Reduction of Thiocyanate, Cyanate, and Carbon Disulfide by Nitrogenase: Kinetic Characterization and EPR Spectroscopic Analysis†

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ABSTRACT: Nitrogenase catalyzes the reduction of N_2, protons, and a number of alternative substrates that contain C−C, C−N, N−N, and N−O double and triple bonds. Recently it has been shown that nitrogenase also reduces the C═S bond of COS and the C═O bond of CO_2. The current work demonstrates that the COS analogs SCN^-, CS_2, and OCNH are novel substrates for nitrogenase and that the reduction of these substrates produces changes in the electron paramagnetic resonance (EPR) spectrum of nitrogenase, providing insight into the mechanism of substrate reduction by nitrogenase. CH_4, HCN, H_2S, and NH_4^+ were detected as products of the nitrogenase-catalyzed reduction of SCN^-. CS_2 was reduced by nitrogenase to H_2S, providing the first demonstration of CS_2 reduction catalyzed by a purified enzyme. CO was detected as a product of KOCN reduction by nitrogenase. Interestingly, the K_m for KOCN reduction to CO decreased at lower pH values, suggesting that OCNH rather than OCN^- was the substrate for nitrogenase. Analysis of the EPR spectra of nitrogenase under turnover conditions in the presence of KOCN, CS_2, or KSCN revealed new EPR signals. Signals with *g*-values corresponding to those reported for CO bound to the iron−molybdenum cofactor of nitrogenase were detected during turnover of nitrogenase in the presence of KOCN. During SCN^- and CS_2 reduction by nitrogenase, novel EPR inflections were observed that appear to report the interaction between nitrogenase and a bound substrate or a transient intermediate produced during the reduction of SCN^- and CS_2.

Nitrogenase catalyzes the reduction of N_2 to NH_3 as summarized in eq 1:

$$N_2 + 8e^- + 8H^+ + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i \quad (1)$$

Recent X-ray crystal structures of the iron (Fe) protein and molybdenum−iron (MoFe)[1] protein of nitrogenase have revealed elegant structural details about the protein components and associated cofactors (Georgiadis et al., 1992; Kim & Rees, 1992a,b; Howard & Rees, 1996). These structures have stimulated investigations elucidating the role of MgATP and protein conformational changes in catalysis, docking interactions between the protein components, and electron transfer pathways within the MoFe protein [Seefeldt, 1994; Ryle & Seefeldt, 1996; Lanzilotta et al., 1996; Peters et al., 1995a; for reviews, see Peters et al. (1995b) and Burgess and Lowe (1996)]. Interestingly, despite these structural models and numerous functional studies of nitrogenase, the mechanism of substrate binding and reduction has remained elusive and is one of the principal outstanding questions in the study of nitrogenase catalysis. While it is generally accepted that substrates bind to a novel mixed metal cofactor called the iron−molybdenum cofactor (FeMoco) (1Mo-7Fe-8S-1homocitrate), there is currently no direct evidence demonstrating that N_2 or any other nitrogenase substrate binds to FeMoco. The X-ray crystal structure of the MoFe protein has produced a model of FeMoco in which 4Fe-3S and 1Mo-3Fe-3S partial cubanes are joined by sulfide ligands with homocitrate bound to the Mo (Kim & Rees, 1992b). On the basis of this structure of FeMoco, at least nine models of substrate binding to nitrogenase have been proposed (Dance, 1996); however, the actual mechanism of substrate binding and activation by nitrogenase has not yet been elucidated.

One strategy for investigating the mechanism of substrate binding is to examine the range of compounds that can be reduced by nitrogenase. Substrates for nitrogenase include N_2, protons, C_2H_2, HCN, N_3^-, NO_2^-, N_2O, cyclopropene, diazirene, *trans*-dimethyldiazirene, and a number of other small molecules that have in common the presence of C−C, C−N, N−N, and N−O double or triple bonds (Burgess, 1985; Burgess & Lowe, 1996; McKenna et al., 1996). It has recently been shown that COS and CO_2 are substrates for nitrogenase (Seefeldt et al., 1995), demonstrating for the first time that nitrogenase reduces C═S and C═O bonds. In addition to revealing the nature of the chemical bonds that can be reduced by nitrogenase, the substrate range also indicates that (i) with the exception of NO_2^- and N_3^-, all known nitrogenase substrates are uncharged molecules and (ii) the active site of nitrogenase must be capable of accommodating small cyclic molecules and molecules as large as C_3H_7CN (Burgess & Lowe, 1996).

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[1] Abbreviations: Fe protein, iron protein of nitrogenase; MoFe protein, molybdenum−iron protein of nitrogenase; CODH, carbon monoxide dehydrogenase; MES, 2-(4-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; EXAFS, extended X-ray absorption fine structure; BSA, bovine serum albumin.

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Kinetic analysis and competition studies among different nitrogenase substrates are consistent with the existence of at least two different substrate binding sites or different oxidation states of the enzyme involved in substrate binding (Burgess, 1985). Indeed, the redox state of the MoFe protein plays a critical role in determining whether a particular substrate binds to the active site. According to the catalytic cycle proposed by Thorneley and Lowe (Thorneley & Lowe, 1996), the MoFe protein can exist in eight different oxidation states designated as E_0 through E_7 with the subscript representing the number of electrons present beyond the dithionite reduced state. In this catalytic model, protons bind to the E_1 and E_2 states of the MoFe protein, while N_2 binds only to the more highly reduced E_3 and E_4 states. HCN binds to a state that is more oxidized than the H_2-evolving state, thus accounting for the ability of HCN to completely eliminate H_2 evolution (Li et al., 1982).

The nature of the interactions between nitrogenase and small molecule ligands has also been investigated using spectroscopic probes to monitor changes in FeMoco during the binding of substrates and inhibitors. Electron paramagnetic resonance (EPR) spectroscopy has revealed the existence of two separate binding sites for C_2H_2 and one binding site for C_2H_4, the product of C_2H_2 reduction (Lowe et al., 1978; Hawkes et al., 1983). EPR analysis has also identified two cyanide binding sites on FeMoco isolated from the MoFe protein (Richards et al., 1994). The most well characterized example of ligand binding to nitrogenase is that of CO, a nonreducible inhibitor of the reduction of all nitrogenase substrates except protons (Hwang et al., 1973). EPR spectroscopy has determined that nitrogenase contains two binding sites for CO (Davis et al., 1979), and recent electron nuclear double resonance (ENDOR) studies have localized the sites of these interactions to FeMoco (Christie et al., 1996). These ENDOR studies have provided the first and only direct evidence thus far for ligand binding to FeMoco under turnover conditions.

To gain further insight into the mechanism of substrate binding and reduction by nitrogenase, the reactivities of thiocyanate (SCN^-), carbon disulfide (CS_2), and cyanate (OCN^-) were investigated using kinetic analysis and EPR spectroscopy. These compounds contain C═S or C═O bonds, which have recently been shown to characterize a new class of nitrogenase substrates, including COS and CO_2. The results have identified pathways of reduction for three novel nitrogenase substrates and have revealed new EPR signals associated with the reduction of these substrates.

EXPERIMENTAL PROCEDURES

Materials

Gases were obtained from Liquid Air Corp. (Walnut Creek, CA). CS_2 (99.99%) was purchased from EM Science (Gibbstown, NJ). Hemoglobin was from Pentex, Inc. (Kankakee, IL). Titanium chloride was from Aldrich Chemical Co. (Milwaukee, WI). *N*-Chloro-4-methylbenzenesulfonamide sodium salt (Chloramine-T), 3-methyl-1-phenyl-2-pyrazolin-5-one, and 3,3′-dimethyl-1,1′-diphenyl-[4,4′-bi-2-pyrazoline-5,5′-dione] were purchased through Fisher Scientific (Pittsburgh, PA). All other chemicals were of reagent grade and were obtained commercially.

Methods

Nitrogenase Proteins. The MoFe protein and Fe protein of nitrogenase were purified from *Azotobacter vinelandii* as described previously (Seefeldt et al., 1992; Seefeldt & Mortenson, 1993). The specific activities of the component proteins were 2280 nmol of C_2H_2 reduced · min^{-1} · (mg of MoFe protein)$^{-1}$ and 2270 nmol of C_2H_2 reduced·min^{-1}·(mg of Fe protein)$^{-1}$. Protein concentrations were determined using a modification of the biuret assay (Chromy et al., 1974) with bovine serum albumin as the standard.

Proton and C_2H_2 Reduction Assays. Proton and C_2H_2 reduction assays were performed at 30 °C as described previously (Seefeldt et al., 1995), except that 100 mM MOPS, pH 7.0, was used as the buffer.

Detection of CH_4 as a Product of KSCN Reduction. The reduction of KSCN to CH_4 was measured at 30 °C under 1 atm of Ar in 10-mL sealed glass vials containing 1.1 mL of assay solution (100 mM MOPS, pH 7.0, 1 mg/mL BSA, 3 mM MgATP, 5 mg/mL phosphocreatine, 0.16 mg/mL creatine phosphokinase, and either 10 mM dithionite or 5 mM titanium citrate (Seefeldt & Ensign, 1994) as the reductant). The concentration of titanium citrate used (5 mM) resulted in the highest amount of nitrogenase-catalyzed CH_4 production during a 10-min assay. KSCN was added from a 500 mM stock solution in water. Each assay contained 256 μg of MoFe protein and was initiated by the addition of 120 μg of Fe protein. After 10 min, the assays were terminated by the addition of 250 μL of 2.5 N H_2SO_4. CH_4 was quantified by gas chromatography after separation on a 30 cm × 0.3 cm Porapak N column fitted to a Shimadzu GC-8A gas chromatograph with a flame ionization detector interfaced to a Hewlett-Packard 3396B integrator. The column temperature was 50 °C, and the injector and detector temperatures were 180 °C. N_2 was the carrier gas at a flow rate of 15 mL/min. CH_4 eluted at 0.81 min. A standard curve was prepared by adding known quantities of CH_4 to acid-quenched nitrogenase assay mixtures in sealed 10-mL glass vials. For determination of the effect of pH on the rate of substrate reduction by nitrogenase, the assay buffer consisted of 33 mM MES, 33 mM MOPS, and 33 mM Tris, adjusted to the appropriate pH with NaOH or HCl.

Measurement of H_2S as a Product of KSCN and CS_2 Reduction. H_2S was detected as a product of the nitrogenase-catalyzed reduction of KSCN and CS_2 following the reaction of H_2S with *N*,*N*-dimethylphenylenediamine hydrochloride to form methylene blue (Ensign, 1995). Substrate reduction assays were performed at 30 °C under 1 atm of Ar in sealed 10-mL glass vials containing 1.1 mL of nitrogenase assay mixture and an empty glass test tube (1 × 3 cm). Titanium citrate (5 mM) was used as the reductant to avoid interference from dithionite in the measurement of H_2S. KSCN was added from a 500 mM stock solution in water, and CS_2 was added from a 1 M solution in ethanol. Each assay contained 256 μg of MoFe protein and 120 μg of Fe protein and was terminated with 100 μL of 17.4 N acetic acid. An aliquot (0.8 mL) of a 1% (w/v) zinc acetate solution was injected into the test tube, and the vial was shaken (120 cycles/min) at 30 °C to trap the H_2S in the zinc acetate solution. After 2 h, the amounts of CH_4 and H_2 in the gas phase were quantified. The test tube containing the zinc acetate solution was placed in a 13-mL glass vial containing 5 mL of water, and the vial was sealed with a butyl rubber stopper. One

milliliter of 0.5% (w/v) *N*,*N*-dimethylphenylenediamine hydrochloride solution in 5 M HCl and 0.2 mL of 23 mM $FeCl_3$ in 1.2 M HCl were injected into the vial. The vials were vortexed vigorously and incubated at room temperature for 30 min. The absorbance at 670 nm was measured using a Hewlett-Packard 8452A diode array spectrophotometer interfaced to a personal computer. A standard curve was made by adding known amounts of 50 mM sodium sulfide to acid-quenched assay solutions. The sodium sulfide solution was standardized by iodometric titration (Swift & Butler, 1972). To correct for the amount of H_2S released from the iron−sulfur-containing centers of nitrogenase upon acid treatment, the nitrogenase components were added to acid-quenched assay solution containing the substrate. The amount of H_2S detected was subtracted from the values obtained under turnover conditions.

Quantitation of HCN. Cyanide was detected as a product of KSCN reduction using a modification of the pyridine−pyrazolone method (Hargis, 1978). KSCN reduction assays were performed at 30 °C in 10-mL vials containing test tubes (1 × 3 cm) as described above for the measurement of H_2S. After the reaction was terminated with the addition of 100 μL of 17.4 N acetic acid, 500 μL of 0.1 N NaOH was injected into the test tubes to trap the HCN. The vials were shaken (120 cycles/min) at 30 °C for 2 h, and CH_4 and H_2 were quantified by gas chromatography. To measure the amount of cyanide trapped in the NaOH solution, 50 μL of the solution was placed in a test tube containing 1.95 mL of ice-cold 0.15 M potassium phosphate, pH 7.5. Chloramine-T (500 μL of a 0.14% (w/v) solution) was added, and the solution was mixed and placed on ice for 5 min. One milliliter of a pyridine−pyrazolone mixture (60.8 mg of 3-methyl-1-phenyl-2-pyrazolin-5-one and 24.2 mg of 3,3′-dimethyl-1,1′-diphenyl[4,4′-bi-2-pyrazoline-5,5′-dione] in 25 mL of pyridine) was added, and the test tube was vortexed and incubated at room temperature for 90 min. The absorbance at 632 nm was determined. The standard consisted of known amounts of KCN prepared in acid-quenched nitrogenase assay mixture.

Hemoglobin Binding Assay for the Detection of CO. The production of CO during cyanate reduction by nitrogenase was quantified using a hemoglobin CO binding assay as described previously (Seefeldt et al., 1995), except that the assay buffer consisted of 83 mM MOPS at pH 7.0 or 83 mM MES at pH 6.0 or 6.5. KOCN was added from a 0.5 M or 2 M stock solution in water.

Quantitation of NH_3. NH_3 produced during the incubation of nitrogenase with 5 mM KSCN and 10 mM dithionite was measured using the phenol−hypochlorite reaction (Conway, 1960). The nitrogenase-catalyzed reduction of KSCN was initiated with the addition of 128 μg of MoFe protein and 60 μg of Fe protein and terminated after 30 min with 250 μL of 2.5 N H_2SO_4. The assay solution was centrifuged in an eppendorf tube for 4 min, and 1 mL of the supernatant was placed in the outer well of a Conway microdiffusion dish (Bel-Art Products, Pequannock, NJ). H_2SO_4 (400 μL of a 0.25 N solution) was added to the center well, followed by the addition of 1 mL of K_2CO_3-saturated water to the outer well. The dish was immediately sealed and placed on a shaker to allow the NH_3 to diffuse into the acid trap. After 2 h the solution in the center well (400 μL) was transferred to a test tube; 1.75 mL of a solution containing 0.14 M phenol and 65 μM sodium nitroprusside and 0.5 mL of alkaline hypochlorite (200 μL of 5% hypochlorite in 10 mL of 0.75 M NaOH) were added. The absorbance at 624 nm was measured after 30 min. The standard curve consisted of known amounts of NH_4Cl added to acid-quenched reaction mixtures containing the nitrogenase components.

EPR Spectroscopy. Samples for EPR analysis were prepared using a 4 to 1 molar ratio of MoFe protein (2.9 mg, 13 nmol) to Fe protein (0.21 mg, 3.3 nmol) in nitrogenase assay solution (250 μL) with 10 mM dithionite in the presence or absence of 3 mM ATP. All of the reaction components except for the Fe protein were placed in a 600-μL eppendorf tube (for HCN, KSCN, and KOCN) or a 1 × 3 cm glass test tube (for CS_2 and H_2S) sealed in a 10-mL glass vial under 1 atm of Ar. The reaction was initiated with the addition of Fe protein. The solution was immediately mixed, and the liquid was drawn up into the Hamilton syringe to allow the reaction to continue in the absence of a gas phase. After 2 min, the assay solution was injected into a septum sealed argon-purged 4-mm quartz EPR tube (Wilmad, Buena, NJ) and frozen in liquid N_2. For CO-treated samples, nitrogenase was incubated with the complete nitrogenase reaction mixture including 3 mM MgATP (turnover conditions) in sealed 10-mL glass vials. The gas phase contained Ar and either 0.0125 atm CO or 0.3 atm CO, corresponding to liquid phase concentrations of 9 μM CO or 216 μM CO, respectively. EPR spectra were recorded on a Bruker ESP300E spectrometer equipped with a dual mode cavity and an Oxford ESR 900 liquid helium cryostat. Unless otherwise noted, the spectra were recorded at 12 K with a microwave power of 3.19 mW and a microwave frequency of 9.64 GHz.

Numeric Constants and Data Analysis. Kinetic constants were determined by nonlinear regression analysis using the program IgorPro (Wavemetrics, Inc., Lake Oswego, OR) to fit the data to the Michaelis−Menten equation (Segel, 1975): $\nu = (V_{max}[S])/(K_m + [S])$, where ν = the reaction velocity and [S] = the substrate concentration. The inhibition constants for KSCN inhibition of C_2H_2 reduction were estimated using nonlinear regression analysis to fit the data to the equation for mixed or noncompetitive inhibition (Segel, 1975; Cleland, 1979; Cornish-Bowden, 1979).

RESULTS

SCN^- Reduction by Nitrogenase. To provide insight into the mechanism of substrate binding and reduction by nitrogenase, the reactivities of SCN^-, CS_2, and OCN^- were investigated by kinetic analysis and EPR spectroscopy. The pathway of SCN^- reduction was predicted to involve the two-electron reduction of the C═S bond to form H_2S and HCN (eq 2) by analogy to the mechanism of COS reduction by nitrogenase (eq 3). HCN is also a substrate for nitrogenase (Li et al., 1982), and the six-electron reduction of HCN would produce CH_4 and NH_3. Therefore, CH_4 production was monitored to test the possibility that SCN^- is reduced as a substrate for nitrogenase.

$$S{=}C{=}N^- + 2e^- + 3H^+ \rightarrow H_2S + HCN \qquad (2)$$

$$S{=}C{=}O + 2\,e^- + 2H^+ \rightarrow H_2S + CO \qquad (3)$$

A time-dependent increase in CH_4 production was observed when nitrogenase was incubated in a complete

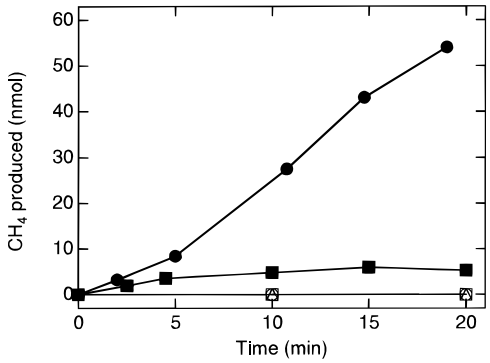


FIGURE 1: KSCN reduction to CH_4 by nitrogenase. Assays were performed at 30 °C in sealed 10-mL glass vials under 1 atm of Ar as described in the Experimental Procedures. The complete nitrogenase assay solution (1.1 mL) contained 100 mM MOPS, pH 7.0, 1 mg/mL BSA, 3 mM MgATP, an ATP-regenerating system (consisting of 5 mg/mL phosphocreatine and 0.16 mg/mL creatine phosphokinase), 10 mM dithionite, and 5 mM KSCN. Each assay contained 256 μg of MoFe protein and was initiated with the addition of 120 μg of Fe protein. At fixed times, the CH_4 content of the gas phase was quantified by gas chromatography. The assay conditions were as follows: complete assay solution (●); assay solution without a regenerating system (■); assay solution without MgATP (○); assay solution without MoFe protein (□); or assay solution without Fe protein (△).

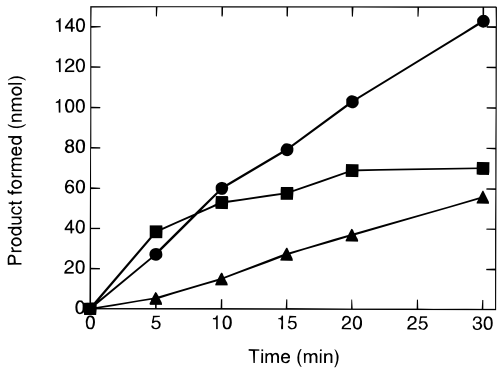


FIGURE 2: Nitrogenase-catalyzed reduction of KSCN to H_2S, HCN, and CH_4. KSCN reduction assays were performed at 30 °C as outlined in the Experimental Procedures. The complete nitrogenase assay solution (1.1 mL; described in the legend to Figure 1) contained 5 mM titanium citrate in place of dithionite as the reductant. Each assay contained 256 μg of MoFe protein and was initiated with the addition of 120 μg of Fe protein. At fixed times, the assays were terminated with 100 μL of 17.4 N acetic acid. HCN and H_2S were quantified in separate assay vials as described in the Experimental Procedures. The symbols represent the amount of each product formed: H_2S (●), HCN (■), and CH_4 (▲).

nitrogenase assay solution containing SCN^-, dithionite, MgATP, and a MgATP-regenerating system (Figure 1). CH_4 was not detected in the absence of SCN^-. To verify that CH_4 production resulted from nitrogenase catalysis rather than the activity of a contaminating protein, SCN^- was added to an assay solution lacking MgATP or one of the protein components of nitrogenase, which are absolute requirements for nitrogenase activity. No CH_4 was produced when MgATP, MoFe protein, or Fe protein was omitted from the assay (Figure 1). As a final test to confirm the role of nitrogenase in SCN^- reduction, the assay was performed in the absence of a MgATP-regenerating system. Because MgADP is an inhibitor of substrate reduction by nitrogenase, phosphocreatine and creatine phosphokinase are typically included in nitrogenase assays to convert the MgADP produced during the reaction back into MgATP. When the MgATP-regenerating system was omitted from the SCN^- reduction assay, CH_4 production was inhibited (Figure 1), consistent with a role for nitrogenase in SCN^- reduction to CH_4. These data taken together demonstrate that SCN^- is a novel substrate for nitrogenase.

To further elucidate the pathway of SCN^- reduction to CH_4, spectrophotometric assays were used to detect H_2S and HCN as potential intermediates. Figure 2 illustrates the time course of the nitrogenase-catalyzed reduction of SCN^- to H_2S, HCN, and CH_4. The rate of H_2S production was essentially constant during the course of the 30-min assay. In contrast, the rate of HCN production decreased substantially during the assay, while CH_4 production exhibited an initial lag, followed by a constant rate of production. One possible explanation for the initial high rate of HCN production is that early in the time course there could be an initial slow release of the two-electron reduction product from the active site before it can be further reduced by nitrogenase. Later in the assay, perhaps corresponding to a gradual increase in more reduced forms of the enzyme, there could be more efficient use of HCN to form the fully reduced product CH_4.

It is interesting to note that the concentration of HCN detected at 10 min was only about 50 μM, which is 2 orders of magnitude lower than the solution K_m for HCN reduction by nitrogenase (4.5 mM HCN) (Li et al., 1982). This suggests that HCN is released from the active site only slowly at neutral pH, but completely upon acid quenching of the reaction. Despite the production of HCN during the 30-min reaction, there appears to be little or no inhibition of total electron flux by CN^- (K_i of 27 μM) (Li et al., 1982). This lack of inhibition could be due to the low amounts of CN^- expected at pH 6.5 (approximately 0.17 μM CN^- at 30 min) or the possibility that HCN is not released from or deprotonated at the active site of nitrogenase.

The amount of NH_3 produced during the nitrogenase-catalyzed reduction of SCN^- was quantified in a separate experiment. During a 30-min assay containing 60 μg of Fe protein, the amount of NH_3 produced (60 ± 9.3 nmol) was approximately 1.3 times the amount of CH_4 detected (47 ± 1.4 nmol). This is the same ratio of NH_3 to CH_4 reported for the reduction of HCN by nitrogenase (Li et al., 1982), supporting the proposal that CH_4 is produced from the reduction of HCN. The excess NH_3 presumably results from the hydrolysis of either an enzyme-bound or free methyleneimine intermediate to form NH_3 and a product at the oxidation level of HCHO (Li et al., 1982). During the reduction of HCN, methylamine (CH_3NH_2), a four-electron reduction product, is formed at a constant ratio of 0.35 mol of CH_3NH_2 to 1 mol of CH_4 (Li et al., 1982); thus, CH_3NH_2 is likely to be a minor product of SCN^- reduction by nitrogenase. If H_2S is the only sulfur-containing product of SCN^- reduction, and CH_4, HCN, HCHO, and CH_3NH_2 are the major carbon-containing products, the amount of H_2S produced should be roughly equivalent to the sum of the amounts of the carbon-containing products. At the 30-min time point, the sum of the amounts of CH_4 plus HCN was 126 nmol, equivalent to 88% of the amount of H_2S produced (143 nmol). HCHO and CH_3NH_2 could account for the remaining 12% of the carbon-containing products. The above results taken together are consistent with a pathway of KSCN reduction in which the C═S bond of SCN^- is

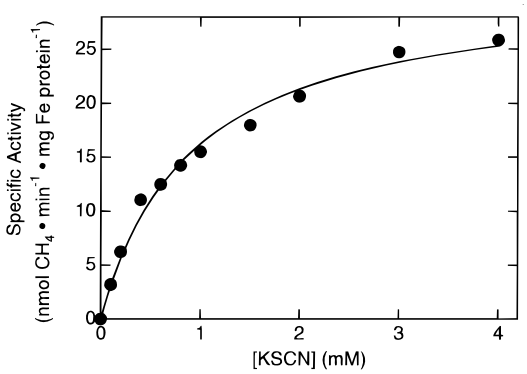


FIGURE 3: Effect of KSCN concentration on the rate of CH_4 formation by nitrogenase. KSCN reduction assays were performed at 30 °C in complete nitrogenase assay solution (1.1 mL), with 100 mM MES, pH 6.5, as the buffer and 10 mM dithionite as the reductant. Each assay contained 256 μg of MoFe protein and 120 μg of Fe protein. After 10 min, the assays were terminated by the addition of 250 μL of 2.5 N H_2SO_4. The K_m (0.9 ± 0.1 mM KSCN) and V_{max} (31 ± 1.2 nmol of CH_4•min^{-1}•mg of Fe $protein^{-1}$) were determined using nonlinear regression analysis to fit the data to the Michaelis−Menten equation as shown: $\nu = (V_{max}[S])/(K_m + [S])$, where ν = the reaction velocity and [S] = the substrate concentration (Segel, 1975). The solid line shows the best fit of the data to a hyperbola calculated using the Michaelis−Menten equation.

initially reduced by two electrons to form H_2S and HCN, followed by the six-electron reduction of HCN to CH_4 and NH_3.

Kinetics of SCN^- Reduction by Nitrogenase. The maximum rate of CH_4 production was obtained at pH 6.5, the same value obtained for the nitrogenase-catalyzed reduction of COS (Seefeldt et al., 1995). At this pH, thiocyanate (pK_b = 0.97; Macintyre, 1992) is present almost exclusively as the anionic species SCN^-; thus, SCN^- is more likely than HSCN to be the substrate reduced by nitrogenase. Interestingly, SCN^- is one of only three anionic compounds, including N_3^- (Dilworth & Thorneley, 1981; Rubinson et al., 1985) and NO_2^- (Vaughn & Burgess, 1989), to be characterized as a substrate for nitrogenase.

The concentration profile for the nitrogenase-catalyzed reduction of SCN^- to CH_4 approached typical hyperbolic saturation kinetics when the KSCN concentration was less than 6 mM (Figure 3). Above 6 mM SCN^-, substrate inhibition of SCN^- reduction to CH_4 occurred (data not shown). A fit of the data to the Michaelis−Menten equation resulted in an apparent K_m of 0.9 ± 0.1 mM SCN^- and an apparent V_{max} of 31 ± 1.6 nmol of CH_4 produced•min^{-1}•(mg Fe protein)$^{-1}$. The kinetic constants are reported as apparent values because the nitrogenase-catalyzed reduction of SCN^- to CH_4 involves multiple steps. The K_m for SCN^- reduction was also determined by measuring the direct product H_2S, and the result was consistent with the K_m determined by measuring CH_4. The K_m for SCN^- reduction is of the same order of magnitude as the K_m reported for the nitrogenase-catalyzed reduction of COS (3.1 mM) (Seefeldt et al., 1995).

Examination of the competition between substrates and inhibitors is one way to investigate the nature of substrate binding to nitrogenase. Thus, the effects of N_2, H_2, and CO on the rate of CH_4 production from SCN^- were analyzed (Table 1). One atmosphere of N_2 inhibited SCN^- reduction to CH_4 by 46%, indicating that N_2 and SCN^- compete as substrates for electrons or for binding to the active site of nitrogenase. H_2, an inhibitor of N_2 reduction but not the reduction of alternative substrates, did not affect the rate of CH_4 production. CO, which inhibits all substrate reduction reactions except for proton reduction, completely inhibited CH_4 production by nitrogenase. A previous report (Li et al., 1982) showed that HCN reduction is not inhibited by N_2. This result was interpreted to mean that HCN and N_2 bind to different sites or oxidation states of nitrogenase. N_2 inhibition of SCN^- reduction indicates that these compounds may compete for binding to the same site or oxidation state; however, the lack of inhibition by H_2 demonstrates that the mechanisms of N_2 and SCN^- binding and reduction are not identical.

Table 1: KSCN Reduction to CH_4 under Different Gas Phases

gas phase	specific activity (nmol of CH_4•min^{-1}•mg of Fe $protein^{-1}$)[a]
Ar	35 ± 2
N_2	19 ± 1
H_2	34 ± 1
CO (10%)	none detected

[a] Values represent the average of three determinations.

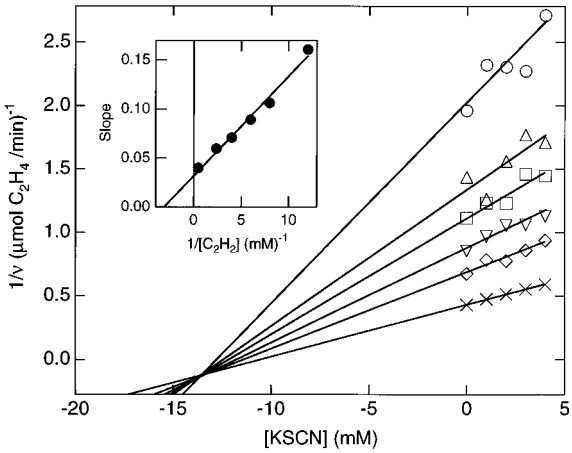


FIGURE 4: KSCN inhibition of C_2H_2 reduction by nitrogenase. The assays were performed at 30 °C in complete nitrogenase assay solution (1.1 mL) containing 100 mM MOPS, pH 7.0, as the buffer and 10 mM dithionite. Various amounts of KSCN (0−4 mM) and C_2H_2 (20−500 μL) were added to a series of assay vials. MoFe protein (256 μg) was added to each vial, and the reaction was initiated by the addition of Fe protein (120 μg). After 5 min, the reactions were terminated by the addition of 250 μL of 2.5 N H_2SO_4. C_2H_4 was measured as described in the Experimental Procedures. The vials contained the following amounts of C_2H_2: 20 (○), 30 (△), 40 (□), 60 (▽), 100 (◇), and 500 μL (×). The data were fit to the equation for mixed inhibition (Cleland, 1979; Cornish-Bowden, 1979), and the following kinetic constants were obtained: K_{i1} = 10.1 ± 1.2 mM KSCN and K_{i2} = 14.7 ± 3.8 mM KSCN. Inset: Plot showing the slope of each line versus the reciprocal of the C_2H_2 concentration, indicating mixed inhibition.

The interaction between SCN^- and C_2H_2 was investigated by examining the kinetics of SCN^- inhibition of C_2H_2 reduction (Figure 4). Analysis of the Dixon plot indicated that SCN^- is a rapid-equilibrium inhibitor of C_2H_2 reduction. To distinguish between competitive and mixed inhibition, the slope of each line of the Dixon plot was plotted against the reciprocal of the C_2H_2 concentration. The resulting line intersected the *y*-axis at a point above 0, indicating mixed-type inhibition (Cornish-Bowden, 1979). A fit of the data to the equation for mixed-type inhibition (Cleland, 1979) resulted in values of K_{i1} of 10.1 ± 1.2 mM SCN^- and K_{i2} of 14.7 ± 3.8 mM SCN^-.

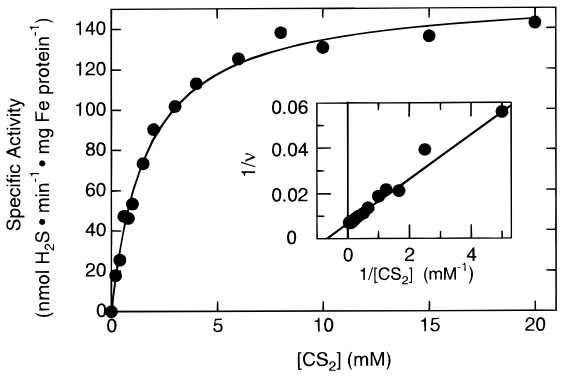


FIGURE 5: CS_2 reduction to H_2S by nitrogenase. CS_2 reduction assays were performed at 30 °C in complete nitrogenase assay solution (1.1 mL) with 100 mM MES, pH 6.5, and 5 mM titanium citrate. The assays contained 256 μg of MoFe protein and were initiated by the addition of 120 μg of Fe protein. After 10 min, the reactions were terminated with 100 μL of 17.4 N acetic acid. H_2S produced during the assays was quantified as described in the Experimental Procedures. The line shows the best fit of the data to a hyperbola calculated using the Michaelis−Menten equation (Segel, 1975). The following kinetic constants were obtained: K_m = 1.7 mM ± 0.1 CS_2 and V_{max} = 157 ± 4 nmol of H_2S·min^{-1}·mg of Fe protein^{-1}. Inset: Plot showing the reciprocal of the velocity ($1/v$) versus the reciprocal of the CS_2 concentration. The reciprocal velocity ($1/v$) is expressed in units of 1/(nmol of H_2S·min^{-1}·mg of Fe protein^{-1}).

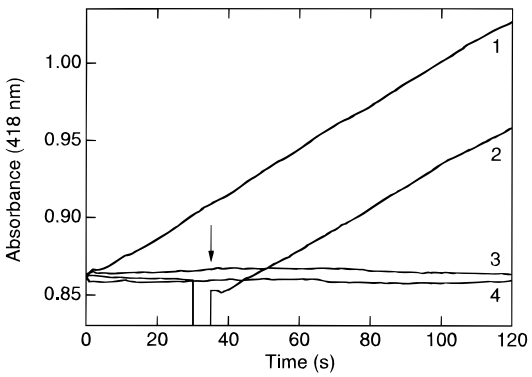


FIGURE 6: Cyanate reduction to CO by nitrogenase. CO produced during the nitrogenase-catalyzed reduction of cyanate was measured spectrophotometrically using the hemoglobin CO binding assay as described in the Experimental Procedures. The assays were performed at 30 °C in 2.2-mL Ar-purged quartz cuvettes containing 1.2 mL of assay solution (83 mM MOPS, pH 7.0, 1 mg/mL BSA, 0.2 mg/mL hemoglobin, 2.7 mM MgATP, 0.15 mg/mL creatine phosphokinase, 4.5 mg/mL phosphocreatine, 8.3 mM dithionite, and 2 mM KOCN). Each assay contained 256 μg of MoFe protein and was initiated by the addition of 120 μg of Fe protein, unless otherwise noted. The assay solutions contained the following components: trace 1, complete assay mixture; trace 2, assay solution without ATP, followed by addition of ATP to 2.7 mM at 30 s (indicated by the arrow); trace 3, assay solution without MoFe protein; trace 4, assay solution without Fe protein.

CS_2 as a Substrate for Reduction by Nitrogenase. CS_2 was previously shown to be an inhibitor of proton reduction by nitrogenase and a rapid-equilibrium mixed-type inhibitor of nitrogenase-catalyzed C_2H_2 reduction (Seefeldt et al., 1995); however, the possibility that CS_2 could be a substrate was not investigated. Given the structural similarity of CS_2 to COS and SCN$^-$, it was predicted that nitrogenase could reduce the C═S bond of CS_2 to form H_2S. When nitrogenase was incubated with CS_2, H_2S was produced through a process requiring CS_2, MgATP, and both components of nitrogenase (data not shown), demonstrating that CS_2 is a substrate for nitrogenase. This result is the first example of CS_2 reduction by a purified enzyme. In the absence of a MgATP-regenerating system, the rate of H_2S production over a period of 10 min was inhibited by 74%, providing additional evidence that nitrogenase is required for CS_2 reduction. The pH optimum for CS_2 reduction was 6.5, and the rate of H_2S production was constant for at least 10 min up to a concentration of 20 mM CS_2.

The effect of CS_2 concentration on the rate of H_2S production by nitrogenase is illustrated in Figure 5. From a fit of the data to the Michaelis−Menten equation, the K_m was estimated to be 1.7 ± 0.1 mM CS_2, and the V_{max} was estimated to be 157 ± 4 nmol of H_2S produced·min^{-1}·(mg of Fe protein)$^{-1}$. The fate of the reduced carbon atom of CS_2 is unknown at this time. CO and CH_4 were not detected as products of CS_2 reduction, and no additional peaks eluting from the Porapak N column were detected using a flame ionization detector.

OCNH Reduction by Nitrogenase. A logical extension of the characterization of nitrogenase substrates containing C═O and C═S bonds was to investigate the reactivity of OCN$^-$, a structural analog of both CO_2 and SCN$^-$. Since OCN$^-$ is a bidentate ligand, two pathways of OCN$^-$ reduction by nitrogenase can be envisioned. In the first pathway, the C═O bond can be reduced to form H_2O and HCN (eq 4), which would be further reduced by nitrogenase to CH_4. In the second pathway, the C═N bond would be

$$O{=}C{=}N^- + 2e^- + 3H^+ \rightarrow H_2O + HCN \qquad (4)$$

reduced to form CO and NH_3 (eq 5) in a manner similar to the reduction of CO_2 (eq 6).

$$O{=}C{=}N^- + 2e^- + 3H^+ \rightarrow CO + NH_3 \qquad (5)$$

$$O{=}C{=}O + 2e^- + 2H^+ \rightarrow CO + H_2O \qquad (6)$$

To distinguish between these pathways, CH_4 production was monitored in a nitrogenase assay containing 5 mM KOCN at pH 7.0. No CH_4 was detected during a 10-min assay. Therefore, to determine if CO was produced from KOCN reduction via C−N bond reduction (eq 5), CO was quantified using a hemoglobin CO binding assay (Seefeldt et al., 1995). In this assay, the binding of CO to hemoglobin results in a change in the visible absorbance spectrum of the heme near 418 nm, which can be monitored spectrophotometrically. The binding of CO to hemoglobin plays a second critical role by removing free CO from the assay solution. This alleviates any potential inhibitory effects of CO on nitrogenase activity. Figure 6 illustrates the time course of the production of CO when nitrogenase was incubated with 2.5 mM KOCN at pH 7.0. In the presence of the complete assay mixture, KOCN was reduced to CO. No CO was detected when MgATP, MoFe protein, or Fe protein was omitted from the assay, demonstrating that nitrogenase is required for KOCN reduction to CO (Figure 6).

The effect of pH on the rate of KOCN reduction to CO by nitrogenase was determined using a mixed buffer system over a range of pH values from 5.5 to 8.5. The pH optimum for the nitrogenase-catalyzed reduction of C_2H_2, COS, SCN$^-$, and CS_2 is 6.5. In contrast, the rate of KOCN reduction by nitrogenase increased with decreasing pH from 7.0 to 5.5.

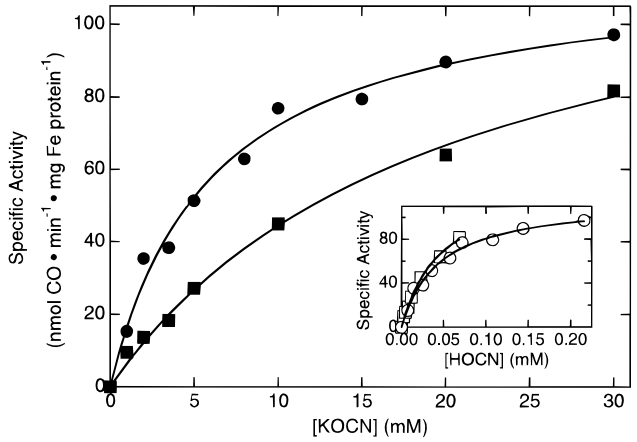


FIGURE 7: Effect of KOCN concentration on the rate of nitrogenase-catalyzed CO production. CO production from the nitrogenase-catalyzed reduction of KOCN was monitored using the hemoglobin CO binding assay as described in the legend to Figure 6, except that the assay buffer was 83 mm MES at pH 6.0 (●) or pH 6.5 (■). The data were fit to the Michaelis−Menten equation (Segel, 1975), and the kinetic constants are reported in the text. *Inset:* Plot of the data as a function of HOCN concentration at each pH. Specific activity is reported as nmol of CO·min^{-1}·mg of Fe protein^{-1} at pH 6.0 (○) and pH 6.5 (□).

Below pH 5.5, the assay mixture was unstable and assay components began to precipitate. Therefore, the kinetic determinations of KOCN reduction were performed at pH 6.0 and 6.5. The K_m of KOCN reduction was dependent on the pH of the assay solution (Figure 7). At pH 6.0 the K_m was 6.1 ± 0.7 mM KOCN and the V_{max} was 116 ± 5 nmol of CO produced·min^{-1}·(mg of Fe protein)$^{-1}$, while at pH 6.5, the K_m was 20 ± 0.7 mM KOCN and the V_{max} was 134 ± 8 nmol of CO produced·min^{-1}·(mg of Fe protein)$^{-1}$. The pH dependence of the K_m suggested that OCNH rather than OCN^- was the substrate for reduction by nitrogenase. To illustrate the effect of OCNH concentration on the rate of CO production, the concentrations of OCNH were calculated using the Henderson−Hasselbalch equation and a pK_a value for OCNH of 3.86 (Macintyre, 1992). When the rates of CO production were plotted as a function of OCNH concentration rather than OCN^- concentration, the fits of the data at pH 6.0 and 6.5 were nearly identical (Figure 7, inset). The corresponding values of the K_m for OCNH reduction by nitrogenase were 46 μM OCNH at pH 6.0 and 44 μM OCNH at pH 6.5. These results are consistent with the proposal that OCNH rather than OCN^- is the substrate for reduction by nitrogenase. Although CH_4 could not be detected in assays without hemoglobin present to trap the CO, small amounts of CH_4 were produced in the assay cuvettes containing hemoglobin. This indicates that both the C−O bond and the C−N bond of OCNH are susceptible to reduction by nitrogenase.

EPR Spectroscopy of Nitrogenase during OCNH, SCN^-, and CS_2 Reduction. To further investigate the interactions of nitrogenase with substrates containing C═S and C═O bonds, the effects of OCNH, KSCN, and CS_2 reduction on the EPR properties of nitrogenase were determined. Figure 8 illustrates the effects of substrate concentration and pH on the EPR spectra of nitrogenase treated with KOCN. Traces 1 and 2 show the EPR spectra of nitrogenase in the absence of MgATP (nonturnover conditions) or in the presence of MgATP (turnover conditions) without added KOCN or CO. When nitrogenase was incubated with 30

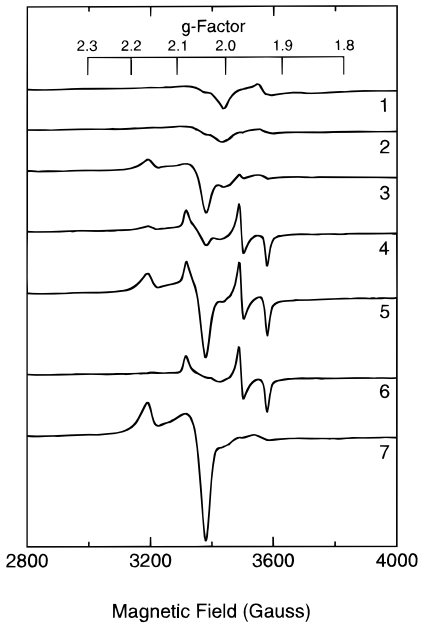


FIGURE 8: Effect of KOCN on the EPR spectrum of nitrogenase under turnover conditions. EPR samples were prepared as described in the Experimental Procedures using a 4 to 1 molar ratio of MoFe protein (2.9 mg, 13 nmol) to Fe protein (0.21 mg, 3.3 nmol). The reaction solution (250 μL) contained 100 mM MES, pH 6.0, (or 100 mM MOPS, pH 7.0), 1 mg/mL BSA, 3 mM MgATP, 5 mg/mL phosphocreatine, 0.16 mg/mL creatine phosphokinase, 10 mM dithionite, and KOCN or CO where indicated. After MoFe protein was added, the reaction was initiated with the addition of Fe protein. The solutions were mixed as described in the Experimental Procedures. Samples were incubated at ambient temperature (22 °C) for 2 min prior to freezing in EPR tubes in liquid N_2. The EPR spectra were recorded at 12 K with a microwave power of 3.19 mW and a microwave frequency of 9.64 GHz. In trace 1, the nitrogenase assay solution contained 30 mM KOCN at pH 6.0 in the absence of MgATP. In traces 2−7, the nitrogenase assay solution contained 3 mM MgATP (turnover conditions) and the following additions: trace 2, no KOCN at pH 6.0; trace 3, 30 mM KOCN at pH 6.0; trace 4, 1 mM KOCN at pH 6.0; trace 5, 30 mM KOCN at pH 7.0; trace 6, 9 μM CO (0.125 atm CO in the gas phase) at pH 6.0; trace 7, 216 μM CO (0.3 atm CO) at pH 6.0.

mM KOCN at pH 6.0 under turnover conditions, a signal with inflections at $g = 2.16$, 2.09, and 2.05 was observed (trace 3). The spectrum in the $g = 4.3$ region was essentially the same as the spectrum of nitrogenase and MgATP without KOCN added, except that a small inflection near g = 4.46 was observed (data not shown). In the absence of MgATP the spectrum was identical to that of nitrogenase without added KOCN, indicating that under nonturnover conditions KOCN does not react with nitrogenase in a way that affects the EPR properties of the metal clusters. When nitrogenase was incubated with 1 mM KOCN under turnover conditions, a dramatically different spectrum was produced (trace 4). The main features of this spectrum were inflections at $g =$ 2.08, 1.98, and 1.92. Minor inflections were evident near $g = 2.16$ and 2.05.

Since CO is a product of KOCN reduction, the spectra of nitrogenase treated with KOCN were compared with the spectra of nitrogenase incubated under turnover conditions with a relatively low concentration of CO (0.0125 atm; 9 μM) (trace 6) or a high concentration of CO (0.3 atm; 216 μM) (trace 7). The spectrum of nitrogenase treated with 30 mM KOCN at pH 6.0 resembled that of nitrogenase treated with a high concentration of CO. This so-called "hi-CO signal" (Christie et al., 1996) reports the binding of two

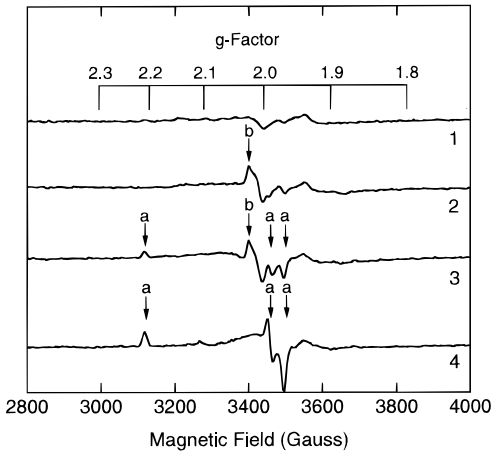
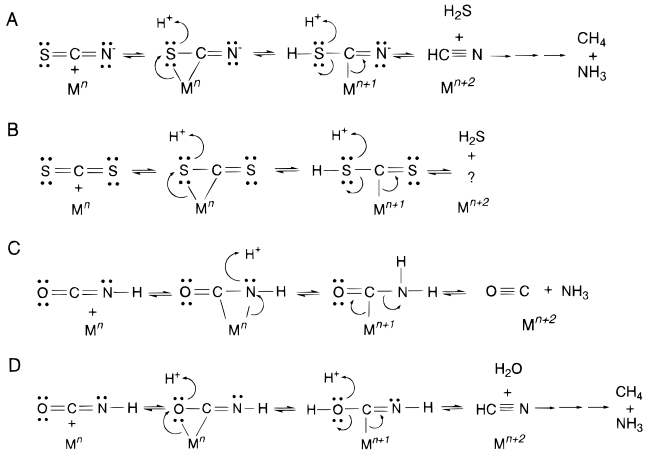


FIGURE 9: Effect of HCN, KSCN, and CS_2 on the EPR spectrum of nitrogenase under turnover conditions. Samples (250 μL) contained MoFe protein (2.9 mg, 13 nmol), Fe protein (0.21 mg, 3.3 nmol), and the complete reaction solution in 100 mM MES, pH 6.5, as described in the legend to Figure 8. Prior to the samples being frozen in liquid N_2, the assay solutions were incubated for 2 min in the presence of 3 mM MgATP and the following components: trace 1, no addition; trace 2, 60 μM HCN; trace 3, 2 mM KSCN; trace 4, 0.6 mM CS_2 and 84 mM DMSO. The EPR spectra were recorded at 12 K with a microwave power of 3.19 mW and a microwave frequency of 9.64 GHz. The arrows indicate inflections associated with the interaction of nitrogenase with (a) a sulfur-containing adduct or (b) cyanide.

molecules of CO to FeMoco, indicating that under the conditions studied the reduction of KOCN resulted in the binding of two molecules of CO to FeMoco. The spectrum of nitrogenase treated with 1 mM KOCN was similar to that of nitrogenase incubated with a low concentration (9 μM) of CO. The inflections near $g = 2.08$, 1.98, and 1.92 report the presence of FeMoco with one CO bound; however, the small inflections at $g = 2.16$ and 2.05 indicate that some of the FeMoco molecules also contained two bound CO molecules. Because the K_m of KOCN reduction is pH-dependent, the effect of pH on the EPR spectrum of nitrogenase treated with 30 mM KOCN was examined (trace 5). The spectrum obtained at pH 7.0 contained both the lo-CO and hi-CO signals. The rate of CO production at pH 7.0 is lower than the rate at pH 6.0, resulting in a CO concentration that is intermediate between the amount of CO required to produce either the lo-CO or hi-CO signal exclusively.

The effects of SCN^- and CS_2 on the EPR spectra of nitrogenase are presented in Figure 9. Under turnover conditions in the presence of 2 mM KSCN, new inflections with *g*-values at 2.21, 2.02, 1.99, and 1.97 were observed (trace 3). The intensity of the inflection at $g = 2.21$ remained constant from 1.5 to 3.5 min, indicating that the signal was saturated during this time period. The intensity decreased to 25% of the maximum value by 6 min and was undetectable by 15 min, suggesting that the enzyme was no longer turning over. Incubation of nitrogenase with 0.6 mM CS_2 and 84 mM DMSO resulted in the production of new EPR inflections (trace 4) that were not present when nitrogenase was incubated with DMSO alone. A simulation of the spectrum of nitrogenase treated with CS_2 fit reasonably well with a model for Fe in a high sulfur coordination environment. Interestingly, the *g*-values of the spectrum obtained with CS_2 ($g = 2.21$, 1.99, and 1.97) were similar to three of the inflections (marked "a") in the spectrum of nitrogenase

Scheme 1



treated with SCN^-, suggesting a common transient intermediate in the reduction of SCN^- and CS_2. Nitrogenase treated with H_2S did not produce the new EPR inflections (data not shown), indicating that the EPR signals did not result from a complex between nitrogenase and the product H_2S. However, nitrogenase treated with 60 μM HCN, a product of SCN^- reduction, produced a signal with a *g*-value at 2.02 (trace 2). This $g = 2.02$ inflection (marked "b") is present in the spectrum of nitrogenase treated with SCN^- (trace 3) but not CS_2 (trace 4), indicating that the SCN^- spectrum can be resolved into two components resulting from the interaction of nitrogenase with a sulfur-containing species (inflections at $g = 2.21$, 1.99, and 1.97) or with a cyanide adduct or reduction intermediate (inflection at $g = 2.02$).

DISCUSSION

The present work describes the kinetic characterization and EPR spectroscopic analysis of three novel substrates for nitrogenase, SCN^-, CS_2, and OCNH, which are members of the new class of nitrogenase substrates that contain C═S or C═O bonds. SCN^- is one of only three anionic compounds that have been characterized as substrates for nitrogenase. The conclusion that SCN^- rather than HSCN is the reducible substrate is based on the low pK_b for SCN^- (0.97) and the observation that the K_m for SCN^- reduction was not dependent on pH. In contrast, the K_m for OCN^- decreased at lower pH values, suggesting that the protonated species OCNH was the actual substrate reduced by nitrogenase. These results are consistent with the observation that the biological effects of cyanates are due primarily to the protonated species, while the biological reactions of thiocyanates most commonly involve the anionic form (Cohen and Oppenheimer, 1977). The reduction of CS_2 by nitrogenase is the first demonstration of CS_2 reduction catalyzed by a purified protein. The bioconversion of CS_2 by a *Thiobacillus* species that apparently uses CS_2 as the sole energy source has been reported (Plas et al., 1993); however, the reaction products and the enzymes required for the degradation of CS_2 by this organism have not been identified.

Pathways of Substrate Reduction. The characterization of SCN^-, CS_2, and OCNH as novel substrates for nitrogenase can be discussed in terms of the mechanism of substrate binding and reduction by nitrogenase. The proposed pathways for the reduction of SCN^-, CS_2, and OCNH are illustrated in Scheme 1. In this scheme, M represents

FeMoco of the MoFe protein of nitrogenase. Note that this is a schematic model of the reactions proposed and is not meant to imply the actual bonding configurations. H_2S, HCN, CH_4, and NH_3 were produced during the nitrogenase-catalyzed reduction of SCN^-. These products are consistent with an initial two-electron reduction of the C═S bond of SCN^- to form H_2S and HCN, followed by the six-electron reduction of HCN to CH_4 and NH_3 (Scheme 1A), presumably through enzyme-bound methyleneimine and methylamine intermediates as proposed by Burgess (1985). The ratio of NH_3 to CH_4 formed from SCN^- reduction (1.3 to 1) was the same ratio observed previously during the reduction of HCN (Li et al., 1982), supporting the proposal that NH_3 and CH_4 were derived from the reduction of HCN. The reduction of CS_2 by nitrogenase produced H_2S through a mechanism involving the two-electron reduction of one of the C═S bonds of CS_2 (Scheme 1B). The carbon-containing product of CS_2 reduction was not detected. CS, the product predicted from analogy to the mechanism of COS and SCN^- reduction, is highly unstable and tends to polymerize in solution (Macintyre, 1992). The major product of OCNH reduction by nitrogenase was CO. This product would result from the two-electron reduction of the C═N bond as shown in Scheme 1C. Small amounts of CH_4 were also detected during the reduction of OCNH, providing evidence for an alternative pathway in which the C═O bond is reduced to form H_2O and HCN, and HCN is reduced to NH_3 and CH_4 (Scheme 1D).

Interactions of SCN^- with Nitrogenase Substrates and Inhibitors. N_2 and CO inhibited the reduction of SCN^- by nitrogenase, while H_2 had no effect on SCN^- reduction. It has been previously shown that N_2 does not inhibit HCN reduction, suggesting that HCN and N_2 bind to different sites or oxidation states of nitrogenase (Li et al., 1982). The inhibition of SCN^- reduction by N_2 indicates that these compounds compete as substrates for nitrogenase; however, since H_2 inhibits the reduction of N_2 but not SCN^-, the mechanisms of N_2 and SCN^- binding and reduction cannot be identical.

SCN^- is a rapid equilibrium mixed-type inhibitor of C_2H_2 reduction by nitrogenase. The classical interpretation of mixed inhibition is that the inhibitor binds with different affinities to two different forms of the enzyme, the free enzyme and the enzyme−substrate complex. The inhibition constants K_{i1} and K_{i2} represent different affinities of the inhibitor for the free enzyme and enzyme-substrate complex, respectively (Segel, 1975). Thus, mixed inhibition of C_2H_2 reduction by SCN^- suggests that SCN^- can bind to both nitrogenase and the nitrogenase−C_2H_2 complex. One possible interpretation of mixed inhibition of C_2H_2 reduction by SCN^- is that the two substrates bind to different locations or different oxidation states of the MoFe protein. If the binding of these substrates occurred at the same site and oxidation state, purely competitive inhibition would be expected. In contrast, mixed or partial inhibition would result if one substrate binds to a more oxidized or more reduced form of the enzyme.

It is interesting to note that SCN^- inhibits both C_2H_2 and proton reduction by nitrogenase, while the closely related compound COS inhibits C_2H_2 reduction but not proton reduction (Madden et al., 1990; Seefeldt et al., 1995). The inability of COS to inhibit proton reduction by nitrogenase could result from two effects of CO, the product of COS reduction. First, in the absence of a hemoglobin CO binding system, CO produced during COS reduction would inhibit the further reduction of COS. Second, since CO does not inhibit the reduction of protons, the effect of small amounts of CO produced during COS reduction would be to allocate all of the electron flux to proton reduction. These two CO effects would prevent COS from inhibiting proton reduction substantially even though COS is a substrate for nitrogenase.

EPR Spectroscopy. Nitrogenase contains two binding sites for CO. Distinct EPR signals are produced when one site is occupied by CO (at low CO concentrations) and when two sites are occupied at high CO concentrations. During the nitrogenase-catalyzed reduction of OCNH to CO, the lo-CO signal was observed under conditions which produced low amounts of CO, while the hi-CO signal was produced in the presence of high OCNH concentrations at pH 6.0. This is the first example of the identification by EPR spectroscopy of a nitrogenase product formed during the reduction of the corresponding substrate. A unique EPR signal has been observed when nitrogenase is treated with C_2H_4 under turnover conditions (Lowe et al., 1978); however, this signal is not detected during the reduction of C_2H_2 presumably because the two compounds compete for a common binding site on FeMoco and C_2H_4 has a relatively low affinity for nitrogenase (K_m = 1.5 mM).

Because an equilibrium may exist between CO binding and diffusion at the active site, it is difficult to predict the effective concentration of CO at the active site of the MoFe protein. However, it is possible to estimate the total amount of CO likely to be produced by nitrogenase in the EPR samples. At 30 °C with 30 mM KOCN at pH 6.5, the rate of OCNH reduction would be approximately 100 nmol of $CO \cdot min^{-1} \cdot (mg\ of\ Fe\ protein)^{-1}$ (Figure 7). During preparation of the EPR samples at 22 °C, the reaction might be expected to proceed at approximately half that rate. The EPR samples contained 0.21 mg of Fe protein in 0.25 mL, and therefore the solution concentration of CO produced in 2 min is expected to be about 84 μM. Since the reaction contained 52 μM MoFe protein, equivalent to 104 μM active sites (two active sites per MoFe protein), there might not be enough CO produced to bind greater than one CO molecule per active site if CO is first released from the active site before binding FeMoco. The hi-CO signal (Figure 8) suggests that at least some of the active sites contain two bound CO molecules; thus, the levels of the EPR signal may not be consistent with the release and rebinding of CO and indicate that the concentration of CO at the active site may be higher than in the total solution. This is possible since CO is generated in the active site of nitrogenase during the reduction of OCNH.

Nitrogenase treated with CS_2 exhibited an EPR signal with *g*-values at 2.21, 1.99, and 1.97, while SCN^- produced an EPR spectrum with the same three inflections plus an additional inflection at g = 2.02. No signal was observed when nitrogenase was treated under turnover conditions with H_2S, a product of the nitrogenase-catalyzed reduction of both SCN^- and CS_2, suggesting that the spectrum did not result from H_2S binding to nitrogenase. The inflections with *g*-values at 2.21, 1.99, and 1.97 exhibited characteristics of Fe in a high sulfur coordination environment and could represent the formation of a nitrogenase−CS_2 complex or a transient intermediate in the reduction of CS_2. The binding of CS_2 and SCN^- is proposed to occur through a common

mechanism involving the binding of C−S to Fe atoms of FeMoco as shown in Scheme 1. The appearance of common EPR inflections during the reduction of CS_2 and SCN^- is consistent with this proposed mechanism of binding.

If the EPR spectrum of nitrogenase treated with CS_2 is due to the binding of a transient intermediate, it is likely that not all of the active sites of the MoFe protein are saturated with the proposed intermediate. While it is difficult to estimate the concentration of a transient intermediate at the active site of nitrogenase, a reasonable estimate of the total amount of CS_2 reduced in the EPR samples can be made by assuming that the rate of CS_2 reduction with 0.6 mM CS_2 at 22 °C is approximately 20 nmol of $H_2S \cdot min^{-1} \cdot (mg\ of\ Fe\ protein)^{-1}$ (about half the rate observed at 30 °C; Figure 5). In 2 min with 0.21 mg of Fe protein, there would be only about 8 nmol of CS_2 reduced compared to 26 nmol of MoFe protein active sites. The amount of the putative intermediate present in the sample is expected to be lower than the total amount of substrate reduced, and therefore even if the binding were tight, only a portion of the active sites would contain a bound intermediate.

The inflection at $g = 2.02$ produced during the reduction of SCN^- (but not CS_2) was also observed when nitrogenase was treated with HCN under identical conditions. Therefore, the $g = 2.02$ inflection observed during SCN^- reduction can be accounted for by the cyanide moiety produced during the reduction of SCN^-. In preliminary EPR studies, it appears that the $g = 2.02$ inflection results from cyanide interaction with the MgATP-bound form of the Fe protein alone.

Modes of Substrate Binding. The site and orientation of substrate binding to nitrogenase remains a topic of considerable controversy. Although a crystal structure of nitrogenase with a substrate bound is not yet available, theoretical models of substrate binding to FeMoco have been proposed, including side-on binding to Fe, end-on binding to Fe, end-on binding to Mo, and binding within the caged structure formed by the six central Fe atoms of FeMoco (Dance, 1996). The side-on binding mode to a rectangular 4-Fe face of FeMoco is favored in a recent review by Dance (1996). Model chemistry studies of CS_2 binding to mononuclear metals demonstrate that the most common binding orientation of CS_2 is through π bonds in a side-on fashion to the metal (Ibers, 1982). Thus, side-on binding to a 4-Fe face of FeMoco is a reasonable mechanism by which CS_2 binds to nitrogenase (Scheme 1A). Model studies demonstrate that the ambidentate molecule SCN^- is capable of binding to metals through either the sulfur or nitrogen atoms (Burmeister, 1975). In nitrogenase the bonding of the more polarizable sulfur atom of SCN^- appears to be preferred (Scheme 1B), forming an enzyme-bound intermediate resembling that of CS_2. The EPR inflections at $g = 2.21$, 1.99, and 1.97 in the spectra of nitrogenase treated with CS_2 or SCN^- support the existence of a common binding mode or transient intermediate.

In model complexes of cyanate with transition metals, the most common binding orientation of cyanate is through the nitrogen atom (Schönherr, 1986; Balahura & Jordan, 1970). Oxygen-bonded cyanate−metal complexes are less frequently observed (Duggan & Hendrickson, 1974). N-binding of OCNH to nitrogenase would result in the reduction of the C═N bond to produce CO and NH_3 (Scheme 1C), while binding through the oxygen atom would account for the reduction of OCNH to H_2O and HCN in the pathway of CH_4 production (Scheme 1D).

Table 2: Kinetic Constants of Selected Nitrogenase Substrates

substrate	k_{cat}[a] (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	reference
COS	0.039	3.1	0.0126	Seefeldt et al., 1995
CO_2	0.00085	23.3	0.0000365	Seefeldt et al., 1995
SCN^-	0.300[b]	0.9	0.333	this work
CS_2	0.167	1.7	0.098	this work
OCNH	0.143	0.045	3.18	this work

[a] k_{cat} is expressed relative to the Fe protein as the number of electron pairs per second used to reduce each substrate. [b] Estimated number of electron pairs per second used to reduce SCN^- to HCN, HCHO, CH_3NH_2, and CH_4, calculated as described in the text.

Kinetic Comparisons of Nitrogenase Substrates. The kinetic constants for selected nitrogenase substrates are presented in Table 2. The K_m values for SCN^- (0.9 mM) and CS_2 (1.7 mM) are similar to the affinities reported for COS (3.1 mM), HCN (4.5 mM) (Li et al., 1982) and C_2H_2 (0.4 mM) (Davis et al., 1975). In contrast, the K_m for OCNH (0.045 mM) was at least 1 order of magnitude lower than the values for these alternative substrates and was close to the values previously reported for N_2 (K_m = 0.08 mM) (Rivera-Ortiz & Burris, 1975), the cyclic molecule diazirene (CH_2N_2) (K_m = 0.05−0.09 mM) (McKenna et al., 1996), and HN_3 (K_m = 0.012 mM) in the six-electron reduction to form N_2H_4 and NH_3 (Rubinson et al., 1985; Dilworth & Thorneley, 1981). One structural feature shared by OCNH, diazirene, and HN_3, is the presence of an R = NH group in one part of the molecule, which could contribute to the high affinity of these substrates for the active site of nitrogenase. It is interesting to note that although the K_m for HCN (4.5 mM) is relatively high, the methyleneimine intermediate (E-CH_2NH) is proposed to be enzyme bound (Li et al., 1982), and therefore methyleneimine appears to have a high affinity for the active site of nitrogenase, possibly conferred by the R = NH group.

In order to compare k_{cat} values for nitrogenase substrates that require different numbers of electrons for complete reduction, the k_{cat} values reported in Table 2 are expressed as the number of electron pairs used to reduce the substrate per second. Because the products of COS, CO_2, OCNH, and CS_2 reduction result from two-electron reduction reactions (one electron pair), the reported k_{cat} values are directly comparable to the amounts of product formed per second. However, the complete reduction of SCN^- to CH_4 requires eight electrons (four electron pairs); in addition, determination of the k_{cat} for SCN^- reduction is further complicated by the release of the partially reduced products HCN (one electron pair), HCHO (two electron pairs), and possibly CH_3NH_2 (three electron pairs). On the basis of the V_{max} of SCN^- reduction to CH_4, the maximum rate of CH_4 production would be 0.033 s^{-1}, but the k_{cat} expressed as the number of electron pairs used to reduce SCN^- could be almost 10 times that rate (about 0.3 electron pairs per second). This estimate is based on the following approximations: (i) the rate of HCN production at 10 min is about 3.5 times the rate of CH_4 production (Figure 2); (ii) the amount of HCHO produced would be equivalent to the amount of excess NH_3 produced during SCN^- reduction (0.3 times the amount of CH_4); and (iii) the amount of CH_3NH_2 produced during SCN^- reduction is likely to be similar to the amount

of CH_3NH_2 formed during HCN reduction (0.35 times the amount of CH_4) (Li et al., 1982). Since SCN^- reduction to CH_4 requires four electron pairs, the number of electron pairs allocated to CH_4 would be 4 times the amount of CH_4 produced. For HCN, HCHO, and CH_3NH_2 the electron pair allocation would be respectively about 3.5, 0.6, and 1.0 times the amount of CH_4 produced. The number of electron pairs allocated to all four SCN^- reduction products is about 9.1 times the amount of CH_4 produced, and therefore, the value of 0.033 molecules of CH_4 per second was multiplied by 9.1 to give an estimated k_{cat} of 0.30 electron pairs allocated to SCN^- reduction per second.

The structural similarities of CO_2, COS, and OCNH provide a unique opportunity to compare the affinities and reactivities of the C═O, C═S, and C═N bonds for reduction by nitrogenase. CO_2 has both the lowest affinity for nitrogenase (K_m = 23.3 mM) and the lowest turnover number (k_{cat} = 0.00085) of the three compounds. The kinetic constants for COS show that replacing the C═O bond with C═S increases the affinity by 7-fold and the turnover number by almost 50-fold. OCNH has the highest affinity (K_m = 0.045 mM) and turnover number (k_{cat} = 0.143). These data indicate that the order of reactivity for the reduction of these bonds by nitrogenase is C═O < C═S < C═N. The rate of CH_4 production from SCN^- is greater than that from OCNH, demonstrating that, in these structurally similar molecules, the C═S bond is also reduced more readily than the C═O bond.

Carbon monoxide dehydrogenase (CODH) catalyzes two of the same reactions as nitrogenase: the reduction of CO_2 to CO and H_2O and the reduction of COS to CO and H_2S (Ensign, 1995). CS_2, SCN^-, and OCN^- act as inhibitors of CODH, and it is therefore of interest to compare the interactions of CODH and nitrogenase with these compounds. The reduction of CS_2, SCN^-, and OCNH is catalyzed by nitrogenase, but none of these compounds are reported to be substrates for CODH from *Clostridium thermoaceticum* (Kumar et al., 1994; Seravelli et al., 1995). CS_2 is a rapid equilibrium competitive inhibitor of CO_2 reduction by CODH from *Rhodospirillum rubrum* (Ensign, 1995) and a noncompetitive inhibitor of CO_2 reduction by CODH from *C. thermoaceticum* (Kumar et al., 1994). SCN^- is a partial mixed inhibitor of CO oxidation by the clostridial CODH (Seravelli et al., 1995). EPR spectroscopy reveals that in the absence of reductant, SCN^- and OCN^- bind to center C, a Ni/Fe−S center involved in CO_2 reduction. In contrast, CS_2 does not bind to the CO_2-reducing center C, but rather to center A, a Ni/Fe−S cluster that is the site of an exchange reaction between CO and acetyl coenzyme A in the pathway of acetyl coenzyme A synthesis (Kumar at al., 1994). The EPR inflections (g = 2.200, 2.087, 2.017) were attributed to CS_2 binding to one of the Fe atoms of center A. It is likely that the EPR inflections observed during the nitrogenase-catalyzed reduction of CS_2 report the binding of CS_2 or a transient reaction intermediate to Fe atoms of FeMoco.

This work provides the first evidence that SCN^-, CS_2, and OCNH are substrates for nitrogenase and identifies novel EPR signals that report the interaction between nitrogenase and a bound substrate or transient intermediate produced during the reduction of SCN^- and CS_2. These C═S and C═O containing substrates have potential for use as probes to examine substrate binding to nitrogenase using spectroscopic or crystallographic methods. Advances using kinetic characterization, spectroscopic analysis, and X-ray crystallography are expected to provide further insights into the mechanism of substrate binding and reduction by nitrogenase.

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