These results can be explained from earlier findings that showed that the binding of MgATP to the Fe protein causes (a) a conformational change in the protein that is characterized by a decrease in the redox potential of the Fe-S center, (b) a change of the rhombic EPR signal to an axial signal, and (c) an increased accessibility of the Fe atoms of the [4Fe-4S] center to chelators. MgADP, on the other hand, was shown to lower the midpoint potential of the Fe-S cluster, but it did not affect the EPR signal or the rate at which chelators removed Fe. The above facts are consistent with our findings that the ADP-Fe protein complex shows a rate of NO inactivation between that of the ATP-Fe protein complex and the nucleotide-free Fe protein.

Meyer (1981) also demonstrated that many of the effects of NO on the Fe protein were indistinguishable from the effects of nitrite. This is consistent with the finding that significant quantities of NO are produced by reduction of nitrite by dithionite, the reductant normally used in the nitrogenase assay. More recently, Vaughn and Burgess (1989) have demonstrated that nitrite can be a substrate of nitrogenase. These workers also reported an irreversible inactivating effect of nitrite on the Fe protein and argued that, in the absence of detectable NO, nitrite is the likely inactivating species. In addition, they reported that the presence of MgATP led to an increase in the rate of inactivation of the Fe protein by nitrite. Since we have demonstrated that the inactivating effects of NO occur at ratios to Av2 approaching one and that the rate of inactivation is accelerated by the presence of MgATP, caution

must be taken in accepting nitrite as a true inactivating species of the Fe protein.

NO inactivation of the Fe protein also affects the ability of the Fe protein to interact with nucleotides. This is of considerable interest since a number of lines of evidence suggest that the Fe-S center and nucleotide interacting regions of the Fe protein are as much as 20 Å from one another on the protein (Zumft et al., 1973; Morgan et al., 1990; Georgiadis et al., 1990). We found that NO-inactivated Av2 no longer bound either MgATP or MgADP arguing that effects at the Fe-S center are communicated to the nucleotide-binding site even though the centers are 19–20 Å apart. This could be rationalized if the NO reaction with Av2 displaces one or more ligands from the Fe-S center which, in turn, alters the nucleotide-binding region of the protein. Further evidence for a conformational change of NO-inactivated Av2 is suggested by experiments in which we find NO-treated protein is unable to compete with equimolar concentrations of native Av2 for binding to untreated Av1 in C_2H_2 -reduction assays. While these data suggest a conformational change of Av2 upon interaction with NO, the change (or changes) does not lead to dissolution of the subunits or aggregation and does not appear to result in the loss of total Fe from the protein.

NO Interaction with Av1. The effect(s) of NO on Av1 can be distinguished from its effect(s) on Av2 by the large difference in the NO concentration required to inactivate the two proteins. One finding in this paper which may have led other workers to think that the MoFe protein does not react with NO is the protective effect dithionite has on the inactivating effect of NO. The effects of NO on Av1 in the presence and absence of dithionite (Figure 8) and the loss of UV absorption at 310 nm (peak of dithionite absorption) associated with the inactivation experiment in the presence of dithionite (Figure 9) strongly suggest that Av1 catalyzes an NO-dependent oxidation of dithionite. We have not determined the product(s) of this reaction. However, Vaughn and Burgess (1989) have suggested that the reduction of NO_2^- to NH_3 may proceed via an enzyme-bound nitrosyl species, followed by a five-electron reduction of the nitrosyl to NH_3 and water. By analogy, the reduction of NO might be expected to produce ammonia or a partially reduced form of nitrogen such as NH_2OH .

Dithionite delays, but does not prevent or alter, inactivation of Av1 by NO. Except for the large concentration difference required for interaction, the overall process of NO inactivation shows similarities to those discussed for Av2. For example, there is a direct correlation between the extent of inactivation of Av1 and the loss of EPR signals. The EPR spectrum of the "as prepared" Av1 results from the paramagnetic MoFe centers. Oxidation of Av1 by chemical oxidants such as thionine leads to a complete loss of the EPR spectrum. A time course of inactivation of Av1 by NO shows a progressive decrease in its EPR spectrum and the appearance of an axial feature at $g = 2.03$ with secondary features at $g = 1.97$ and $g = 1.94$. As we have described for Av2, the $g = 2.03$ signal is consistent with the formation of a dinitrosyl-iron complex. In contrast, the signals at $g = 1.97$ and 1.94 are characteristic of iron-sulfur centers. There are several explanations for the appearance of the latter signals including a rearrangement of the M center or the detection of an altered form of its normally EPR-silent Fe centers. Like Av2, addition of dithionite to the NO-treated Av1 EPR samples showed no recovery of native Av1 spectra.

Although it is not possible to unequivocally state which center(s) in Av1 undergoes reactions with NO, one cannot assume that NO is a general and nonspecific reactant with

all Fe-S centers. To the contrary, evidence indicates that the effects of NO can be preferentially directed at single Fe-S centers in enzymes which contain multiple Fe-S centers. For example, the soluble hydrogenase from *Alcaligenes eutrophus* H16 contains 2 Ni, three [4Fe-4S] centers, one [2Fe-2S] center, and FMN. The effects of NO on this enzyme are complex, but it was found that treatment with NO under nonturnover conditions prevents the enzyme from being activated by H₂ in the presence of NADH (Hyman & Arp, 1988). This same treatment did not affect the enzyme's ability to oxidize H₂ or its diaphorase activity. The latter results are compatible with the selective nitrosylation and destruction of a coupling [4Fe-4S] center (Hyman & Arp, 1988) but not the FeS and Ni centers required for H₂ activation.

General Consideration of NO Interaction with Fe-S-Containing Proteins. The effects of NO on nitrogenase (Liang & Burris, 1989), the soluble hydrogenase from *A. eutrophus* H16 (Hyman & Arp, 1988), and the membrane-associated hydrogenase from *A. vinelandii* (Hyman & Arp, 1991) differ when NO is added under turnover versus nonturnover conditions. For nitrogenase, the effects of NO under turnover conditions indicate that the Fe protein component is extremely sensitive to inactivation by NO (Liang & Burris, 1989). On the basis of results from this paper, their description of NO as a noncompetitive inhibitor of nitrogenase activity can be explained by the fact that catalytically active Fe protein is "removed" from the reaction mixture. Liang and Burris (1989) further observed that the effects of NO on the Fe protein diminished under conditions in which the nitrogenase components are mixed and presumably bound to one another. This suggests that the binding of the Fe protein to the MoFe protein affords some protection of the [4Fe-4S] center against NO, consistent with an earlier report (Walker & Mortenson, 1974) that showed that the rate of MgATP-dependent release of Fe from the [4Fe-4S] center of the Fe protein to chelators was diminished by the presence of the MoFe protein. Since we have demonstrated that NO inactivation of Av2 severely compromises its ability to complex "effectively" with Av1, insight into the site of the effects of NO on Av2 could be gained by rigorously examining the protective effects of Av1. Such experiments could examine homologous mixtures of nitrogenase components or mixtures of Cp2 with Av1 that are known to produce catalytically inactive "tight" complexes (Emerich et al., 1978).

Over the last four years, NO has been recognized as an important messenger in cells (Barinaga, 1991). The mechanism of NO action in cell message transmission is unknown, although the known effects of NO on Fe-S proteins coupled with the extreme sensitivity of Fe protein of nitrogenase discovered in our work suggest that one possible mode of action of NO might be an interaction with Fe-S clusters of important enzymes.

REFERENCES

- Barinaga, M. (1991) *Science* 254, 1296-1297.
- Bolin, J. T., Ronco, A. E., Mortenson, L. E., Morgan, T. V., Williamson, M., & Xuong, N. H. (1990) in *Proceedings of the 8th International Congress on Nitrogen Fixation* (Gresshoff, P. M., Roth, L. E., Stacey, G., & Newton, W. E., Eds.) pp 117-124, Chapman and Hall, New York.
- Bui, P. T., & Mortenson, L. E. (1968) *Proc. Natl. Acad. Sci. U.S.A.* 61, 1021-1027.
- Burgess, B. K. (1985) in *Molybdenum Enzymes* (Spiro, T. G., Ed.) pp 161-219, John Wiley & Sons, New York, NY.
- Burgess, B. K., Jacobs, D. B., & Stiefel, E. I. (1980) *Biochim. Biophys. Acta* 614, 196-209.
- Butler, A. R., Glidewell, C., & Li, M.-H. (1988) *Adv. Inorg. Chem.* 32, 335-393.
- Dilworth, M. J. (1966) *Biochim. Biophys. Acta* 127, 285-294.
- Emerich, D. W., Ljones, T., & Burris, R. H. (1978) *Biochim. Biophys. Acta* 527, 359-369.
- Georgiadis, M. M., Chakrabarti, P., & Rees, D. C. (1990) in *Proceedings of the 8th International Congress on Nitrogen Fixation* (Gresshoff, P. M., Roth, L. E., Stacey, G., & Newton, W. E., Eds.) pp 111-116, Chapman and Hall, New York.
- Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem.* 177, 751-766.
- Hyman, M. R., & Arp, D. J. (1988) *Biochem. J.* 254, 469-475.
- Hyman, M. R., & Arp, D. J. (1991) *Biochim. Biophys. Acta* 1076, 165-172.
- Jensen, B. B., & Burris, R. H. (1988) *Biochemistry* 25, 1083-1088.
- Kelly, M., & Lang, G. (1970) *Biochim. Biophys. Acta* 223, 86-104.
- Liang, J., & Burris, R. H. (1989) *Indian J. Biochem. Biophys.* 25, 636-641.
- Lockshin, A., & Burris, R. H. (1965) *Biochim. Biophys. Acta* 11, 1-10.
- Lovenberg, W., Buchanan, B. B., & Rabihowitz, J. C. (1963) *J. Biol. Chem.* 238, 3899-3913.
- Meyer, J. (1981) *Arch. Biochem. Biophys.* 210, 246-256.
- Michalski, W. P., & Nicholas, D. J. D. (1987) *Arch. Microbiol.* 147, 304-308.
- Morgan, T. V., McCracken, J., Orme-Johnson, W. H., Mims, W. B., Mortenson, L. E., & Peisach, J. (1990) *Biochemistry* 29, 3077-3082.
- Mortenson, L. E., & Thorneley, R. N. F. (1979) *Annu. Rev. Biochem.* 48, 387-418.
- Orme-Johnson, W. H. (1985) *Annu. Rev. Biophys. Chem.* 14, 419-459.
- Schollhorn, R., & Burris, R. H. (1967) *Proc. Natl. Acad. Sci. U.S.A.* 58, 213-216.
- Trinchant, J. C., & Rigaud, J. (1980) *Arch. Microbiol.* 124, 49-54.
- Trinchant, J. C., & Rigaud, J. (1982) *Appl. Environ. Microbiol.* 44, 1385-1388.
- Vaughn, S. A., & Burgess, B. K. (1989) *Biochemistry* 28, 419-424.
- Walker, G. A., & Mortenson, L. E. (1974) *Biochemistry* 13, 2382-2388.
- Zumft, W. G., Palmer, G., Mortenson, L. E. (1973) *Biochim. Biophys. Acta* 292, 413-421.