Reduction of Cyclic and Acyclic Diazene Derivatives by *Azotobacter vinelandii* Nitrogenase: Diazirine and *trans*-Dimethyldiazene†

Charles E. McKenna,* Anton M. Simeonov,‡ Henry Eran, and Marjorie Bravo-Leerabhandh

Department of Chemistry, University of Southern California, Los Angeles, California 90089-0744

Received May 1, 1995; Revised Manuscript Received September 18, 1995⊗

ABSTRACT: Nitrogenase reduces N_2 to NH_3, but the mechanistic details are unclear. Diazene (N_2H_2), a proposed $2e^-/2H^+$ intermediate on the reduction pathway, is labile under typical enzyme assay conditions, and no firm evidence is available on whether or not it can be reduced by or inhibit nitrogenase. In this paper, we compare the interactions of *Azotobacter vinelandii* (*Av*) nitrogenase with two diazene analogues: diazirine, a photolabile diazene containing the azo (−N═N−) group in a strained, three-membered ring, and *trans*-dimethyldiazene, a diazene containing an unstrained *trans*-disubstituted N═N bond. Diazirine is reduced by nitrogenase under specific conditions to methane, methylamine, and ammonia in a ratio of ca. 1:2:4−5 with a K_m value for all three products similar (0.05−0.09 mM) to that of dinitrogen (0.06−0.12 mM). The K_m value of diazirine does not depend on the ratio of nitrogenase Fe protein (Av2) to nitrogenase MoFe protein (Av1) at Av2:Av1 ratios of 0.71 and 14.9. Diazirine potently and competitively inhibits acetylene reduction by *Av* nitrogenase with $K_i = 0.03$ mM and is predicted to inhibit H_2 evolution completely at pressures $\gg K_m$. The experimental Henry's Law constant (1.50 M/atm) determined for *trans*-dimethyldiazene in H_2O shows that it has about 20-fold higher solubility than diazirine in water at 30 °C. *trans*-Dimethyldiazene is reduced by nitrogenase under specific conditions to ammonia, methane and methylamine in a ratio of ca. 1:1:1 with K_m values for the three products of 0.51−0.58 M. The product ratio does not change significantly when the component ratio (Av2:Av1) is varied over 2.06−13.62. *trans*-Dimethyldiazene reduction is inhibited non-competitively by CO and C_2H_2 with K_i values of ca. 0.0008 and 0.006 atm, respectively. The results are discussed with respect to the stereoelectronic differences between the two azo substrates. A "random-edge" reduction is compared with alternative schemes for the diazirine reduction. For *trans*-dimethyldiazene, initial C−N cleavage is proposed to yield CH_4 and a bound CH_3N_2H species, which is then reduced to CH_3NH_2 and NH_3.

Nitrogenase (EC 1.18.6.1) is the enzyme responsible for biological nitrogen fixation, which can be represented by the following equation (Burris, 1991, Yates, 1992):

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

In the absence of exogenous reducible substrates, nitrogenase functions as a hydrogenase (Yates, 1992). Besides the enzyme, sources of energy (ATP),[1] electrons (dithionite), and protons are required for *in vitro* activity. Standard nitrogenases from different types of azotrophs all consist of two metalloproteins: an iron (Fe) protein (dinitrogenase reductase) and a molybdenum−iron (MoFe) protein (dinitrogenase). In the aerobe *Azotobacter vinelandii*, the Fe protein is a γ_2 dimer [∼60 kDa (Georgiadis *et al.*, 1992)] and the MoFe protein is a $\alpha_2\beta_2$ tetramer [∼240 kDa (Kim & Rees, 1992a)]. The X-ray structures of both proteins (Av2, 2.9 Å and Av1, 2.7 Å) are now available (Chan *et al.*, 1993; Georgiadis *et al.*, 1992). Although both nitrogenase components are necessary for enzyme activity, their functions differ. As implied by the nomenclature given above (Burris, 1991), the Fe protein transfers electrons to the MoFe protein which is believed to contain the active site of N_2 reduction. This active site is thought to include the FeMo-cofactor (Shah & Brill, 1977), a unique cluster of one Mo atom, 6−8 Fe atoms, 8−9 S atoms, and one homocitrate ligand (Hoover *et al.*, 1989).

Models of the FeMo-cofactor have been proposed on the basis of X-ray crystallographic electron densities observed at the location of the cluster which is ∼10 Å below the protein surface (Bolin *et al.*, 1993; Chan *et al.*, 1993; Kim & Rees, 1992b). In one model (Chan *et al.*, 1993), the cofactor is shown to contain two clusters of composition 4Fe-3S and 1Mo-3Fe-3S that are bridged by two putative nonprotein S^{2-} and a low density ligand, possibly a well-ordered S, a N, or an O. Besides its protein ligands [a Cys (α275) and a His (α442); a Gln (α191) and a His (α195) are proximal], the cofactor is coordinated through its Mo atom to homocitrate. The proposed intracluster cavity in the cofactor model is too small by 0.5 Å to accommodate N_2, but it has been suggested that the cofactor metal cage expands on reduction to admit the substrate (Chan *et al.*, 1993). Several alternative modes of binding between the cofactor (Mo or Fe site) and N_2 have been proposed (e.g., end-on or side-on) (Coucouvanis, 1993; Hardy, 1979; Leigh, 1995), but none has been unequivocally verified.

† This work was supported in part by grants from NIH (GM 31716) and NRICGP/CREES/USDA (95-37305-2068) and by a Dean's Innovative Research Award to C.E.McK.

* Corresponding author.

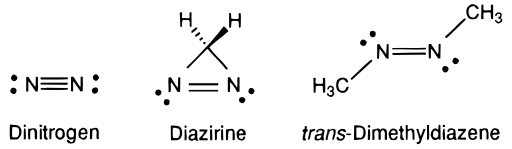
‡ 1994−1995 Oakley Fellow.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1995.

[1] Abbreviations: Av1 and Av2, *A. vinelandii* dinitrogenase and dinitrogenase reductase, respectively; DT, sodium dithionite; ATP, adenosine 5′-triphosphate; HPLC, high-performance liquid chromatography; GC, gas chromatography.

0006-2960/96/0435-4502$12.00/0

Scheme 1



The chemical mechanism of N_2 reduction to NH_3 (e.g., via intermediates corresponding to three $2H^+/2e^-$ steps or a $4H^+/4e^-$ step followed by a $2H^+/2e^-$ step (Hardy, 1979) is not yet well understood in terms of specific enzyme moieties and well-defined intermediates. The feasibility of an enzyme-bound form of diazene (HN═NH), a $2e^-/2H^+$ intermediate, remains a subject of active inquiry by model chemists (Sellmann, 1993), and hydrazine (H_2N-NH_2), a $4e^-/4H^+$ intermediate, has been detected in acid- or base-quenched nitrogenase systems (Thorneley *et al.*, 1978). A metal-bound diazene tautomer (e.g., $M{=}N-NH_2$) has also been postulated as an intermediate on the pathway of N_2 reduction by nitrogenase (Chatt, 1980).

Alternative substrates and inhibitors are of value as probes for elucidating the mechanism of nitrogen fixation (Yates, 1992). The majority of these molecules contain a reducible triple bond (HCN, MeNC, HN_3/N_3^-, C_2H_2; CO is an inhibitor which is not reduced). Some of the substrates have side reactions with nitrogenase, e.g., CN^- is a potential modifier of the enzyme binding site (Ljones, 1973; Lowe *et al.*, 1989; Wallace & Rabinowitz, 1971), and nitrite inactivates the Fe protein (Meyer, 1981; Vaughn & Burgess, 1989). Chemical probes containing a −N═N− double bond analogous to that in diazene are clearly of interest (Scheme 1). The cyclic azo compound diazirine was proposed as a possible substrate and active site probe after the discovery that the structurally related cyclopropene (McKenna *et al.*, 1976) is a nitrogenase substrate with unique properties, although unstrained acyclic alkenes such as ethylene and noncumulated dienes are not reduced by wild-type Mo nitrogenases. Diazirine contains the −N═N− (double) bond in a strained, three-membered ring. In addition, it is photolabile (Amrich & Bell, 1964) and therefore is a potential candidate as a photolabel for the nitrogenase active site. Shortly thereafter, diazirine was briefly reported to be an inhibitor (McKenna *et al.*, 1981) of nitrogenase and also a substrate reduced to methane (McKenna & Eran, 1982; Orme-Johnson *et al.*, 1981), ammonia (McKenna & Eran, 1982), and methylamine (McKenna *et al.*, 1984). It is not clear whether the special structural and electronic properties conferred by the small-ring geometry of diazirine are essential to its interaction with nitrogenase, as is the case with cyclopropene vs ethylene or propene: no acyclic azo compound has yet been demonstrated to be reduced by nitrogenase. The recent availability of the X-ray crystallographic structures of Av1 and the subsequently developed FeMo-co model stimulates further examination of alternative substrates as probes for nitrogenase reduction mechanisms. In this paper, we describe some details of diazirine−nitrogenase interactions. To obtain a better understanding of the role of steric and electronic effects in these interactions, we have also investigated a second diazene analogue as a nitrogenase substrate, *trans*-dimethyldiazene. In contrast with diazirine, *trans*-dimethyldiazene is an unstrained, acyclic structure which, however, has a less compact steric profile, with its two methyl groups stretching in opposite directions at a 111.9° angle from the −N═N− bond axis (Chang *et al.*, 1970), within a common molecular plane. *trans*-Dimethyldiazene also differs electronically from diazirine, in terms of the orientation of its lone pair electrons, their coordination aptitude, and in the absence of the Walsh-like C−N orbitals present in the cyclic substrate (Baird, 1987). By analogy with diazirine, *trans*-dimethyldiazene reduction could lead to methylamine, ammonia, methane, and/or other products. We present here a comparative account of *A. vinelandii* nitrogenase interactions with these two diazene derivatives, including documentation that *trans*-dimethyldiazene is a new reduction substrate for the enzyme.

MATERIALS AND METHODS

Reagents. Chemicals and biochemical reagents used in nitrogenase assays were obtained from Sigma/Aldrich Co. in the purest grade available. CO, H_2, N_2, and acetylene were obtained in 99.9% purity from MG Industries or Gilmore Liquid Air Co. Methane (99.0%) and ethylene (99.5%) GC standards were from MG Industries. Ethane (99.99%) was purchased from Matheson. 1,2-Dimethylhydrazine dihydrochloride (99+%) and dansyl chloride (98%) were purchased from Aldrich. Mercuric oxide (reagent grade), magnesium chloride, and sodium tetraborate decahydrate (both analytical grade) were procured from Mallinckrodt. For diazirine synthesis, butyl ether (Aldrich) was purified by fractional distillation at 75 °C and 80 mm Hg. Sodium dithionite (Sigma) used in the *trans*-dimethyldiazene assays was purified by 2-fold recrystallization using a method described elsewhere (McKenna *et al.*, 1991).

Analytical Methods. IR and UV spectra were measured on Perkin-Elmer 281 IR and Beckman Acta VI or Shimadzu UV-260 UV−visible spectrophotometers, respectively. ^1H NMR spectra were recorded at 250.13 or 360.13 MHz and ^{13}C NMR spectra at 62.89 MHz on Bruker AM-250 or Bruker AM-360 spectrometers, unless noted otherwise. Identification of CH_4 as a reduction product was performed by mass spectrometry on an LKB 9000 mass spectrometer at 70 eV for diazirine reduction and on a Hewlett-Packard 5989A GC/MS coupled to an HP 5965B IRD for dimethyldiazene reduction. Gas chromatography was performed on a Varian 2400 or Shimadzu 14A GC equipped with dual flame ionization (FI) detectors, or a Varian 3700 GC equipped with both FI and thermal conductivity (TC) detectors. GC peaks were integrated using Varian 485 or Hewlett-Packard 3390A recording integrators, or using the Rainin Dynamax MacIntegrator I system. Chromatograms were also recorded on a Varian A-5 recorder. The HPLC system used to determine ammonia and methylamine has been previously described (Bravo *et al.*, 1988). Calculations were performed with an IBM personal computer using a Lotus 1-2-3 spreadsheet or on Apple Macintosh computers using a Microsoft Excel 4.0 spreadsheet.

Synthesis of Diazirine and Its Precursors: General Comments. Diazirine was synthesized from dichloroamine (prepared *in situ* from sodium hypochlorite and ammonium chloride in sodium formate buffer) and *tert*-octylazomethine by an adaptation of the method of Graham (1965). We find that diazirine gas is stable in the dark over saturated aqueous Na_2SO_4 (see below); however, it must be handled on the vacuum line with the utmost caution, and close attention must be given to reproducing exactly the procedure described (see also important safety note in section *Synthesis and Purification of Diazirine* below).

Synthesis of tert-Octylazomethine (Campbell et al., 1944). Aqueous formaldehyde (0.68 mol, 37% solution) was added dropwise to an equimolar amount of *tert*-octylamine (Hurwitz, 1952) at 10 °C with vigorous stirring over 2 h. The upper layer was allowed to stand at 4 °C over KOH overnight. The crude product (stench) was purified by distillation at 54 °C (30 mm Hg), lit. b.p. 50−52 °C (13 mm Hg) (Emmons, 1957), and identified by IR and ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.88 (s, 9 H), 1.15 (s, 6 H), 1.58 (s, 2 H), 7.35, 7.23 (2d, $^2J_{HH}$ = 16.0 Hz); the ^{13}C NMR (63 MHz) spectrum of this compound was also determined: δ 28.85, 29.93, 31.58, 32.39, 55.67, 147.49.

Synthesis and Purification of Diazirine. The synthetic apparatus (assembled in a well-ventilated hood) consisted of a three-necked 1 L round-bottomed flask (thermometer, stir bar) fitted with a 500 mL addition funnel, connected through a series of four U-tube traps to a vacuum pump. Each trap could be individually isolated by *teflon-sealing* glass vacuum stopcocks (10 mm bore) and were cooled as follows: (a) −35 °C (2-propanol slush bath, minimal CO_2); (b) −80 °C (2-propanol/CO_2); (c) −142 °C (methylcyclopentane/liquid N_2 slush bath; Vigreux-type inner tube); and (d) −196 °C (liquid N_2). The bath temperatures were verified with a Cu−constantan thermocouple connected to a Varian A-5 recorder. The reference temperatures were ice water (0 °C) and liquid N_2 (−196 °C). *Important safety note: hazardous procedure.* We stress that the vacuum line employed used only teflon-sealing type glass stopcocks. Because of the explosive nature of neat diazirine in condensed phase, all glassware was covered with protective wire mesh reinforced heavily with plumber's tape, and the entire apparatus was placed behind a protective shield. A face shield and gloves should always be worn by the operator. Extreme caution should be exercised, and we recommend that the reaction scale should not be increased beyond that specified here. Under no circumstances should additional liquid N_2 be added to the methylcyclopentane slush bath once diazirine generation or purification is underway, as explosion in this trap, possibly caused by sudden crystallization of solid diazirine below the normal slush bath temperature, may ignite the slush solvent.

The sodium formate buffer (6.75%, 75 mL), ammonium chloride solution (21.4%, 75 mL), and butyl ether (75 mL) were mixed and added with stirring at 5 °C (ice-NaCl bath) to a three-necked 1 L round-bottomed flask. The sodium hypochlorite solution (0.4 N, 150 mL) was placed in the addition funnel. The entire apparatus including the traps was flushed briefly with Ar. *tert*-Octylazomethine (3.53 g) was then added to the reaction vessel and the system was opened to vacuum (<1 mm Hg). The entire sodium hypochlorite solution was added to the reaction mixture over 6 min with magnetic stirring. The reaction mixture foamed and its temperature rose to 14 °C. Five minutes after the reaction subsided (∼15 min), the reaction vessel was isolated from the traps. After evacuation (10 min), the traps were individually isolated and disconnected. The trap at −142 °C was connected to the vacuum line for further purification of diazirine through the carbon tetrachloride/dry ice and methylcyclopentane/liquid N_2 traps. The purified diazirine was expanded in a 500 mL volume on the vacuum line, which has been described in detail previously [Figure 2 of McKenna *et al.* (1980)]. Usually, a pressure of 40 mm Hg diazirine was observed (0.05 atm, 1.1 mmol of diazirine). Diazirine is a photolabile gas which decomposes when irradiated at 313 nm with a Hg lamp (Amrich & Bell, 1964; Frey & Stevens, 1962) and was therefore handled under minimal light and stored in the dark.

Long-Term Storage of Diazirine. A dual-bulb gas storage apparatus [Figure 1 in Burris (1972)] was adapted for long term storage of diazirine. The apparatus was attached to the vacuum line and evacuated. Diazirine was expanded in the vacuum line and the lower bulb of the apparatus. A degassed solution of sodium sulfate was added to the upper bulb and then allowed to enter the lower bulb, thereby trapping the diazirine gas in the upper portion of the lower bulb. For more dilute mixtures, He or Ar was introduced as desired to bring the total gas pressure to 1 atm. The sodium sulfate solution ensured that no vacuum was created in the lower bulb as diazirine was sampled through a septum-sealed opening in the apparatus, using a Hamilton gastight syringe. All the above operations were performed under reduced lighting. Storage of diazirine in methanol solutions at −85 °C (Orme-Johnson *et al.*, 1981) was considered as an alternative. However, we found that 100 μL of methanol inhibited nitrogenase activity in our standard assay by as much as 30% (data not shown), and quantitative addition to give exactly known molar diazirine concentrations in assays proved more difficult, because the effect of variably added methanol on diazirine solubility in the liquid phase had to be taken into account.

Characterization and Quantitation of Diazirine. Diazirine was characterized in the gas phase by IR and UV spectroscopy. A GC method for analysis of diazirine was also established.

(a) IR Spectrum of Diazirine. On the vacuum line, diazirine was expanded (approximately 0.04 atm) into a pre-evacuated cylindrical gas IR cell with NaCl windows (5 cm path length, 20 mL capacity). The IR spectrum (data not shown) was in good agreement with those previously published (Ettinger, 1964; Graham, 1962). In particular, characteristic C−H absorptions at 3000−3200 cm^{-1} and multiple bands at 1610−1660 cm^{-1} (tentatively assigned to N═N stretching vibrations) were observed. Unlike the reference spectrum (Ettinger, 1964), our spectrum showed no CO_2 impurity at 2300 cm^{-1} [strong asymmetric stretching vibration (Mecke & Langenbucher, 1965)]. When obtained over 1 month at a sampling rate of one spectrum every 2 days, the IR spectrum of diazirine stored in the IR cell (dark, room temperature) remained stable. There was no evidence of photodecomposition in the samples when exposed to subdued room lighting over several hours.

(b) UV Spectrum of Diazirine. An anaerobic, saturated sodium sulfate solution was displaced from a septum-sealed cylindrical quartz cell (1 cm path, 3 mL capacity) with diazirine from the dual-bulb storage device (0.04 atm in Ar) using a 3 mL plastic syringe (Becton Dickinson). A similar, empty cell was used as reference. The UV spectrum was in good agreement with a published spectrum (Graham, 1962). In particular, several sharp and regularly spaced peaks (288, 294, 301, 308.5, 313, 316.5, and 322.5 nm) were observed between 282 and 324 nm. The molar absorptivity (ϵ) of 176 L mol^{-1} cm^{-1} at 308.5 nm (Graham, 1962) was used to calculate diazirine concentrations in the gas phase. The UV spectrum was stable for at least 1 month when diazirine was stored in the dark. Irradiation of diazirine in the cuvette for 5 min by a Hg lamp (679A Hanovia medium pressure, total energy output 175.8 W) caused the spectrum to disappear.

(c) GC Quantitation of Diazirine and Its Reduction Products. Diazirine was separated by GC from methane (one of the nitrogenase-catalyzed reduction products), ethylene, and acetylene on a 183 × 0.32 cm stainless steel column of Porapak N (Analabs) at 40 °C and detected by FI. The He carrier gas flow rate was 60 mL/min. A fast flow rate and low oven temperature were preferred to minimize the possibility of thermal decomposition of the diazirine samples. Typical retention times for methane, ethylene, acetylene, and diazirine were 26, 46, 112, and 270 s, respectively. The diazirine GC peak was verified by UV spectroscopy: 75% photodecomposition of diazirine by UV irradiation (5 min, Hg lamp) was concurrent with a corresponding decrease in the area of the GC peak at 270 s.

(d) Diazirine Purity: To quantitate the levels of H_2, O_2, or N_2 in diazirine, a 183 × 0.32 cm copper GC column filled with 5 Å molecular sieve was used with TC detection. GC peaks corresponding to H_2, O_2, and N_2 appeared at 40, 60, and 110 s, respectively (diazirine was not detected). The Ar carrier gas flow was 25 mL/min, the oven temperature 40 °C, and the TC filament current 107 mA. We established by this method that less than 0.4% of O_2 or N_2 was present in diazirine stored for 1 month in the dual-bulb device.

Synthesis, Purification, and Handling of trans-Dimethyldiazene. *trans*-Dimethyldiazene was prepared from 1,2-dimethylhydrazine by the method of Renaud (Renaud & Leitch, 1954). The product was stored in gas tubes (Pyrex, 14/20 ground glass joint) kept in a liquid nitrogen refrigerator. All gas handling was done on the vacuum line referred to above. Typical yields of crude product were between 65 and 70%, as determined by the pressure and volume of the expanded gas product at room temperature. For IR spectroscopy, *trans*-dimethyldiazene was expanded into a pre-evacuated gas cell with NaCl windows (74 mm path length, 30 mL capacity) at partial pressures of 0.066−0.4 atm. IR spectra obtained from several samples were fully congruent with the published spectrum (Durig *et al.*, 1972), displaying a prominent ensemble of three strong doublets centered at 2926 cm^{-1} (symmetric stretching mode of the methyl groups), and singlets at 1440 and 1450 cm^{-1} (methyl group bending vibrations). NMR samples (ca. 1 M) were prepared by condensing 1 L of gas at $P \sim 0.02$ atm into a 5 mm NMR tube containing 0.6 mL of degassed $CDCl_3$ and cooled in a diethyl malonate slush bath (−51.5 °C). All product spectra showed the expected singlet at 3.67 ppm (Freeman, 1963) with impurities at 2.90−2.95 ppm totaling ≤1%. Crude *trans*-dimethyldiazene was 99.2% pure by GC analysis. Purification by vacuum distillation at −74 °C and 75−100 mm (56% yield) in an apparatus described previously (McKenna *et al.*, 1980) increased the purity to 99.6% (GC). *trans*-Dimethyldiazene was quantitated by GC on a Chromosorb P column (80−100 mesh, 150 cm × 3.2 mm) at 0 °C, with a helium flow rate of 40 mL/min; typical retention times were 5.48−5.65 min. *trans*-Dimethyldiazene was used in assays either in an undiluted form or diluted with Ar. *trans*-Dimethyldiazene at 1 atm was stable by GC and IR for the duration of the experiments (5−6 h) under indoor fluorescent lights.

Diazirine and trans-Dimethyldiazene Solubility in Water. Diazirine was previously reported to be insoluble in water (Schmitz, 1963). However, we found that it was slightly soluble in water at 30 °C. A GC method was developed to compare the solubility of diazirine with that of ethane (Wilhelm *et al.*, 1977). Briefly, known mixtures of ethane and diazirine were added to either empty glass vaccine bottles or to bottles containing 1 mL of water. Analysis of the first set of bottles established the ratio of diazirine to ethane in the gas phase. Analysis of the second set of bottles established the ratio of the two gases in the aqueous phase. The ratio of the second set of values to the first set was proportional to the ratio of the solubility of diazirine to ethane. As the solubility of ethane in water was known [1.63×10^{-3} M/atm (Wilhelm *et al.*, 1977)], the solubility of diazirine could be calculated accordingly.

The water solubility of *trans*-dimethyldiazene was determined by adding varying amounts of the gas to vented vials (5 mL) containing Ar and degassed water (1.0 mL) which were subsequently incubated at 30 °C. Gas (10 μL) and liquid (1 μL) injections into the GC were used to determine the partitioning of *trans*-dimethyldiazene between the two phases. The molar amount of *trans*-dimethyldiazene in each sample and phase was determined using a standard curve. A "reverse" experiment was also performed to verify the solubility results in which a sample of the liquid phase from an equilibrated water/dimethyldiazene system was transferred into an empty, Ar-filled vial, allowed to reach a new equilibrium, and reanalyzed for *trans*-dimethyldiazene partitioning. When stock solutions of higher concentrations (up to 0.91 M) were prepared, the molarity of *trans*-dimethyldiazene was determined from the UV spectrum of the solution using an ϵ_{343} of 25 (Hutton & Steel, 1964). *trans*-Dimethyldiazene stability ($P = 0.434$ atm in argon) in the presence of assay components (see below) was verified by GC analysis.

Nitrogenase Proteins. The Av1 and Av2 proteins were purified according to a previously published procedure (McKenna *et al.*, 1982). The protein specific activities were 1000−1600 and 1800−2500 for Av2 and Av1, respectively. The molecular masses used were 60 and 240 kDa, respectively (Georgiadis *et al.*, 1992; Kim & Rees, 1992a). The protein component ratio used in this work is defined as the molar ratio of Av2 to Av1.

Acetylene Reduction Assays. This assay is commonly used to measure nitrogenase activity (Hardy *et al.*, 1973). The method described here and the ones to follow have several elements in common: (a) vaccine bottles (21 and 5 mL, Wheaton) sealed with flanged rubber septa (Sigma/Aldrich); (b) an ATP-generating solution containing adenosine triphosphate (5 μmol, $Na_2ATP{\cdot}2H_2O$, Sigma), creatine phosphate (25 μmol, disodium salt, Pierce), creatine phosphokinase (8 units, Sigma), $MgCl_2$ (5 μmol, Mallinckrodt), and Hepes buffer (25 μmol, titrated to pH 7.3 with NaOH, Calbiochem); (c) a degassing protocol in which vaccine bottles were attached to a vacuum manifold through 22G needles. The bottles were evacuated three times to replace air with Ar; (d) the reductant, consisting of 0.25 mL of 0.08 M dithionite prepared anaerobically; (e) an incubation protocol (in this case, 10 min at 30 °C in a shaker bath at 100−200 rpm); (f) a procedure for terminating the assay, in this case, by injection of 0.25 mL of a 10% TCA solution. The following protocol was used: the ATP-generating solution and buffer (used to normalize all the assay volumes to 1 mL) were added to the vaccine bottles which were then sealed and degassed on a manifold. Dithionite was then added. Acetylene (1 mL) purified through a 2-propanol/CO_2 trap was added. The reaction was started by addition of dinitrogenase reductase. The reaction mixture was then incubated at 30 °C for 10 min in a shaker bath. A 10% TCA solution (0.25 mL) was

added to stop the reaction. For the NH_3 assays, the reaction was terminated with 0.1 mL HCl in saturated KIO_3 (Bravo *et al.*, 1988). Analysis of acetylene and its reduction product, ethylene, was performed using FI detection, with the GC operated at 50 °C, on a 50 × 0.32 cm stainless steel column of Porapak N eluted with He at flow rate of 30 mL/min. The nitrogenase specific activity was defined as units·(mg of protein)$^{-1}$. A unit of activity was defined as 1 nmol of ethylene formed per min. Acetylene was used as the internal standard (McKenna *et al.*, 1976). The ratio of the relative sensitivities of the GC to ethylene compared to acetylene (response ratio) was 1.2. The specific activity was calculated as described previously (McKenna *et al.*, 1976).

Diazirine and trans-Dimethyldiazene Reduction Assays. A protocol similar to that used for acetylene reduction assays (see above) was followed. The diazirine partial pressure inside the dual-bulb storage device was usually 0.25 atm in Ar. Volumes of 0.2−1.0 mL were withdrawn with an Ar-flushed disposable plastic syringe and injected into the assay bottles. Similarly, stock solutions of pure *trans*-dimethyldiazene in H_2O were injected into the vials. Reaction times varied between 5−30 min. Reactions were carried out under reduced lighting to ensure the absence of substrate photodecomposition and were terminated as described above. When several reduction products (gaseous or dissolved) were analyzed, the gaseous components (e.g., diazirine, *trans*-dimethyldiazene, acetylene, ethylene, methane, or H_2) were quantitated first by GC methods. Typically, a gas aliquot (0.05−0.2 mL) was withdrawn from assay bottles and simultaneously replaced by an equivalent volume of Ar. The soluble components (e.g., methylamine or ammonia) were analyzed last using microdistillation or HPLC methods.

Quantitation of Ammonia and Methylamine. Ammonia was detected in early experiments by an indophenol method (Chaney & Marbach, 1962; Chaykin, 1969). Typical values for the NH_3 standard curve were 10.1 μg of NH_3/AU (slope) and 0.02 AU (intercept). The indophenol method was found to be at least four times more sensitive than the Nessler assay for NH_3 (Dilworth *et al.*, 1965). In later experiments HPLC analysis was preferred using a dansyl chloride precolumn derivatization (Bravo *et al.*, 1988). The HPLC method afforded higher sensitivity with a lower limit of detection (0.5 nmol NH_3/mL), while simultaneously providing analysis of any methylamine formed. The same method was very sensitive for methylamine (lower limit of detection of 0.025 nmol/mL). The assay components of the nitrogenase mixture did not cause any serious interference in the HPLC measurements but did contribute a background of 0.1−0.15 nmol NH_3 per assay.

Quantitation of H_2 and CH_4. H_2 evolution was measured by GC as described above (see section on diazirine purity). Standard samples contained 0−3 μmol of H_2 gas (Airco), purified through a Deoxo cartridge (Engelhard) to remove traces of O_2. The standard bottles also contained 1 mL of nitrogenase assay mixture without the enzyme. After incubation for 10 min at 30 °C, the assay bottles received an injection of 0.1 mL of a 10% (w/v) trichloroacetic solution (TCA) to stop the enzymatic reaction. An aliquot of the gas phase (0.2 mL) was injected into the GC. The lower limit of detection by this method was 10 nmol of H_2. Quantitation of methane was done on the Porapak N GC system for quantitation of ethylene and acetylene described earlier. A standard curve was constructed prior to each experiment by assaying gas aliquots from reaction vials incubated with the complete assay system without the substrate and containing 0−400 nmol of CH_4.

Attempted Detection of Diaziridine and Formaldehyde. According to the inorganic model chemistry of Zones *et al.* (1978), diaziridine is a possible product of nitrogenase-catalyzed reduction of diazirine. We therefore attempted to design a detection method for diaziridine in our nitrogenase assay. We used 3,3-pentamethylenediaziridine (Schmitz & Ohme, 1961) as a model product containing the diaziridine ring. An iodine test was designed by adding varying amounts of the diaziridine to a mixture containing 1 mL of a KI solution (2.5 g of KI in 40 mL of sulfuric acid), 0.2 mL of carbon tetrachloride, and 1 mL of water. The iodine formed from oxidation of I^- by sulfuric acid was soluble in carbon tetrachloride and gave rise to a violet-colored solution. Diaziridine is oxidized by iodine causing a decrease in color intensity.

A method (Bricker & Vail, 1950) was evaluated to detect formaldehyde as a possible product of the nitrogenase-catalyzed reduction of diazirine. Chromotropic acid (0.1 g, Aldrich) and formaldehyde (1 mL of a 1−10 mM solution) were heated to dryness, cooled, and then treated with 5 mL of concentrated sulfuric acid. The mixture was gently boiled for 30 min, cooled, and diluted to 50 mL with deionized water. The resultant purple color was measured spectrophotometrically at 570 nm. A lower limit of 1 μmol of formaldehyde could be detected by this method.

Detection of HD Formation in the Presence of Diazirine. This experiment was designed to test for nitrogenase-catalyzed HD evolution in the presence of diazirine and D_2 in an H_2O-based reduction assay reduction system, following the studies of Burris (Guth & Burris, 1983). We have developed a convenient procedure for direct GC separation and detection of D_2 and HD based on a modification of the method of Ohkoshi *et al.* (1958). Varian 3700 GC, equipped with a TC detector, and a 183 × 0.32 cm copper or stainless steel molecular sieve (5 Å, 60−80 mesh) column immersed in liquid N_2 (−196 °C) was used with H_2 as a carrier gas at a flow rate of 25 mL/min. Under these conditions, the respective retention times for HD and D_2 were 150 and 200 s. The GC parameters were as follows: injector T 40 °C, detector T 60 °C, column oven temperature off, filament T 150 °C, filament current 266 mA. The HD standard was prepared by slow addition of D_2O to $LiAlH_4$ in an evacuated flask just prior to use. Determination of D_2/HD was done immediately after the nitrogenase reaction was terminated. Assays with gas phase mixtures of 50% D_2 (Airco or Aldrich 99.8%)/40% N_2/10% Ar and 50% D_2/50% Ar served as positive and negative controls, respectively.

Controls and Standards for Diazirine and trans-Dimethyldiazene Reduction Assays. Several sets of controls and standards were included in each reduction experiment. Bottles contained the complete assay mixture *without nitrogenase* but with one of the following components: (a) Ar; (b) diazirine gas or *trans*-dimethyldiazene, to monitor any nonenzymatic reduction; (c) ammonium chloride and methylamine, to generate NH_3 and CH_3NH_2 standard curves with 0−40 nmol of each; (d) methane gas, to generate a CH_4 standard curve with 0−400 nmol of CH_4; (e) H_2 gas, to construct a H_2 standard curve with 0−32 nmol of H_2. In addition, two sets of controls containing the complete assay mixture with the enzyme were used to verify (a) H_2 evolution in the absence of exogenous substrates and (b) acetylene reduction.

RESULTS

Aqueous Solubility of Diazirine and trans-Dimethyldiazene. The aqueous solubility of diazirine at 30 °C was calculated to be 0.078 M atm^{-1}. Diazirine thus displays sparing solubility in water, and the percent mole fraction in the liquid phase of the total gas originally introduced into an assay vial is relatively small under our conditions (9% in a 21 mL assay vial/1 mL liquid). As a result, only a minor adjustment to the initial value of the gas partial pressure is involved (not applied to our data).

The solubility of *trans*-dimethyldiazene in water at 30 °C was 1.50 M atm^{-1}, showing that *trans*-dimethyldiazene has a much higher water solubility than diazirine, nitrogen (0.006 M atm^{-1}) or acetylene (0.035 M atm^{-1}) (Wilhelm *et al.*, 1977). A plot of molar concentrations of *trans*-dimethyldiazene versus its partial pressures gave a regression line which passed through the origin with a good linear correlation ($r^2 = 0.998$) over the entire data range (up to 0.169 atm *trans*-dimethyldiazene); distribution of *trans*-dimethyldiazene between the gas and liquid phases reached equilibrium within <10 min (GC, data not shown).

Due to the relatively high aqueous solubility of the *trans*-dimethyldiazene molecule, a substantial percent mole fraction would be transferred to the liquid phase in our assays, lowering the partial pressure significantly. We therefore used the Henry's Law constant we obtained for *trans*-dimethyldiazene to calculate its final liquid phase molar concentration (C_∞) and its final gas phase partial pressure (P_∞, atm) in assay bottles after equilibrium was established. Let V_i = volume (liters) of *trans*-dimethyldiazene gas at atmospheric pressure P (atm) injected into an assay vial with gas and liquid phase volumes V_g and V_l (liters), respectively, and n_g and n_l be the actual amounts (mol) of *trans*-dimethyldiazene present at any moment in the gas and liquid phases respectively. Then, at a given moment,

$$n_g = \frac{PV_i}{RT} - n_l \tag{1}$$

and P_∞ is

$$P_\infty = \frac{n_g RT}{V_g} \tag{2}$$

The initial estimate of n_l using $P_\infty = P$ will be too high, since n_g falls as the solute dissolves. The relative pressure change corresponding to a new estimate is

$$\frac{\Delta P}{P_\infty} = \frac{P_\infty - P'_\infty}{P_\infty} \tag{3}$$

where P'_∞ is the new partial pressure obtained from the Henry's Law and the first estimate of n_l:

$$P'_\infty = \frac{C_\infty}{K} = \frac{n_l}{KV_l} \tag{4}$$

using the known value of the Henry's Law constant K for *trans*-dimethyldiazene.

Since Henry's Law relates molarity of a gas in a solvent with its momentary partial pressure, to determine P_∞ and n_l after dissolution has reached equilibrium one must use iterative methods. The value of n_l was allowed to vary (Goal Seek function, Excel 4.0 spreadsheet) until the condition $\Delta P/P_\infty \times 100 \leq 1\%$ was fulfilled. The values C_∞ and P_∞ in the assay bottle were then calculated using eq 4 and setting $P_\infty = P'_\infty$.

Reduction of Diazirine to Methane, Methylamine, and Ammonia: Product Detection and General Properties. In a standard assay, nitrogenase reduced diazirine (2×10^{-2} atm) to methane (40 nmol), methylamine (88 nmol), and ammonia (~200 nmol) in 5 min. A control assay gave 1.35 μmol of C_2H_4 from C_2H_2 (0.05 atm). The identity of the methane was initially established by comparing GC traces obtained from the complete assay gas phase sample and from a sample of pure CH_4 (lecture bottle). It was later confirmed by GC separation of the assay gas phase and MS analysis of the products (plot of *m/z* 16 and 14 vs time). CH_3NH_2 and NH_3 were confirmed as reduction products by subjecting an aliquot from the assay mixture to the derivatization protocol (see detection of ammonia and methylamine in Materials and Methods) and matching the retention times (ca. 2.3 min for NH_3 and ca. 3.0 min for CH_3NH_2) of the peaks observed from a complete diazirine reduction assay with retention times for added standards. No reduction product (<1.0 nmol of CH_4 or CH_3NH_2, <5 nmol of NH_3) could be detected when any of the following assay components was omitted: (a) ATP; (b) dithionite; (c) Av2; (d) Av1; or (e) diazirine. Reduction time course experiments were carried out to establish the limits of linearity of the reactions under standard assay conditions. No lag phase was observable and the formation of methane, methylamine, and ammonia remained linear for at least 10−15 min. In two experiments with Av1:Av2 = 2, the average product ratio was 1.0 CH_4:2.2 CH_3NH_2:4.5 NH_3 ±10%. These results were used to establish the optimal reaction time for all subsequent experiments.

When, in search of other reduction products, the assay for diaziridine (see Materials and Methods) was performed in the nitrogenase assay mixture, dithionite interfered with the color development. Distillation of the substituted diaziridine onto etched glass rods (see indophenol assay for NH_3) failed to give a positive iodine test. The iodine test was therefore found to be inconclusive for the presence of diaziridine in the assay mixture. The chromotropic acid test for ≥1 μmol of formaldehyde hydrate in the diazirine reduction assay was negative (see, however, Discussion).

Reduction of Diazirine to Methane, Ammonia, and Methylamine: Kinetics. Diazirine pressure was varied and methane formation was measured in assay mixtures in which either Av2 or Av1 was limiting (Figure 1, only Av1 limiting data are shown). The Lineweaver−Burk plots for these two conditions showed that the K_m values were similar: 1.11×10^{-3} atm or 0.0863 ± 0.0142 mM (Av2 limiting) and 0.71×10^{-3} atm or 0.0551 ± 0.0277 mM (Av1 limiting). The corresponding V_m values (÷ mg of enzyme) were 9.4 ± 1.0 and 17.0 ± 4.2 nmol CH_4/min·mg Av1. We also obtained protein component titration curves in which either methane (diazirine reduction assays) or ethylene (acetylene reduction assays) was measured in assay mixtures in which the protein component ratio was varied (not shown). The profiles of the two curves were similar, CH_4 being steeper. These saturation curves were used to determine the correct protein ratio for either limiting Av2 or Av1 protein conditions. The K_m values for reduction of diazirine to ammonia were not very different for Av1-limited (Figure 1) (0.63×10^{-3} atm or 0.0492 ± 0.0377 mM) and Av2-limited conditions (1.19×10^{-3} atm or 0.0926 ± 0.0350 mM). The corresponding

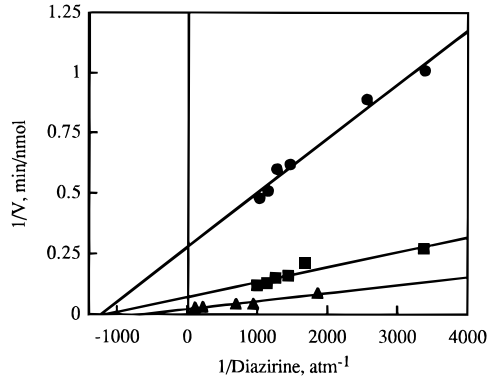


FIGURE 1: Lineweaver−Burk plot of kinetic data for reduction of diazirine to methane (●), methylamine (▲) and ammonia (■) by *Av* nitrogenase. Protein component ratios (Av2:Av1) were 16, 0.217 mg of Av1, 10 min assay (methane and ammonia) and 3.7, 0.612 mg of Av1, 15 min assay (methylamine).

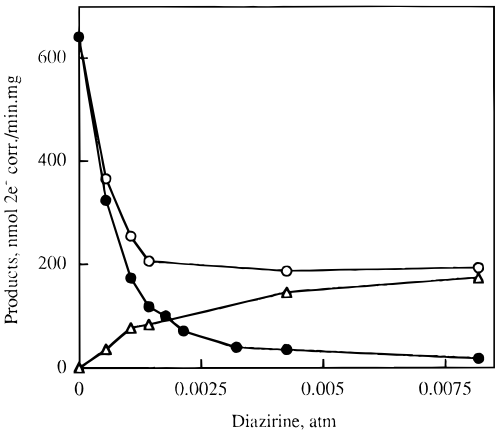


FIGURE 2: Dependence of product formation on diazirine partial pressure. The products are reported as nmol of electron pairs per min per mg of limiting protein: (●) H_2; (△) diazirine reduction products calculated as ($4CH_4 + 3CH_3NH_2$); and (○) total product evolution as ($H_2 + 4CH_4 + 3CH_3NH_2$). The relationship $2e^-/H_2$, $8/CH_4 + 2NH_3$, and $6/CH_3NH_2 + NH_3$ was used in calculating electron flow ("nmol $2e^-$ corr.") from the products. The protein component ratio (Av2:Av1) was 3.3, 0.6 mg of Av1.

V_m values were 73.6 ± 12.6 and 20.6 ± 4.12 nmol of NH_3/min·mg of Av1. When methylamine was quantitated, the reaction was carried out under conditions of limiting Av1 (i.e., excess dinitrogenase reductase) only. The K_m and V_m were 0.87×10^{-3} atm (0.0679 ± 0.0163 mM) and 41.3 ± 3.1 nmol CH_3NH_2/min·mg Av1 (Figure 1).[2]

Inhibition of Hydrogen Evolution and Electron Balance for Diazirine Reduction. Data obtained under conditions where Av1 is limiting and in the presence of $0-8 \times 10^{-3}$ atm diazirine indicate that sufficiently high pressures of diazirine ($\gg K_m$) will completely suppress H_2 evolution (Figure 2). In this respect diazirine resembles C_2H_2 and differs from N_2, which is unable to suppress more than 75% of electron flux to H_2 (Rivera-Ortiz & Burris, 1975). Diazirine thus might appear to have an extrapolated electron allocation coefficient of ~1.0. The electron allocation coefficient (EAC) is defined as the ratio of electrons transferred/unit time to an exogenous substrate to the total electron flux rate, including H_2 evolution (Hardy, 1979).

[2] The V_m for CH_3NH_2 from Figure 1 is not directly comparable to the other two V_m values given above (unified conditions data set not available). The value given here is estimated for a 1.0 CH_4:2.4 CH_3NH_2 ratio. The CH_4:NH_3 V_m product ratios are, Av1 limited, 1:4.35; Av2 limited, 1:2.2.

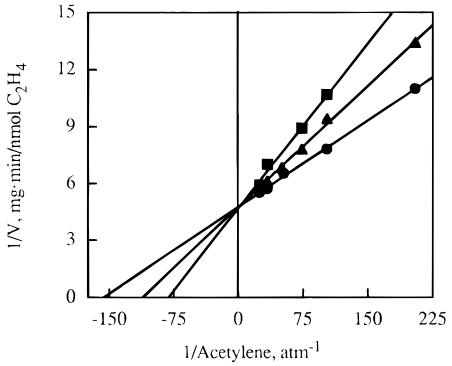


FIGURE 3: Diazirine inhibits acetylene reduction competitively. Acetylene reductions were carried out for 15 min in the presence of 0 (●), 0.13×10^{-3} atm [10.1 μM (▲)], and 0.35×10^{-3} atm [27.3 μM (■)] of diazirine. The protein component ratio (Av2:Av1) was 0.8. $1/V$ values are $\times 10^3$.

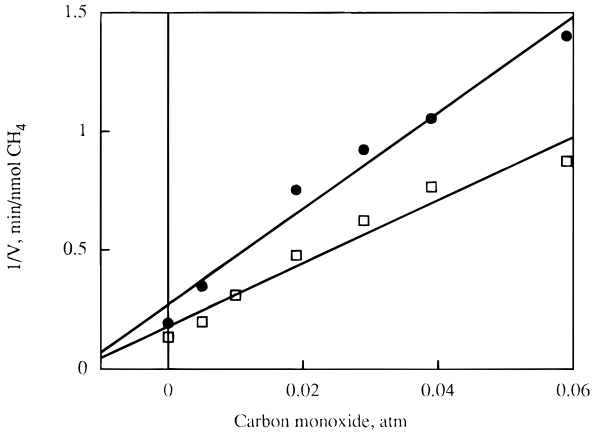


FIGURE 4: CO inhibits the reduction of diazirine to methane. Reactions were run for 30 min with a protein ratio (Av2:Av1) of 0.73. The following partial pressures of diazirine were used: 0.0016 atm (●) and 0.0031 atm (□).

However, the electron balance of all products of nitrogenase activity (H_2, methane, methylamine, and ammonia) only accounted for 30% of the total electrons available (measured in the absence of any exogenous substrate). Although this could be due to an undetected product, it is equally consistent with an inhibitory effect by diazirine on total electron flux, as has been observed with some other nitrogenase substrates.

Diazirine Inhibits Acetylene Reduction. As shown in Figure 3, diazirine is a potent competitive inhibitor of acetylene with a K_i of 0.4×10^{-3} atm (0.03 mM). The K_m of acetylene in the absence of diazirine was 6.7×10^{-3} atm (0.25 mM), which is similar to previously reported values from our laboratory (McKenna, 1980).

CO Inhibits Diazirine Reduction to Methane, Ammonia, and Methylamine. Formation of methane (Figure 4), ammonia (data not shown), and methylamine (data not shown) in the presence of 0−0.06 atm of CO was measured. In the case of methane, a set of control bottles containing the assay reagents, CO, and diazirine (but no nitrogenase) was used to correct for the contribution by CO to the methane GC peak. A linear data fit suggests that CO is a noncompetitive inhibitor of diazirine reduction to methane with a K_i of 0.0139 ± 0.007.4 atm; however, the linearity of the plots is not very satisfactory. If the plots are taken to be nonlinear, then the inhibition is better described as partial noncompetitive; however, a correction was made in the methane GC data which increased with the amount of CO, which might increase the error at high CO. CO also appears to be a

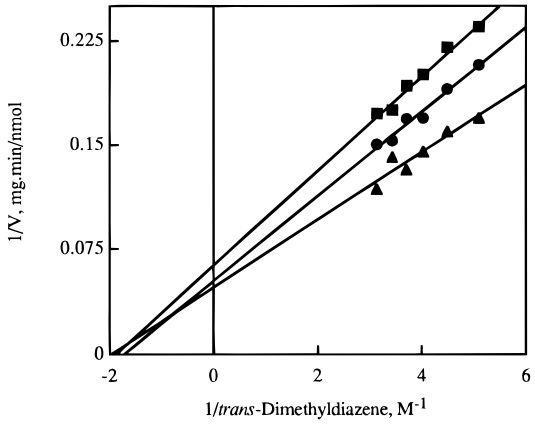


FIGURE 5: Lineweaver−Burk plot of kinetic data for reduction of *trans*-dimethyldiazene to methylamine (▲), methane (●), and ammonia (■). The reactions were run for 30 min in the presence of 0.10 mg Av1 at a protein ratio (Av2:Av1) of 6.6.

noncompetitive inhibitor of diazirine reduction to ammonia and methylamine (data not shown) with K_i values of 0.0121 ± 0.00356 and 0.0315 ± 0.0178 atm, respectively. Diazirine reduction thus appears to be less affected by CO than the reductions of acetylene (K_i of 12.9 × 10^{-4} atm) and dinitrogen (K_i of 4.7 × 10^{-4} atm) (Hwang *et al.*, 1973).

No H_2 and D_2 Effects in Diazirine Reduction. In experiments carried out to detect H_2 inhibition of diazirine reduction, a very large K_i (3.38 atm) was obtained (data not shown), with a data scatter suggesting that this apparent effect could fall in the range of the standard deviation of the regression line. A *t*-test (Zar, 1974) designed to compare the V_m and K_m values (Eadie−Hofstee format where *V* plotted against *V*/*S* yields $-K_m$ and V_m as the slope and the *y*-intercept, respectively) showed that these values in the presence or absence of H_2 were indistinguishable at the 99% confidence level. When the nitrogenase-catalyzed diazirine reduction was carried out in the presence of D_2 as described in Materials and Methods, no HD formation comparable to that observed with 40% N_2 (5× background HD in 90 min assay) was detected.

Nitrogenase-Catalyzed Reduction of trans-Dimethyldiazene. *trans*-Dimethyldiazene is reduced in the presence of nitrogenase to methylamine, methane, and ammonia. Omission of any required component from the enzyme assay (Av1 and/or Av2, ATP generator, reducing agent) resulted in detection of background levels of the three products similar to those obtained with diazirine. Time course experiments with *trans*-dimethyldiazene concentrations in the liquid phase ranging from 0.17 to 0.318 M showed that the evolution of the three products was linear for up to 35 min with no lag phase observed; therefore, the steady-state experiments used a 30 min incubation time to allow for accumulation of the highest possible amounts of products (see below). The K_m and V_m values obtained for the three products (Figure 5) are 0.51 ± 0.035 M and 21.0 ± 2.04 nmol/mg·min, 0.58 ± 0.0451 M and 19.2 ± 1.97 nmol/mg·min, and 0.53 ± 0.048 M and 15.7 ± 2.11 nmol/mg·min for methylamine, methane, and ammonia, respectively.

Inhibition of Hydrogen Evolution and Electron Balance for trans-Dimethyldiazene Reductions. In an attempt to determine the effect of *trans*-dimethyldiazene on nitrogenase-catalyzed proton reduction, *trans*-dimethyldiazene reduction products were quantitated together with H_2 in a series of assays with variable substrate concentration. The results

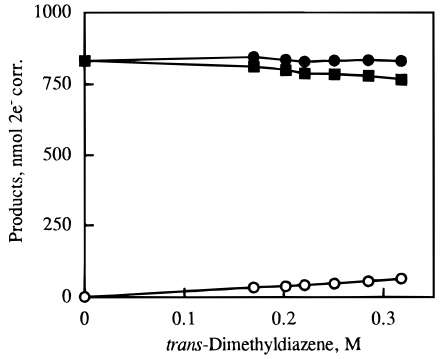


FIGURE 6: Inhibition of H_2 evolution by *trans*-dimethyldiazene. The assay conditions were the same as in Figure 5. The total nmol of electron pairs allocated to product formation ("nmol corr.") was calculated based on the relationships $2e^-/H_2$, $3e^-/NH_3$, $2e^-/CH_3$-NH_2, and $1e^-/CH_4$. Plots show electron pairs allocated to total product formation from the diazene substrate (○), H_2 evolution (■), and the sum of both (●).

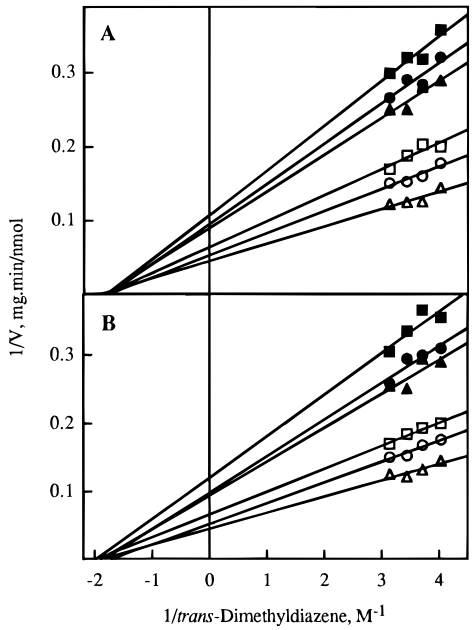


FIGURE 7: Inhibition of *trans*-dimethyldiazene reduction by CO (A) and acetylene (B). The assay conditions and the plot symbols for the three products were the same as in Figure 5. Open symbols: control (no CO/C_2H_2), filled symbols: 0.0006 atm CO (A)/0.005 atm C_2H_2 (B).

(Figure 6) show that *trans*-dimethyldiazene causes a weak inhibition of H_2 evolution and that the decrease in the amount of H_2 produced corresponds to the amounts of methane, methylamine, and ammonia produced within the error of the experiment.

Effects of Acetylene, Carbon Monoxide, and Hydrogen on trans-Dimethyldiazene Reduction. Reduction of *trans*-dimethyldiazene is inhibited noncompetitively by acetylene and carbon monoxide. Preliminary experiments showed that both gases at low pressures (∼0.008 atm for C_2H_2 and ∼0.001 atm for CO) inhibited the reduction of *trans*-dimethyldiazene at 0.318 M sufficiently to impair quantitation of the three products. The estimated inhibition constant of CO for all three products is 0.0008 atm (Figure 7A). A similar plot (Figure 7B) was obtained when the reduction was measured in the absence and the presence (0.005 atm) of acetylene, from which an estimated K_i of 0.006 atm for all three products was obtained. When, in separate assays, *trans*-dimethyldiazene (0.17 M) reduction was performed in the

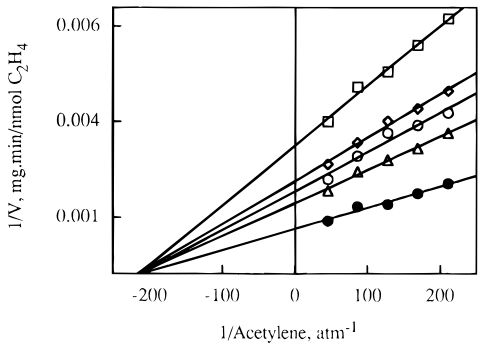


FIGURE 8: Inhibition of acetylene reduction by *trans*-dimethyldiazene. The assay conditions were the same as in Figure 5 with exception of the reaction time which was 10 min. The *trans*-dimethyldiazene concentrations were 0 (●), 0.05 M (△), 0.08 M (○), 0.09 M (◇), and 0.17 M (□).

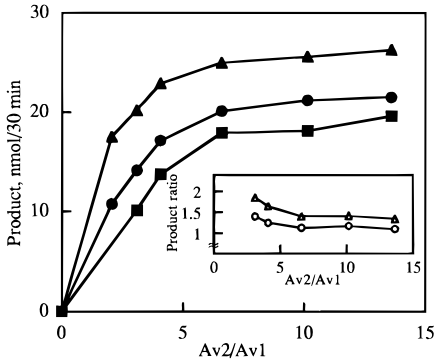


FIGURE 9: Electron allocation to alternative products of *trans*-dimethyldiazene reduction as a function of the Av2:Av1 ratio. Reactions were carried out in presence of 0.10 mg Av1 and variable amounts of Av2. The incubation time was 30 min, and the *trans*-dimethyldiazene concentration was 0.32 M. Plot symbols for the three products are the same as in Figure 5. (Inset) Dependence of the product ratios CH_4/NH_3 (○) and CH_3NH_2/NH_3 (△) on the ratio Av2:Av1.

absence and the presence of 0.10 and 0.25 atm H_2, the changes in the amounts of products evolved were <1.9% (data not shown), which places the lower K_i limit at 3.9 atm.

Weak Inhibition of Acetylene Reduction by trans-Dimethyldiazene. *trans*-Dimethyldiazene was evaluated as an inhibitor of acetylene reduction. The Lineweaver−Burk plot of the data, shown in Figure 8, demonstrates that the inhibition mode is noncompetitive with a K_i value of 0.093 ± 0.0061 M. No curvature was observed when the slopes and *y*-intercepts from Figure 8 were replotted against the inhibitor concentration (not shown). Both N_2 and diazirine are competitive inhibitors of C_2H_2, and it is possible that the acyclic azo compound has a second, nonspecific binding interaction with the enzyme; it may also affect C_2H_2 solubility.

Effect of the Protein Component Ratio on the trans-Dimethyldiazene Reduction Product Distribution. Protein component titration curves for the three products were obtained by carrying out the reduction assays at different Av2 to Av1 ratios (Figure 9). As evidenced by these results, the ratio methylamine/methane/ammonia depends little on the protein component ratio in the range 3.1−13.62. Attempts to determine the product distribution at *Av*2 limiting conditions were unsuccessful because of the dramatic drop in the activity below a protein ratio of 2 (Figure 9).

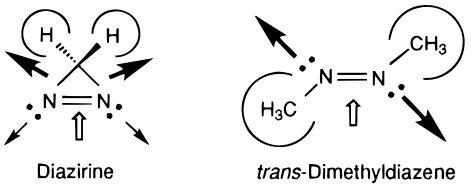
DISCUSSION

Experimental Aspects of Diazirine and trans-Dimethyldiazene as Nitrogenase Probes. Once prepared, purified, and stored over saturated aqueous Na_2SO_4, diazirine is readily and safely manipulated for nitrogenase assays, in which it is shown to be a substrate of the enzyme. Because its molar K_m and K_i values are quite low and its water solubility as measured in our experiments is ca. 100-fold greater than that of N_2, diazirine is a convenient substrate to use at low partial pressures in He or Ar, and its major reduction products are readily determined by GC (CH_4) or HPLC (CH_3NH_2, NH_3). Nevertheless, its aqueous solubility is not so high as to require more than a modest correction in the final partial pressure in standard assays; the partial pressure is readily verified by GC or UV analysis of a gas phase aliquot. Diazirine is stable to the assay reagents (minus enzyme) at 30 °C. With *trans*-dimethyldiazene, the aqueous solubility increases another 20-fold, and a substantial fraction of total substrate is found in the liquid phase, requiring iterative solutions to find the final partial pressure. It is fortunate that molar concentrations of this substrate can be attained in assay mixtures, as its K_m is quite high (see below). Both diazirine and *trans*-dimethyldiazene are sensitive to UV light but are handled readily under ambient lighting, it being recommended to minimize exposure in the case of diazirine. Both compounds are chemically stable on prolonged storage in contact with H_2O.

Relevant Structural Features of Diazirine and trans-Dimethyldiazene. Before considering the behavior of the two azo compounds as nitrogenase substrates, it is useful to summarize certain of their molecular properties that may be pertinent to their differing interactions with the presumptive active metal center in nitrogenase. A key feature of diazirine is its unique structure as a diazene having the −N═N− group confined within a highly strained, three-membered ring. Sterically, this results in a compact molecule similar to N_2 in its NN edge dimension, with the nitrogen atom unshared electron pairs nominally in orbitals oriented *cis* at an angle of ca. 120° to the NN bond axis, similarly to those in *cis*-N_2H_2.

Bonding in diazirine has been well characterized experimentally and theoretically (Baird, 1987; Frey & Stevens, 1962; Kisch, 1987; Kochanski & Lehn, 1969; Liu & Stevens, 1987; Von Niessen *et al.*, 1981). The N−N distance of 1.228 Å in diazirine is close to that expected for a double bond, and the C−N distance of 1.482 Å is similar to analogous values for acyclic systems (Baird, 1987). The highest occupied MO (HOMO) in diazirine combines the N−N lone pair orbitals with a somewhat Walsh-like orbital which is C−N bonding and N−N antibonding. Electron donation via ligation would thus strengthen the N−N bond and weaken the C−N bonds, tending to enhance ring-opening chemistry. The HOMO corresponding to the asymmetric combination of the two lone-pair orbitals has ca. 30% C−N and ca. 70% lone-pair character in a six-membered cyclic diazene, but 68% and 32%, respectively, in diazirine (Baird, 1987). This would be expected to modify the donor ligand properties of diazirine in its interactions with unoccupied, acceptor orbitals in site metal center(s), although it evidently does not preclude formation of 3,3-disubstituted diazirine complexes with $Fe_2(CO)_9$, for example, in which the diazirine bridges two Fe atoms, with preservation of the azo N═N bond (Kisch, 1987). In other Fe carbonyl complexes and in Ru carbonyl

Scheme 2: Comparison of Steric Profiles and Electron Donor Properties of Diazirine and *trans*-Dimethyldiazene[a]



[a] Solid arrows depict σ (or σ-like) electron donor sites. Unfilled arrows symbolize availability of π^* electron acceptor sites for occupied metal d-orbital backbonding (geometry not specified).

complexes with diazirine the N═N bond is cleaved (Kisch, 1987).

In contrast to diazirine with its unusual ring bonding, *trans*-dimethyldiazene is a conventional acyclic azo compound, but the *trans*-geometry orients the two lone pair orbitals in opposite directions within the molecular plane, thus allowing only one *syn* interaction with a metal site. Weaker metal coordination by *trans* vs *cis* azo derivatives has been reported (Kisch, 1987). Although diazirine is a polar compound with a dipole moment of 1.59 D (Von Niessen *et al.*, 1981), its solubility in H_2O is considerably lower than that of *trans*-dimethyldiazene, which has zero dipole moment. One explanation for the observed difference might be the greater basicity of the more localized two lone pairs of electrons in the acyclic diazene, resulting in enhanced hydrogen bonding to the protic solvent. Differences and similarities in the steric profiles and ligand electron donor properties are summarized in Scheme 2 (differences in the LUMOs are probably also relevant, but are not represented for simplicity).

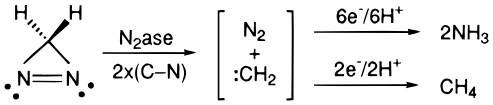
The two substrates should also present quite different steric profiles to the enzyme active site. As pointed out already, diazirine is similar in size to N_2 for an N−N edge-on approach to the site and will be only slightly more hindered in a CN edge-on approach, owing to the presence of the two methylene hydrogen atoms at one end of the edge. Edge-approach by *trans*-dimethyldiazene will be made awkward by the protruding methyl groups *vicinal* to either coordinating N. End-on (or molecular plane-perpendicular) approaches to the site could also yield significantly different steric interactions with site moieties neighboring the approach path required for binding and reduction of each molecule.

Substrate Reduction Pathways. Azo substrate interactions with nitrogenase can be characterized on several bases: (a) the products formed, the maximal velocities for formation of each, and their ratios, corresponding to absolute and relative rates of cleavage for C−N and N═N bonds; (b) the K_m values for the substrates; (c) inhibition by or of other compounds bound by the enzyme; (d) effects on H_2 formation and electron flux.

Beginning with the diazirine reduction, we note that *formally* this strained-ring diazene could be reductively cleaved in several different ways (in schemes where we depict methyl diazene intermediates, it should be stressed that tautomeric methylaminoimine intermediates are not excluded; in addition, for simplicity we do not consider the possibility of odd electron steps in the reduction process):

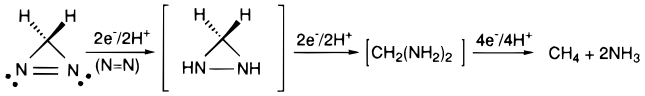
(1) Nonreductive, exclusive cleavage of both C−N bonds, to generate a (metal-bound) carbene, plus N_2, followed by separate reduction of the two fragments (Scheme 3). The initial step of this process is analogous to the photochemical dissociation of diazirine (Liu & Stevens, 1987). If the N_2 were co-bound (implying >1 metal coordination site), the

Scheme 3: Diazirine Reduction: Path 1 (Initial Cleavage to N_2 and Methylene)[a]



[a] Bond(s) attacked are shown in parentheses. Square brackets enclose enzyme-bound species.

Scheme 4: Diazirine Reduction: Path 2, Specific N═N Edge Reductive Cleavage

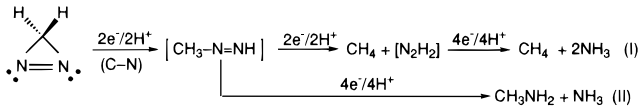


reduction products would be CH_4 ($2e^-$, $2H^+$) and $2NH_3$ ($6e^-$, $6H^+$). The idea that N_2, formed *in situ* from a precursor substrate, can be retained and reduced at the active site rather than released in free form has precedent from studies on azide reduction (Rubinson *et al.*, 1985). However, this mechanism is not supported for diazirine reduction: it does not account for CH_3NH_2 production and predicts the wrong NH_3/CH_4 ratio (2:1 vs 4:1 observed). Alternatively, it could be considered as one component of a multipath binding and reduction scheme, wherein a second process accounts for the CH_3NH_2 and modifies the product ratio to that observed experimentally. However, one might still expect to observe features considered unique to N_2, such as inhibition by H_2 (Hwang *et al.*, 1973) and enzyme-dependent HD formation in D_2/H_2O assays (Guth & Burris, 1983), some of which were observed for azide reduction to N_2 (Rubinson *et al.*, 1985). We were unable to detect either phenomenon in diazirine reduction by nitrogenase. A further argument against two completely separate and independent reduction processes is the observation that formation of all three products from diazirine exhibits the same K_m value, within the experimental error. This argument could of course fail in the face of two processes which coincidentally had very similar kinetic parameters (see further discussion below). We also have no evidence (<5% of CH_4 detected) for formation of C_2 products (C_2H_4 or C_2H_6) from carbene insertion chemistry, as has been reported in the nitrogenase-catalyzed reduction of methyl isocyanide (Rubinson *et al.*, 1983).

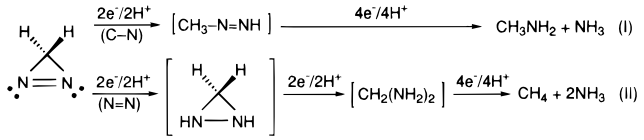
(2) Exclusive reduction of the N═N bond in two $2e^-$ steps or one $4e^-$ step (Scheme 4). This mechanism could thus proceed via bound diaziridine and/or diaminomethane intermediates. Here, reduction properties unique to N_2 are not required, but again the same, incorrect NH_3/CH_4 ratio (2:1) is predicted by this proposed path.

(3) One could envisage a $2e^-$ reduction of one CN bond, generating a bound monomethyldiazene (or tautomeric methylaminonitrene) intermediate (Scheme 5). This intermediate conceivably could partition between a $2e^-$ process (I) to form methane and bound diazene (or its tautomer), followed by a $4e^-$ reduction of the latter to yield $2NH_3$ or a $4e^-$ process (II) to form CH_3NH_2 and NH_3. In the former case, HD formation from D_2 would be predicted by the diazene-intermediate hypothesis, although not necessarily by the alternative hypotheses of Lowe-Thorneley and of Burris (Yates, 1992). Depending on the partitioning ratio, the products would be $CH_4 + 2NH_3$ (100% I) or $CH_3NH_2 + NH_3$ (100% II). Thus, a blend of 1:2 (I:II) between the two processes could account for the observed product ratio (rounded to whole numbers). However, the observed

Scheme 5: Diazirine Reduction: Paths 3-I and 3-II. Specific C−N Edge Reductive Cleavage



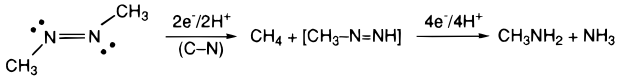
Scheme 6: Diazirine Reduction: Paths 4-I and 4-II ("Random-Edge" Reductive Cleavage)



absence of HD formation would rule out path 3-I, once again depending on whether the diazene hypothesis explaining this phenomenon is correct. We have no evidence at this time for a variant in this mechanism, in which methylhydrazine is produced via a $2e^-$ reduction of the initial intermediate, but we note that depletion of the initial intermediate by this additional path would not affect the product ratio obtained by combining 3-I and 3-II.

(4) Yet another mechanism can be postulated, based on the "random-edge binding" mechanism proposed to explain the cyclopropene product ratio obtained with wild-type nitrogenases (McKenna *et al.*, 1976). In this mechanism, the triangular cyclopropene molecule was postulated to be reduced equivalently at its two C−C and one C═C edges, producing a statistical 2:1 ratio of the propene/cyclopropane products as found experimentally, even *in vivo* (McKenna & Huang, 1979). An implication of this model is the presence of more than one coordination site for the ligand, such that the reduction does not greatly depend upon the coordination of a single, specific edge. Otherwise, we would expect some discrimination between a C═C and C−C edge, unless only Walsh orbitals common to the three edges were involved in coordination. "Random-edge" cleavage of diazirine can be elaborated in the following manner. A $2e^-$ reduction of either C−N single bond would give the bound CH_3N_2H intermediate[3] discussed in (3) above, whereas a $2e^-$ reduction of the N═N bond would result in a bound form of diaziridine. Assume that the first reduction ultimately leads to 1 CH_3NH_2 and 1 NH_3, whereas the second reduction yields, via bound diaminomethane, 1 CH_4 and 2 NH_3 (Scheme 6). Then, application of a statistical factor of 2 to the first process predicts a final product ratio of 1 CH_4:2 CH_3NH_2:4 NH_3 if both processes occur at equal rates, in fair agreement with our experimental ratios. In terms of total electron flux, the methane-forming reaction consumes $8e^-$, and the methylamine-forming reaction consumes $6e^-$ (McKenna & Eran, 1982). A parallel stoichiometry has been

Scheme 7: *trans*-Dimethyldiazene Reduction: Path with Initial Release of CH_4



proposed for the products from reduction of cyanamide by *Kp* nitrogenase (Miller & Eady, 1988). In this report, it was suggested that the enzyme-catalyzed reduction of cyanamide (a constitutional isomer of diazirine, it should be noted) could in principle involve diaminomethane as an intermediate (free or enzyme-bound) (Miller & Eady, 1988). Our experiments with simultaneous measurement of product formation and diazirine consumption (data not shown) indicate that there is no major reduction product other than the three already reported. Failure to detect formaldehyde, a possible product of diaminomethane hydrolysis, may not bear definitively on this point in view of the rapid reaction of formaldehyde with both sulfite and dithionite (Li *et al.*, 1982).

In light of the above analysis, it is instructive to consider the evidence for particular pathways for reduction of *trans*-dimethyldiazene and attempt to correlate the results for both azo substrates. The *trans*-diazene is reduced to NH_3, CH_4, and CH_3NH_2 in almost equal amounts. As discussed below, formation of all three products is characterized by very similar K_m values, consistent with a bound intermediate common to whatever pathways lead to the different products. If the *trans* geometry leads to a preferred active site interaction at only one N for the reasons discussed at the beginning of this section, we propose that an initial $2e^-$ reduction produces CH_4 and bound methyldiazene (or equivalent tautomer). This then undergoes a $4e^-$ reduction to CH_3NH_2 and NH_3, predicting an experimental 1:1:1 product ratio (Scheme 7). It will be noticed that this analysis is consistent with our analysis of the diazirine results, in predicting a CH_3N_2H intermediate which is reduced to CH_3NH_2 and NH_3 (rather than to $CH_4 + 2NH_3$).

The complexity of the chemical mechanism is unknown, but a well-known overall model for nitrogenase function (Lowe & Thorneley, 1984; Thorneley & Lowe, 1984) includes 17 kinetic parameters (without detailed identification of specific reduction intermediates). In such circumstances, it would not be surprising if the experimental K_m represented a complicated constellation of rate constants, rather than a good estimate of K_s for dissociation of an enzyme−substrate complex—making the further assumption that the complex does form reversibly, prior to reduction steps. Comparison of experimental K_m values for diazirine and *trans*-dimethyldiazene should prove instructive on this point, if considered together with other features of the reductions and also with kinetic parameters obtained from experiments in which they are forced to compete at the active site with a non-azo substrate, such as acetylene. The K_m values measured for the three detected diazirine reduction products under "normal" (Av1-limited) fixing conditions were identical within the errors of the values (0.05−0.09 mM), suggesting that they originate from a common enzymatic process. This was also a feature of the K_m values determined for the two products from nitrogenase-catalyzed cyclopropene reduction (McKenna *et al.*, 1976). The molar K_m value for diazirine reductions is much lower than the K_m values of most other second row triatomic substrates [e.g., 5 mM for N_2O (Jensen

[3] A reviewer (Yates, 1992) of our preliminary account (McKenna *et al.*, 1984) has stated that absence of HD exchange rules out a methyldiazene intermediate. In our account, we pointed out, as we elaborate here, that the diazene-intermediate mechanism of HD formation would strictly rule out (absent HD) a $2e^-/2H^+$ C−N cleavage pathway from $[CH_3N_2H]$ to $CH_4 + [N_2H_2]$, but not necessarily a $4e^-/4H^+$ N═N cleavage pathway via $[CH_3N_2H]$ to $CH_3NH_2 + NH_3$. A concerted disproportionation reaction of $D_2 + CH_3N_2H$ to form $HD + CH_3D + N_2$ analogous to the proposed reaction of $D_2 + N_2H_2$ to form $2HD + N_2$ is not known and would involve an energetically unfavorable seven-membered ring in the transition state. Thermodynamically, at 298 K diazene disproportionation is exothermic by −181 kcal/mol, whereas the methyl diazene process is endothermic by +33 kcal/mol (homolytic bond energies from CRC Handbook, 53rd ed., 1972, p F-183).

& Burris, 1986), 1.35 mM for N_3^- (Rubinson *et al.*, 1985),[4] and 10−20 mM for NO_2^- (Vaughn & Burgess, 1989)] and comparable to that of N_2 itself (ca. 0.1 mM) (Hwang *et al.*, 1973). If this low value reflects a high affinity of the enzyme for diazirine, at least in part, a comparably low K_i value would be reasonably expected for competitive inhibition of other substrates by diazirine. Diazirine is a competitive inhibitor of acetylene reduction to ethylene by nitrogenase and exhibits a K_i of 0.03 mM, about 2-fold lower than the K_m of diazirine (the C_2H_2 K_m value is ca. 0.2 mM as determined in our experiments).

For *trans*-dimethyldiazene, the same K_m value (within error) is again obtained for all three of its detected reduction products. However, its magnitude is 10^4 larger than that found for the K_m of diazirine. This dramatic difference could reflect either a steric or an electronic effect on coordination affinity at the site or some combination of these effects. In principle, a variation in reduction rates could also contribute to this difference. The strain energy released by a ring-opening cleavage of the diazirine ring, if coupled to reduction, should produce a large rate enhancement. To address this issue, one can examine V_m values for reduction of the two substrates. The V_m values we obtained for the diazirine reduction products: CH_4, 17; NH_3, 74; and CH_3-NH_2, 41 nmol/mg·min may be normalized to $2e^-/2H^+$ reduction values for C_2H_2 (est. V_m, Figure 1 conditions for $CH_4 + NH_3$, 1.40×10^2) using proposed mechanism 4, resulting in the following values: CH_4 path, 68; CH_3NH_2 path, 123 for a cumulated $2e^-/2H^+$-normalized specific activity of 191 or 14% of the C_2H_2 activity. Thus, although its $K_m \simeq K_m$ (N_2) and $\leq K_m$ (C_2H_2), diazirine is less effective in utilizing the available electron flux. A similar calculation for *trans*-dimethyldiazene shows that its $2e^-/2H^+$-normalized "total" V_m represents <5% of the "standard" activity. These findings show that the strained-ring energy of diazirine is not dramatically expressed in the substrate reduction rate, underscoring the fact that substrate reduction is not normally the rate-determining step in nitrogenase reduction (Thorneley & Lowe, 1984).

According to the results obtained in this study, diazirine at $P \gg K_m$ should completely shut down H_2 evolution. However, measurement of concurrent H_2 evolution and $2e^-/2H^+$-normalized diazirine reduction indicates that the total electron flux is lower than the maximum determined by H_2 evolution in the absence of the substrate. This does not necessarily indicate the existence of "missing" diazirine reduction products, because similar electron flux "deficits" have been reported with other substrates, including N_2 itself (Hageman & Burris, 1980; Rubinson *et al.*, 1983). Although we do not report DT oxidation (Li *et al.*, 1982) or ATP hydrolysis data here, we attempted to address the question of diazirine reduction product stoichiometry by measuring diazirine consumption (GC) and comparing it with the amounts of reduction products formed. The comparison demonstrated a rough balance in the consumption/formation stoichiometry, consistent with the absence of a major undetected product that could account for apparent low total electron flux. The ability of diazirine to completely suppress H_2 evolution differentiates it from N_2, which is unable to decrease H_2 evolution below ca. 25% of electron flux to H_2 + NH_3 (Hardy, 1979), even at high pressures (Simpson & Burris, 1984). This key difference between N_2 and other known nitrogenase substrates has been explained on the basis that N_2 interacts with an oxidation state of the MoFe protein that is more reduced than the oxidation state(s) involved in H_2 evolution or alternative substrate reduction (Rivera-Ortiz & Burris, 1975).

What may most fundamentally determine such differences is the chemical inertness of N_2 vis-à-vis alternative substrates. If the same site, but with variable electron (or H^-) content, is universally involved in binding all substrates, then N_2 alone would fail to react with the less-reduced states of that site. The absence of a serious steric impediment to side-on access by diazirine vs N_2 is suggested by their similar molecular dimensions across the N═N or N≡N bond, consistent with their similar, low K_m values. Like N_2, diazirine is a competitive inhibitor of C_2H_2 reduction. The similarity of the C_2H_2 K_i (0.03 mM) and the K_m (0.05 mM) for diazirine indicates that these values represent at least approximations of binding affinity.

Inability to completely suppress H_2 evolution, HD formation in D_2/H_2O assays, and sensitivity to inhibition by H_2 are special characteristics of nitrogenase-catalyzed reduction that are not shared by diazirine, and the *trans*-dimethyldiazene reductions are also insensitive to H_2. All three characteristics point to a site interaction, or intermediate, that is specific to N_2 among known nitrogenase substrates. Elucidation of the process or entity involved is essential to solving the chemical mechanism by which nitrogenase reduces its natural substrate. Alternative nitrogenase substrates and inhibitors should continue to serve as useful adjuncts to solving this fundamental problem in enzymology.

[4] In the same report, Rubinson and co-workers stated that the K_m value for the undissociated species, HN_3, was in the micromolar range (ca. 12 μM).

ACKNOWLEDGMENT

We thank M. C. McKenna for technical assistance and helpful advice.

REFERENCES

Amrich, M. J., & Bell, J. A. (1964) *J. Am. Chem. Soc. 86*, 292−293.

Baird, N. C. (1987) in *Chemistry of Diazirines* (Liu, M. T. H., Ed.) pp 1−17, CRC Press, Inc., Boca Raton, FL.

Bolin, J. T., Ronco, A. E., Morgan, T. V., Mortenson, L. E., & Xuong, N.-h. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1078−1082.

Bravo, M., Eran, H., Zhang, F. X., & McKenna, C. E. (1988) *Anal. Biochem. 175*, 482−491.

Bricker, C. E., & Vail, W. A. (1950) *Anal. Chem. 22*, 720−722.

Burris, R. H. (1972) *Methods Enzymol. 24*, 415−431.

Burris, R. H. (1991) *J. Biol. Chem. 266*, 9339−9342.

Campbell, K. N., Sommers, A. H., & Campbell, B. K. (1944) *J. Am. Chem. Soc. 66*, 82−84.

Chan, M. K., Kim, J., & Rees, D. C. (1993) *Science 260*, 792−794.

Chaney, A. L., & Marbach, E. P. (1962) *Clin. Chem. 8*, 130−132.

Chang, C. H., Porter, R. F., & Bauer, S. H. (1970) *J. Am. Chem. Soc. 92*, 5313.

Chatt, J. (1980) in *Molybdenum Chemistry of Biological Significance* (Newton, W. E., & Otsuka, S., Eds.) pp 241−254, Plenum Press, New York.

Chaykin, S. (1969) *Anal. Biochem. 31*, 375−382.

Coucouvanis, D. (1993) in *Molybdenum Enzymes, Cofactors, and Model Systems* (Stiefel, E. I., Coucouvanis, D., & Newton, W. E., Eds.) pp 304−331, American Chemical Society, Washington, D.C.

Dilworth, M. J., Subramanian, D., Munson, T. O., & Burris, R. H. (1965) *Biochim. Biophys. Acta 99*, 486−503.

Durig, J. R., Pate, C. B., & Harris, W. C. (1972) *J. Chem. Phys. 56*, 5652−5662.
Emmons, W. D. (1957) *J. Am. Chem. Soc. 79*, 5739−5754.
Ettinger, R. (1964) *J. Chem. Phys. 40*, 1693−1698.
Freeman, J. P. (1963) *J. Org. Chem. 28*, 2508−2511.
Frey, H. M., & Stevens, I. D. R. (1962) *Proc. Chem. Soc.*, 79−80.
Georgiadis, M. M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J., & Rees, D. C. (1992) *Science 257*, 1653−1659.
Graham, W. H. (1962) *J. Am. Chem. Soc. 84*, 1063−1064.
Graham, W. H. (1965) *J. Org. Chem. 30*, 2108.
Guth, J. H., & Burris, R. H. (1983) *Biochemistry 22*, 5111−5122.
Hageman, R. V., & Burris, R. H. (1980) *Biochim. Biophys. Acta 591*, 63−75.
Hardy, R. W. F. (1979) in *A Treatise on Dinitrogen Fixation* (Hardy, R. W. F., Bottomley, F., & Burns, R. C., Eds.) pp 515−568, Wiley-Interscience, New York.
Hardy, R. W., Burns, R. C., & Holsten, R. H. (1973) *Soil Biol. Biochem. 5*, 47−81.
Hoover, T. R., Imperial, J., Ludden, P. W., & Shah, V. K. (1989) *Biochemistry 28*, 2768−2771.
Hurwitz, M. D. (1952) *US Patent 2,582,128*.
Hutton, R. F., & Steel, C. (1964) *J. Am. Chem. Soc. 86*, 745−746.
Hwang, J. L., Chen, C. H., & Burris, R. H. (1973) *Biochim. Biophys. Acta. 292*, 256−270.
Jensen, B. B., & Burris, R. H. (1986) *Biochemistry 25*, 1083−1088.
Kim, J., & Rees, D. C. (1992a) *Nature 360*, 553−560.
Kim, J., & Rees, D. C. (1992b) *Science 257*, 1677−1682.
Kisch, H. (1987) in *Chemistry of Diazirines* (Liu, M. T. H., Ed.) pp 101−109, CRC Press, Inc., Boca Raton, FL.
Kochanski, E., & Lehn, J. M. (1969) *Theor. Chim. Acta 14*, 281−304.
Leigh, G. J. (1995) *Eur. J. Biochem. 229*, 14−20.
Li, J., Burgess, B. K., & Corbin, J. L. (1982) *Biochemistry 21*, 4393−4402.
Liu, M. T. H., & Stevens, I. D. R. (1987) in *Chemistry of Diazirines* (Liu, M. T. H., Ed.) pp 111−60, CRC Press, Inc., Boca Raton, FL.
Ljones, T. (1973) *Biochim. Biophys. Acta 321*, 103−113.
Lowe, D. J., & Thorneley, R. N. F. (1984) *Biochem. J. 224*, 895−901.
Lowe, D. J., Fisher, K., Thorneley, R. N., Vaughn, S. A., & Burgess, B. K. (1989) *Biochemistry 28*, 8460−8466.
McKenna, C. E. (1980) in *Molybdenum and Molybdenum-Containing Enzymes* (Coughlan, M. P., Ed.) pp 439−462, Pergamon Press, Oxford.
McKenna, C. E., & Huang, C. (1979) *Nature 280*, 609−611.
McKenna, C. E., & Eran, H. (1982) *Fed. Proc. 41*, 891.
McKenna, C., McKenna, M.-C., & Higa, M. T. (1976) *J. Am. Chem. Soc. 98*, 4657−4659.
McKenna, C. E., Nakajima, T., Jones, J. B., Huang, C., McKenna, M. C., Eran, H., & Osumi, A. (1980) in *Molybdenum Chemistry of Biological Significance* (Newton, W. E., & Otsuka, S., Eds.) pp 39−57, Plenum Publishing Co., New York.
McKenna, C. E., Eran, H., Nakajima, T., & Osumi, A. (1981) in *Current Perspectives in Nitrogen Fixation* (Gibson, A. H., & Newton, W. E., Eds.) p 358, Australian Academy of Science, Canberra, Australia.
McKenna, C. E., Nguyen, H. T., Huang, C. H., McKenna, M. C., Jones, J. B., & Stephens, P. J. (1982) in *From Cyclotrons to Cytochromes* (Kaplan, N. O., & Robinson, A., Eds.) pp 397−416, Academic Press, New York.
McKenna, C. E., Stephens, P. J., Eran, H., Luo, G.-M., Zhang, F. X., Ding, M., & Nguyen, H. T. (1984) in *Advances in Nitrogen Fixation Research* (Veeger, C., & Newton, W. E., Eds.) pp 115−122, Martinus Nijhoff/De Junk, Wageningen, The Netherlands.
McKenna, C. E., Gutheil, W. G., & Song, W. (1991) *Biochim. Biophys. Acta 1075*, 109−117.
Mecke, R., & Langenbucher, F. (1965) *Infrared Spectra of Selected Chemical Compounds*, Vol. 6, Heyden & Son Ltd., London.
Meyer, J. (1981) *Arch. Biochem. Biophys. 210*, 246−256.
Miller, R. W., & Eady, R. R. (1988) *Biochim. Biophys. Acta 952*, 290−296.
Ohkoshi, S., Fujita, Y., & Kwan, T. (1958) *Bull. Chem. Soc. Jpn. 31*, 770−771.
Orme-Johnson, W. H., Lindahl, P., Meade, J., Warren, W., Nelson, M., Groh, S., & Orme-Johnson, N. R. (1981) in *Current Perspectives in Nitrogen Fixation* (Gibson, A. H., & Newton, W. E., Eds.) pp 79−83, Elsevier/North Holland, Inc., New York.
Renaud, R., & Leitch, L. C. (1954) *Can. J. Chem. 32*, 545−549.
Rivera-Ortiz, J. M., & Burris, R. H. (1975) *J. Bacteriol. 123*, 537−545.
Rubinson, J. F., Corbin, J. L., & Burgess, B. K. (1983) *Biochemistry 22*, 6260−6268.
Rubinson, J. F., Burgess, B. K., Corbin, J. L., & Dilworth, M. J. (1985) *Biochemistry 24*, 273−283.
Schmitz, E. (1963) in *Advances in Heterocyclic Chemistry* (Katrizky, A. R., Boulton, A. J., & Lagowski, J. M., Eds.) pp 83−130, Academic Press, New York.
Schmitz, E., & Ohme, R. (1961) *Ber. Dtsch. Chem. Ges. 94*, 2166−2173.
Sellmann, D. (1993) in *Molybdenum Enzymes, Cofactors, and Model Systems* (Stiefel, E. I., Coucouvanis, D., & Newton, W. E., Eds.) pp 332−345, American Chemical Society, Washington, D.C.
Shah, V. K., & Brill, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3249−3253.
Simpson, F. B., & Burris, R. H. (1984) in *Advances in Nitrogen Fixation Research* (Veeger, C., & Newton, W. E., Eds.) p 243, Martinus Nijhoff/De Junk, Wageningen, The Netherlands.
Thorneley, R. N. F., & Lowe, D. J. (1984) *Biochem. J. 224*, 903−9.
Thorneley, R. N. F., Eady, R. R., & Lowe, D. J. (1978) *Nature 272*, 557−558.
Vaughn, S. A., & Burgess, B. K. (1989) *Biochemistry 28*, 419−424.
Von Niessen, W., Kraemer, W. P., & Schirmer, J. (1981) *J. Chem. Soc. Faraday Trans. II 77*, 1461−71.
Wallace, E. F., & Rabinowitz, J. C. (1971) *Arch. Biochem. Biophys. 146*, 400−409.
Wilhelm, E., Battino, R., & Wilcock, R. J. (1977) *Chem. Rev. 77*, 219−262.
Yates, M. G. (1992) in *Biological Nitrogen Fixation* (Stacey, G., Burris, R. H., & Evan, H. J., Eds.) pp 685−735, Chapman & Hall, New York.
Zar, J. H. (1974) *Biostatistical Analysis*, Prentice-Hall, Inc., Englewood Cliffs, NJ.
Zones, S. I., Palmer, M. R., Palmer, J. G., Doemeny, J. M., & Schrauzer, G. N. (1978) *J. Am. Chem. Soc. 100*, 2113−2121.

BI950964G