Nitrogenase Reactivity: Methyl Isocyanide as Substrate and Inhibitor†

Judith F. Rubinson, James L. Corbin, and Barbara K. Burgess*

ABSTRACT: We have examined the interaction of methyl isocyanide with the purified component proteins of *Azotobacter vinelandii* nitrogenase (*Av*1 and *Av*2). CH_3NC was shown to be a potent reversible inhibitor (K_i = 158 μM) of total electron flow, apparently uncoupling magnesium adenosine 5′-triphosphate hydrolysis from electron transfer to substrate. CH_3NC is a substrate (K_m = 0.688 mM at *Av*2/*Av*1 = 8), and extrapolation of the data indicates that at high enough CH_3NC concentration, H_2 evolution can be eliminated. The products are methane plus methylamine (six electrons) and dimethylamine (four electrons). There is an excess (relative to methane) of methylamine formed, which may arise by hydrolysis of a two-electron intermediate. A rapid high-performance liquid chromatography/fluorescence method was developed for methylamine determination. The products C_2H_4 and C_2H_6 appear to be formed via a reduction followed by an insertion mechanism. CH_3NC appears to be reduced at an enzyme state more oxidized than the one responsible for H_2 evolution or N_2 reduction. Other substrates ($C_2H_2 > N_2 \simeq$ azide $> N_2O$) all both relieve CH_3NC inhibition and inhibit CH_3NC reduction. Both effects occur in the same relative order, implying productive (substrate) and nonproductive (inhibitor) modes of binding of CH_3NC to the same site.

The enzyme nitrogenase is composed of two separately purified proteins, called the molybdenum–iron protein (MoFe protein) and the iron protein (Fe protein), whose properties have been recently reviewed (Orme-Johnson et al., 1977; Mortenson & Thorneley, 1979; Smith, 1983). Nitrogen fixation and all other reductions catalyzed by the nitrogenase system require both component proteins, a source of reducing equivalents, protons, and MgATP[1] (Bulen & LeComte, 1966). The Fe protein is generally accepted as a specific electron donor to the MoFe protein (Hageman & Burris, 1978a,b; Ljones & Burris, 1978a,b; Braaksma et al., 1982), which is believed to contain the site of substrate reduction (Shah et al., 1973; Hageman & Burris, 1979). In addition to N_2, nitrogenase catalyzes the reduction of protons, nitrous oxide, acetylene, azide, cyanide, alkyl cyanides, alkyl isocyanides, hydrazine, cyclopropene, and allene. In recent years, a number of studies have focused on alternative substrates as probes for the number and nature of sites of substrate interaction on nitrogenase and the types of intermediates that might be formed during N_2 reduction. This paper describes recent studies on the interaction of methyl isocyanide with the purified component proteins of *Azotobacter vinelandii* nitrogenase.

Methyl isocyanide reduction was first demonstrated by Kelly et al. (1967). Using crude extracts of the N_2-fixing organism *Azotobacter chroococcum* (*Ac*), they showed that CH_3NC was reduced by six electrons to give methane plus methylamine as products. Like N_2 fixation, the reaction required MgATP and a reductant and was inhibited by carbon monoxide. CH_3NC reduction was later shown to be a general property of N_2-fixing organisms [e.g., Hardy & Jackson (1967), Kelly (1968), Munson & Burris (1969), and Biggins & Postgate (1969)].

Kelly et al. (1967) examined CH_3NC reduction in D_2O and demonstrated that all methane appeared as CD_4 and thus arose from the isocyanide carbon. They therefore suggested CH_3NC bound end-on to nitrogenase through the terminal carbon atom rather than the nitrogen atom. An interesting aspect of CH_3NC reduction by nitrogenase is that the C_2 products, ethylene and ethane, are formed (Kelly et al., 1967; Hardy & Jackson, 1967; Kelly, 1968). Using D_2O, Kelly (1968) demonstrated that both carbon atoms in the C_2 products arose from the isocyanide carbon. Although it was originally suggested that the C_2 products were formed by reaction of two enzyme-bound C_1 radicals (Kelly et al., 1967), further experimentation (Kelly, 1968; Hardy & Jackson, 1967) and kinetic analysis (Hardy, 1979) showed that C_2 product formation was dependent upon both CH_4 formation and the free CH_3NC concentration. On the basis of this information, Hardy (1979) suggested a reduction followed by an insertion mechanism whereby C_2 products are formed by interaction of a single bound C_1 radical with a free molecule of CH_3NC.

Using *Ac* crude extracts, Kelly et al. (1967) observed that although carbon monoxide (0.1 atm) inhibited CH_3NC reduction to methane by 90%, it actually stimulated C_2H_4 formation and decreased C_2H_6 formation by only 50%. When the experiments were repeated with partially purified component proteins of nitrogenase, Kelly (1969a) observed that carbon monoxide inhibited methane and ethane production to the same extent but had little effect on ethylene production. He interpreted these results in terms of two different mechanisms for the formation of ethylene and ethane. Observations of CO stimulation of C_2 product formation were later interpreted (Hardy, 1979) as an indication that CO might be inserted to form C_2 products. The C_3 products propylene and propane were also formed during CH_3NC reduction (Kelly, 1968; Hardy, 1979), albeit in very small amounts.

Methyl isocyanide concentration dependence experiments were complicated by the observation that the rate of methane formation began to decrease above 5 mM CH_3NC (Kelly, 1968; Hwang & Burris, 1972). Total electron flow was not measured so it was not clear if the decrease in methane formation was being offset by an increase in hydrogen evolution or if total electron flow was being inhibited (Hwang & Burris, 1972). Reported K_m values for CH_3NC reduction by nitrogenase range from 0.18 mM for *Ac* (Kelly, 1968) to 1.96

† From the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received May 26, 1983.* This is Contribution No. 816. This study was supported by Projects 79-59-2394-01-383-1 and 82-CRCR-1-1029 from SEA/CGO of the U.S. Department of Agriculture.

[1] Abbreviations: MgATP, magnesium adenosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; Tes, 2-[[tris(hydroxymethyl)methyl]-amino]ethanesulfonic acid; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; N_2ase, nitrogenase.

0006-2960/83/0422-6260$01.50/0

mM for *A. vinelandii* (*Av*) nitrogenase (Hwang & Burris, 1972). There are conflicting reports on the effect of CH_3NC on MgATP hydrolysis by nitrogenase. With *Ac*, it was reported (Kelly et al., 1967; Kelly, 1968) that CH_3NC had no effect on the rate of MgATP hydrolysis up to 5 mM and decreased the rate at higher concentrations. With *Av* (Hwang & Burris, 1972; Hwang et al., 1973), CH_3NC stimulated the rate of MgATP hydrolysis up to 10 mM with decreased enhancement at higher concentrations.

Kelly (1969a) studied the effect of changing the molar ratio of the Fe protein (*Ac*2) to the MoFe protein (*Ac*1) and found that CH_3NC reduction was maximized at a lower *Ac*2/*Ac*1 ratio than was N_2 fixation. This result was also obtained for *Klebsiella pneumoniae* (*Kp*) and *Bacillus polymyxa* (Kelly, 1969b) and interpreted as evidence against CH_3NC being a good model substrate for N_2 fixation.

Conflicting reports have appeared on the effects of other nitrogenase substrates and inhibitors on CH_3NC reduction. Kelly (1968) reported that N_2 and H_2 were both competitive inhibitors of CH_3NC reduction, whereas Hwang et al. (1973) observed that H_2 did not inhibit CH_3NC reduction. The latter investigators also reported that CH_3NC was a competitive inhibitor of azide reduction and suggested that azide, cyanide, and methyl isocyanide all bound to the same site on nitrogenase, which is distinct from the N_2 reduction site. Further studies probing methyl isocyanide reduction with the purified component proteins of *Av* nitrogenase are presented here.

Materials and Methods

Reagents and Chemicals. ATP, creatine phosphokinase, and Tes were obtained from Sigma Chemical Co.; NaN_3 was from MCB. *o*-Phthalaldehyde was from Aldrich Chemical Co. CH_3NC was prepared by a published procedure (Reisburg & Olson, 1980) and stored in a freezer (−20 °C) under argon. It was redistilled after several months as a precautionary measure even though we could detect no evidence of decomposition. K_2CO_3 and $KHCO_3$ were MCB reagents (0.001% N). Creatine phosphate was prepared according to a published procedure (Li et al., 1982) or was from Sigma. High-purity H_2, C_2H_2, and Ar were from AGA Burdox; N_2O and zero-grade N_2 were purchased from Matheson.

Nitrogenase Assay. A. vinelandii MoFe and Fe proteins, designated *Av*1 and *Av*2, respectively, were purified and analyzed as described elsewhere (Burgess et al., 1980). Specific activities of the proteins were ca. 1900 nmol of H_2 min^{-1} (mg of *Av*2)$^{-1}$ and 2900 nmol of H_2 min^{-1} (mg of *Av*1)$^{-1}$. Unless otherwise indicated, all assays were performed at 30 °C in 9.5-mL calibrated hypovials (Pierce Chemical Co.), fitted with butyl rubber serum caps, containing Ar. The 1.0-mL reaction mixture contained 30 mM Tes–KOH, pH 7.3, 2.5 mM ATP, 5.0 mM $MgCl_2$, 30 mM creatine phosphate, 5–10 mM neutralized $Na_2S_2O_4$, and 2.5 units of creatine phosphokinase.

The vessel containing the reaction mixture was degassed and filled with the appropriate gas mixture on a previously described apparatus (Corbin, 1978), $Na_2S_2O_4$ was added, and the mixture was incubated at 30 °C for 5 min. CH_3NC was added where appropriate, followed by *Av*1, and the reaction was started by adding *Av*2 to give the appropriate molar ratio of the two components. A total of 1 mg of protein per 1-mL reaction was used to avoid complications introduced by large protein concentrations (Wherland et al., 1981). Unless otherwise indicated, all experiments were performed at an *Av*2/*Av*1 molar ratio of 8. Molar ratios are based on molecular weights of 64000 for *Av*2 and 230000 for *Av*1. Molar ratio experiments were performed as described elsewhere (Wherland et al., 1981). For assays involving NaN_3, a 0.2 M stock solution was used, and the NaN_3 was added prior to degassing. Unless otherwise indicated, all reactions were run for 5 min, and all product formation was linear with time. Unless otherwise indicated, reactions were terminated with 0.1 mL of 1.5 M $K_2CO_3/KHCO_3$, pH 9.5, because of CH_3NC lability (see CH_3NH_2 determination). Reactions were terminated with 0.1 mL of 37% HCHO when dithionite was to be determined. When ammonia was to be determined, reactions were terminated with 0.4 M EDTA, pH 7.4 (0.1 mL). All termination methods were equally effective.

Preparation of CH_3NC Stock Solution. A total of 21.7 μL of the pure CH_3NC (0.4 μmol) was added to 4.0 mL of cold, degassed buffer and kept on ice. The solution was freshly prepared each day and used directly for high-concentration experiments or diluted appropriately when lower concentrations were desired.

Product Analysis. With the exception of dithionite-utilization studies (see below), *all products were measured on the same reaction vial.* All data points represent means of (typically) triplicate determinations (see paragraph at end of paper regarding supplementary material). Gas samples, 200 μL at bottle pressure, were taken with a pressure-lock syringe (Precision Sampling). Hydrocarbons were analyzed by using a Hewlett-Packard 5750 gas chromatograph equipped with a H_2 flame detector (Porapak N column, He). H_2 was detected by using a home-built gas chromatograph with a thermal conductivity detector (molecular-sieve 5-Å column, Ar). Attempts to measure C_3H_6 and C_3H_8 used a 13-Å molecular sieve at 150 °C. Measurements of C_2H_4 and C_2H_6 in the presence of CO were complicated by trace amounts of the C_2 compounds being present in the CO. To correct for this, we ran controls in triplicate with the appropriate concentrations of CO, with the complete nitrogenase system, but in the absence of CH_3NC to obtain background values. Phosphate was determined by use of a published procedure (Fiske & Subbarow, 1925).

Methylamine. The determination of methylamine was complicated by the lability of CH_3NC in acid solution, which leads to more of the amine by hydrolysis. More than a few minutes at pH 5–6 is sufficient to produce methylamine in amounts larger than that formed enzymatically. Fortunately, CH_3NC is stable in alkaline solution (Sidgwick, 1937). Rather than use our previous method for CH_3NH_2 (Li et al., 1982), which although sensitive, involved an acidic iodate treatment to remove dithionite, as well as a laborious derivatization and extraction procedure, we looked for a more direct method.

Primary amino acids react with borate-buffered mercaptoethanol–*o*-phthalaldehyde reagent (OPT) to form highly fluorescent products (Roth, 1971), which are suited for HPLC work (Lindroth & Mopper, 1979). For CH_3NH_2, we found that while a blue fluorescent product formed rapidly, the intensity of the fluorescence faded with time. However, the stability improved considerably when one worked at 0 °C, although fading still occurred. The reaction was not affected by CH_3NC, dithionite, or any other components of the enzyme assay. Thus, a sample could be mixed with the OPT reagent and, after a short reaction time, injected directly for HPLC analysis. It was very sensitive, and reproducibility was assured by using exact reaction times.

A Waters 244 instrument (U6K injector, 6000A pump) and Model 440 (absorbance) or 420-AC (fluorescence) detector were used. The column was a μBondapak C_{18} (3.9 × 30 cm). For the preparation of the OPT reagent, mercaptoethanol (0.20 mL) was added to a solution of *o*-phthaldehyde (270 mg), and then, 45 mL of 0.1 N sodium borate buffer (pH 9.5) was

added. It gave reproducible results after about 1 h for CH_3NH_2 rather than the 24 h suggested for "aged" reagent (Lindroth & Mopper, 1979). It was stable for several days in a Hypovial under argon. The sample to be analyzed (50 μL) was mixed with OPT reagent (0.50 mL) that had been prechilled in an ice bath. After exactly 2 min in ice, a 10-μL sample was analyzed by HPLC (1.5 mL/min, 65:35 methanol–0.05 M pH 5 sodium citrate, 338/425-nm excitation/emission filters). The peak corresponding to CH_3NH_2 emerged in ca. 5 min. Response was linear to at least 0.75 mM CH_3NH_2 in the original enzyme assay solution. The background level was ca. 0.004 mM.

We have since found that a small C_{18} guard column (Bio-Rad Bio-Sil ODS 5S) works very well for this analysis, allowing it to be completed in ca. 1 min. For this, the solvent is 55:45 methanol–0.05 M pH 5.0 sodium citrate at 1.8 mL/min. We have also observed that the derivatization reaction can be done at pH 7.3 as well as 9.5.

Dimethylamine. We looked for dimethylamine by HPLC with the dansyl and dabsyl (Li et al., 1982) techniques. Some was always detected, but quantitation was impossible because of the high background levels of dimethylamine arising from decomposition of the reagents (Seiler, 1970) (both reagents possess dimethylamino groups). NBD-Cl reagent (Van Hoof & Heyndrickx, 1974) also showed (HPLC) dimethylamine present, but was affected by the presence of dithionite and/or sulfite.

Reasonable quantitation was finally achieved by modifying the copper dithiocarbamate method (Karwelk & Meyers, 1979) and by using HPLC. No interference by methylamine (1 mM), methyl isocyanide, dithionite, or any other components of the enzyme assay mixture was evident. The reagent consisted of 0.25 M copper nitrate in 0.5 M NH_4OH–HCl, pH 9.5, and the extraction solvent was 2% CS_2 in $CHCl_3$. The sample to be analyzed (0.40 mL), reagent (0.75 mL), and solvent (1.0 mL) were shaken (4-mL screw-capped vials with Teflon cap liners) on a Burrell Model 75 wrist-action shaker for 1 h. After a brief centrifugation (clinical centrifuge) to separate the phases, 10 μL of the lower ($CHCl_3$) phase was analyzed by HPLC (1.4 mL/min, $CHCl_3$, 436-nm filter). The peak emerged in ca. 2 min.

Ammonia. Ammonia was determined by fluorescence, with a method similar to that of Taylor et al. (1974) but that uses HPLC. It is rapid (no microdiffusion step needed) and very sensitive. Details of this new procedure will appear elsewhere.

Dithionite Determination. In these experiments, H_2, CH_4, C_2H_4, and C_2H_6 production and $S_2O_4^{2-}$ utilization were measured on the same reaction vial. Dithionite was determined by using a published procedure (Li et al., 1982). The accuracy was enhanced by using a dithionite level such that one-fifth to one-third was consumed in the reaction. That amount was 10 mM $Na_2S_2O_4$ at CH_3NC concentrations below 0.3 mM CH_3NC and 5 mM at higher concentrations.

Data Treatment. Calculation of the total amounts of H_2, CH_4, C_2H_4, and C_2H_6 was based on the calibrated vial volume minus 1.1 mL of liquid phase and then expressed as nanomoles per minute per milligram of total protein. No correction for solubility of the gases in the liquid phase was applied. Total electron flow was obtained either by the amount of dithionite consumed or by the amounts of products formed. Dithionite consumption was determined in the reaction mixture from that found in a control reaction where *Av*2 was added after termination with HCHO. The relationships two electrons/H_2, six electrons/(CH_4 + CH_3NH_2), four electrons/CH_3NHCH_3, and two electrons/excess CH_3NH_2 are used in calculating electron flow from the products formed.

Phenanthroline Method. To examine the effects of bathophenanthrolinesulfonate on *Av*2, we used the method of Blair & Diehl (1961), as modified for the nitrogenase system by Ljones & Burris (1978a,b).

Table I: Product/Electron Balance

products (nmol)[a]				electron pairs as products (nmol)[b]	nmol of $S_2O_4^{2-}$ consumed[c]
H_2	CH_4	CH_3NH_2	CH_3NHCH_3		
229	172	207	27	833 ± 35	785 ± 70

[a] The following conditions were used: 2.5 mM CH_3NC, Ar atmosphere, 5-min reactions, average of six reactions. [b] Assuming two electrons for excess CH_3NH_2. If six electrons are given to excess CH_3NH_2, it would be 904. [c] By titration (Li et al., 1982).

Results

Reduction Products of CH_3NC. Electron Balance. Using highly purified component proteins, we have confirmed the findings of Kelly et al. (1967) that most of the CH_3NC is reduced (by six electrons) to give CH_4 and CH_3NH_2. We have also identified a previously unrecognized, four-electron product, CH_3NHCH_3. The ratio of CH_3NHCH_3 to CH_4 is constant at 0.16 ± 0.02, regardless of substrate concentration at *Av*2/*Av*1 = 8.

Table I gives a typical balance of products and electrons and shows that the CH_3NH_2/CH_4 ratio is greater than the anticipated 1:1. By analogy to the finding of excess NH_3 over CH_4 in the HCN reduction system (Li et al., 1982), it seemed to us that this CH_3NH_2 could arise by hydrolysis of a two-electron intermediate and that HCHO was likely to be the missing carbonaceous product. Unfortunately, HCHO cannot be successfully quantitated at these levels in N_2ase assays (Li et al., 1982). The data in Table I are consistent with the excess CH_3NH_2 being produced by a two-electron pathway, although the argument here is less definitive than for the HCN system (Li et al., 1982). The data are not inconsistent with a two-step hydrolysis reaction, catalyzed by the *complete* N_2ase system, whereby CH_3NC is first hydrolyzed to *N*-methylformamide and then to CH_3NH_2 + HCO_2H. Surprisingly, the CH_3NH_2 to CH_4 ratio varies with substrate concentration (see below).

Hydrogen (in variable amounts) is formed concurrently as a two-electron product. The formation of all CH_3NC reduction products is dependent on the presence of MgATP, dithionite, and both component proteins.

The data in Table I clearly show the equivalence of products formed to electrons consumed, and thus all major products are accounted for. Ethylene and ethane, products of CH_3NC reduction (Kelly et al., 1967), were detected in very small, variable amounts (see below). These products are insignificant with respect to electron–product balance and also cannot account for the excess CH_3NH_2. Propylene and propane have been reported as products of CH_3NC reduction (Kelly, 1968). We were unable to detect these products above background levels in any of our assays (see Materials and Methods).

CH_3NC Inhibition of Total Electron Flow. Nitrogenase turnover has been reported to be essentially independent of the substrate being reduced [e.g., Watt & Burns (1977)]. However, Figure 1 shows a plot of the rate of total electron flow through nitrogenase as a function of CH_3NC concentration and demonstrates that the substrate CH_3NC is a potent inhibitor of total electron flow. A Dixon plot of the data in Figure 1 is linear (see Figure 1 of supplementary material) and gives an apparent K_i of 158 ± 6 μM CH_3NC. That the

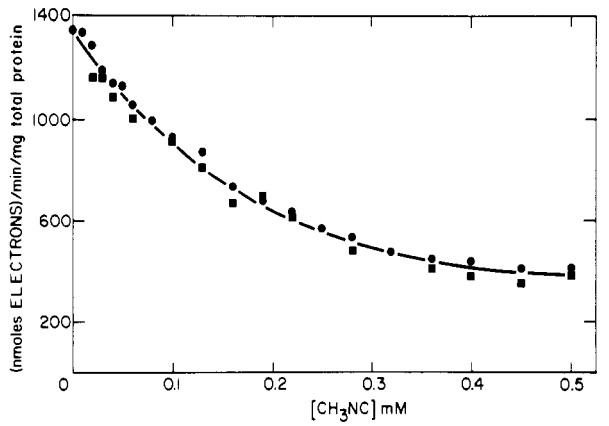

FIGURE 1: Plot of rate of total electron flow through nitrogenase vs. CH_3NC concentration: (●) nmol of electrons min^{-1} (mg of total protein)$^{-1}$ calculated from relationship $2H_2 + 6CH_4 + 4CH_3NHCH_3$ vs. [CH_3NC] in mM; (■) 2(nmol of dithionite consumed) min^{-1} (mg of total protein)$^{-1}$. Assay conditions and calculations are as described under Materials and Methods.

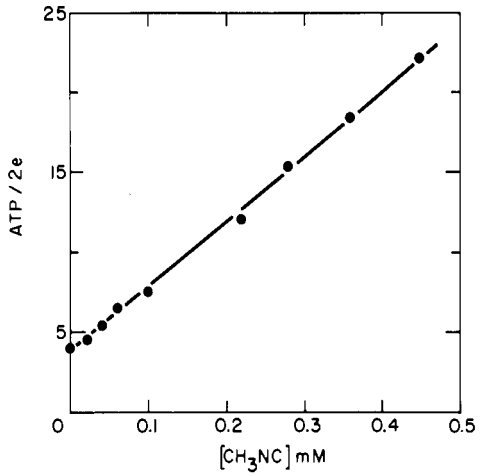

FIGURE 2: Plot showing how many MgATPs are hydrolyzed to get an electron pair transferred through nitrogenase to substrate vs. [CH_3NC]. Assay conditions and calculations are as described under Materials and Methods.

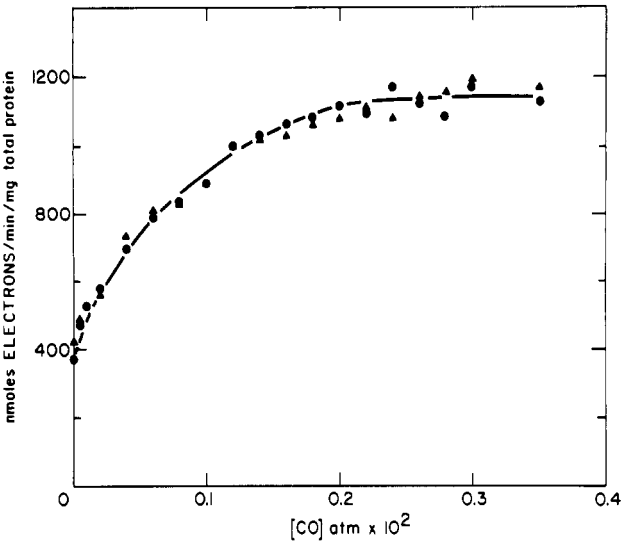

FIGURE 3: CO relief of CH_3NC inhibition: (●) nmol of electrons min^{-1} (mg of total protein)$^{-1}$ calculated from relationship $2H_2 + 6CH_4 + 4CH_3NHCH_3$ vs. [CO] in atm; (▲) 2(nmol of dithionite consumed) min^{-1} (mg of total protein)$^{-1}$. All with 0.4 mM CH_3NC. Controls with no CH_3NC present under 1 atm of Ar gave the same electron flow as experimental reactions under 1 atm of CO with 0.4 mM CH_3NC present [1300 ± 44 nmol of electrons min^{-1} (mg of total protein)$^{-1}$].

Table II: Effect of Various Substrates and Inhibitors on Electron Flow in the Presence of CH_3NC

gas phase[a]	[(nmol of electrons)/2] min^{-1} (mg of total protein)$^{-1}$ [b]
Ar	195 ± 20
H_2	195 ± 21
N_2O	319 ± 19
N_2	354 ± 26
Ar + 10 mM NaN_3	348 ± 29
C_2H_2	440 ± 42
CO	662 ± 24

[a] All at 1 atm except C_2H_2 (0.5 atm) with 0.4 mM CH_3NC (~2.5K_i). [b] $S_2O_4^{2-}$ utilization, sextuplicate.

observed inhibition involved nitrogenase and not the ATP-generating system was demonstrated by increasing the creatine phosphate, creatine phosphokinase, and MgATP concentrations 3-fold with no resultant increase in electron flow (2.5 mM CH_3NC).

CH_3NC Uncouples MgATP Hydrolysis and Electron Transfer. We have previously demonstrated that CN^- is an inhibitor of total electron flow (Li et al., 1982). The finding that CN^- also uncouples MgATP hydrolysis and electron transfer (Li et al., 1982) necessitated testing CH_3NC for its effect on the ATP/two electron ratio. Figure 2 is a plot of the number of MgATPs hydrolyzed for every electron pair consumed by nitrogenase. The data show that like CN^-, CH_3NC causes a dramatic increase in the MgATP/two electron ratio that is equivalent to a decrease in the efficiency of electron transfer through nitrogenase. It should be noted that, while the rate of electron flow through nitrogenase decreases (Figure 1), the rate of MgATP hydrolysis remains fairly constant with increasing CH_3NC at 3024 ± 241 nmol of P_i min^{-1} (mg of total protein)$^{-1}$.

Reversibility of CH_3NC Inhibition. Because CN^- inhibition of total electron flow through nitrogenase could be reversed by CO (Li et al., 1982), we tested the effect of CO on CH_3NC inhibition. Figure 3 is a plot of electron flow through nitrogenase as a function of CO concentration. The data demonstrate that the majority of the observed CH_3NC inhibition does not represent irreversible damage to nitrogenase because it can be reversed by CO. Figure 3 shows that in the presence of 0.4 mM CH_3NC, total electron flow is almost completely counteracted by 0.002–0.003 atm of CO to give electron flow nearly that expected in the absence of CH_3NC. Even if nitrogenase is allowed to turn over for 2 min under argon at 0.4 mM CH_3NC and then the atmosphere is switched to CO, total electron flow recovers at 1200 ± 22 nmol of electron min^{-1} (mg of total protein)$^{-1}$. CO relieves not only inhibition of total electron flow but also the effect on the ATP/two electron ratio. An ATP/two electron ratio of 4.02 ± 0.05 is obtained at 0.4 mM CH_3NC with 1 atm of CO present, in good agreement with the zero CH_3NC value shown in Figure 2. Surprisingly, as shown in Table II, a number of other nitrogenase substrates ($C_2H_2 > N_2 \simeq$ azide $> N_2O$) partially relieve CH_3NC inhibition of total electron flow. Only the nitrogenase inhibitor H_2 had no effect on total electron flow in the presence of CH_3NC.

In order to determine if CH_3NC had a differential effect on *Av*1 or *Av*2, experiments were performed where *Av*2 alone, *Av*1 alone, or the MgATP-generating system were preincubated with 0.4 mM CH_3NC for 15 min. Then, either the reaction was started by adding the appropriate components or the atmosphere was switched to CO (1 atm) and then the

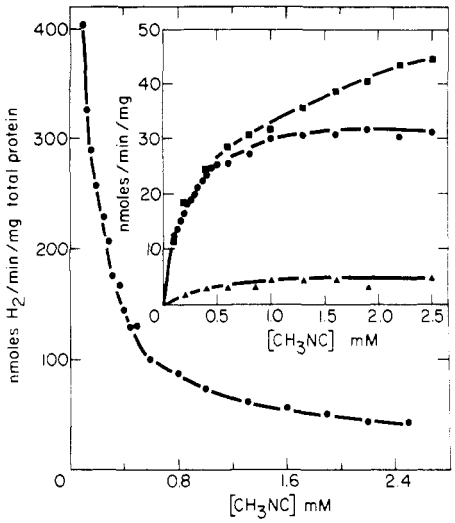

FIGURE 4: Plot of rate of H_2 evolution (●) in nmol min^{-1} (mg of total protein)$^{-1}$ vs. $[CH_3NC]$ in mM. Inset is plot of rates of CH_4 (●), CH_3NH_2 (■), and CH_3NHCH_3 (▲) formation, all in nmol min^{-1} (mg of total protein)$^{-1}$ vs. $[CH_3NC]$ in mM.

reaction was started. Electron flow was unaffected by all preincubation, before or after switching to CO. Thus, this experiment was uninformative. We also monitored changes in the reactivity of the Fe_4S_4 center in *Av*2 toward bathophenanthrolinesulfonate before and after addition of MgATP by using the method of Ljones & Burris (1978b). There was *no difference* in this reaction when *Av*2 was preincubated with 2 mM CH_3NC for 30 min, when compared to preincubation of *Av*2 in the absence of CH_3NC. We may have some evidence for slow irreversible damage to nitrogenase by high concentrations of CH_3NC because the rate of total electron flow through the enzyme decreases with time (ca. 15% in 5 min) at concentrations of substrate above about 1.8 mM. The small changes in electron flow did not affect product distribution and no corrections were applied for this observation.

CH_3NC as Substrate. Figure 4 shows all of the major products of nitrogenase turnover as a function of the CH_3NC concentration. As expected, the rates of formation of all CH_3NC reduction products increase with increasing substrate concentration with a concomitant decrease in the rate of H_2 evolution (Figure 4). Although the rate of CH_4 and CH_3NHCH_3 formation parallel each other, the rate of CH_3NH_2 formation follows a different curve. In a number of experiments, we find that the ratio of CH_3NH_2 to CH_4 is about 1:1 at CH_3NC concentrations below 1 mM. However, at substrate concentrations above 1 mM, the rate of CH_4 formation levels off while the rate of CH_3NH_2 formation continues to increase. All three products begin to decrease in the range 5–10 mM CH_3NC due to a dramatic decrease in total electron flow.

Previous attempts to determine a K_m for CH_3NC reduction relied on the rate of CH_4 formation only and did not consider the inhibition of total electron flow described above. As is shown in the inset to Figure 4, the rate of CH_4 formation does not follow Michaelis–Menten saturation kinetics. CH_4 formation appears to level off at about 1 mM CH_3NC and actually decreases at concentrations above 2.5 mM. This phenomenon has been termed substrate self-inhibition but is actually an artifact caused by CH_3NC inhibition of total electron flow. A better way to treat the data is to plot the percentage of total electrons being used to reduce CH_3NC vs. the substrate concentration. A second problem in determining the K_m is that at low CH_3NC a large proportion of the substrate is being consumed in our 5-min reactions. For example, at a 0.16 mM initial CH_3NC concentration, 57% of the substrate is consumed

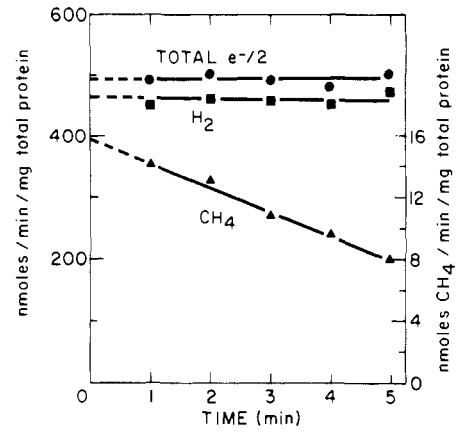

FIGURE 5: Plots of rates of electron pair consumption (●), H_2 evolution (■), and CH_4 production (▲), all in nmol min^{-1} (mg of total protein)$^{-1}$ vs. time in min. Assays in the presence of 0.08 mM CH_3NC.

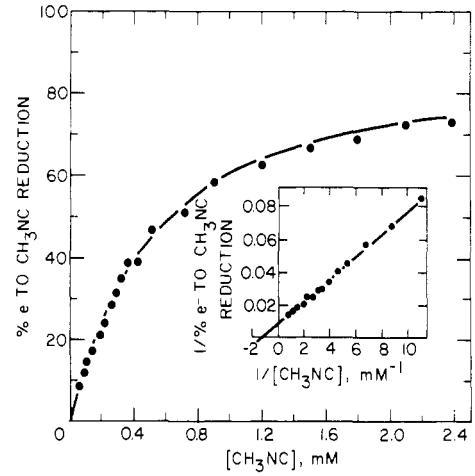

FIGURE 6: Plot of percentage of total electrons being used to produce CH_4, CH_3NH_2, and CH_3NHCH_3 vs. calculated CH_3NC concentration. CH_3NC concentration is taken as $([CH_3NC]_{initial} + [CH_3NC]_{final})/2$. Inset is double-reciprocal plot with CH_3NC concentration in the range $(0.2–2)K_m$ (Segel, 1975). The line is a computer fit to the Michaelis–Menten equation. $K_m = 0.688 \pm 0.016$ mM CH_3NC; $V_{max} = 103.5 \pm 6.7\%$. Assay conditions and calculations are as described under Materials and Methods.

in 5 min, while at 1.9 mM, only 12% is used. This problem is illustrated in Figure 5, which shows that at low CH_3NC concentrations its reduction decreased dramatically with time. A more realistic approach is to use the average CH_3NC concentration [(initial + final)/2] instead of the initial CH_3NC concentration (shown in Figure 6). If all data points in the CH_3NC concentration range (0.2–2) K_m are used, the data fit Michaelis–Menten kinetics, and an apparent K_m of 0.688 ± 0.016 mM CH_3NC is obtained (Figure 6 inset). The y intercept indicates that at infinite CH_3NC, 100% of the electrons will be used to reduce CH_3NC (i.e., H_2 evolution will be eliminated). At 10 mM CH_3NC we actually measure 86% of the electrons going to CH_3NC reduction.

C_2 Product Formation. Figure 7 shows that the rates of formation of the C_2 products do not follow saturation behavior. For both C_2H_4 and C_2H_6, their rates of formation appear second order in CH_3NC at low substrate concentrations. At higher concentrations, when the rate of formation of the C_1 product (CH_4) is saturated, the rates of formation of C_2H_4 and C_2H_6 become linear or first order in $[CH_3NC]$. These data are fit to the following general kinetic equation (Hardy, 1979):

rate of C_2 product formation =

$$k(\text{rate of } C_1 \text{ product formation})[CH_3NC] \quad (1)$$

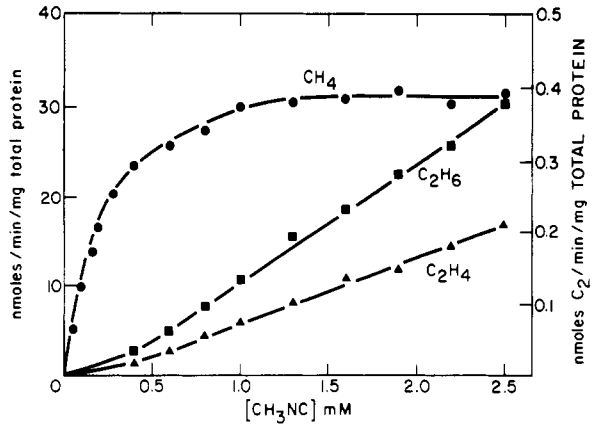

FIGURE 7: Plot of rates of formation of C_1 and C_2 products: (●) CH_4, (■) C_2H_6, and (▲) C_2H_4, all in nmol min^{-1} (mg of total protein)$^{-1}$ vs. $[CH_3NC]$ in mM.

Treating our data in this way, we obtain constant values of $k(C_2H_6) = 5.03 \pm 0.34\ M^{-1}$ and $k(C_2H_4) = 2.67 \pm 0.19\ M^{-1}$ for all CH_3NC concentrations in the range 0.2–to 10 mM. The amounts of C_2H_4 and C_2H_6 formed below 0.2 mM CH_3NC were below our level of detectability. It should be emphasized that C_2H_4 and C_2H_6 formations *do not* account for the majority of the observed excess CH_3NH_2. At 2.5 mM CH_3NC, the C_2 products only account for 10% of the excess CH_3NH_2.

Previous reports have suggested that CO stimulates the rate of C_2 product formation (Kelly, 1969a; Hardy, 1979). In contrast to these reports, we find that CO (0.05–10%) decreases the rates of formation of CH_4, C_2H_4, and C_2H_6 concomitantly, such that $k(C_2H_4)$ and $k(C_2H_6)$ are unaffected by the presence of CO.

Component Ratio Titration. Figure 8 shows the rate of product formation vs. the molar component ratio of *Av*2 to *Av*1. In this experiment, CH_3NC reduction to CH_4 + CH_3NH_2 peaks at a ratio of about 3 as does formation of C_2H_4 and C_2H_6. CH_3NC reduction to CH_3NHCH_3 peaks at a ratio of about 2 while H_2 evolution peaks at a ratio of about 4. This trend is different from that seen in previous experiments for H_2 evolution, N_2 fixation, and HD formation where all products peak at the same ratio (Wherland et al., 1981) while similar to the HCN reduction system (Li et al., 1982).

Previous studies (Silverstein & Bulen, 1970; Davis et al., 1975) with nitrogenase have demonstrated that, in general, H_2 evolution is favored over other substrate reductions (especially N_2) at low Fe/MoFe protein molar ratios where the system is electron limited. For N_2 reduction (Wherland et al., 1981), the higher the *Av*2/*Av*1 ratio the greater the percentage of total electrons going to N_2 reduction. A different trend is seen for cyanide reduction (Li et al., 1982) and methyl isocyanide reduction (Figure 2a of supplementary material), where high ratios favor H_2 evolution over HCN or CH_3NC reduction. The trend of high ratios favoring H_2 evolution over substrate reduction has previously been reported for hydrazine reduction (Wherland et al., 1981). Interestingly, the CH_3NHCH_3/CH_4 ratio also changes dramatically with the *Av*2/*Av*1 ratio. In the *Av*2/*Av*1 ratio range 1–30, the CH_3NHCH_3/CH_4 ratio decreases from about 0.2 to 0.02. No CH_3NHCH_3 is detected at higher ratios. With 1 mM CH_3NC present, the CH_3NH_2/CH_4 ratio is 0.98 ± 0.08 with no trend seen with changes in *Av*2/*Av*1. Because excess CH_3NH_2 over CH_4 is only observed at higher substrate concentrations, a component ratio titration experiment was performed at 2.5 mM CH_3NC. Again, no trend was observed with the CH_3NH_2/CH_4 ratio being 1.25 ± 0.07 regardless of the

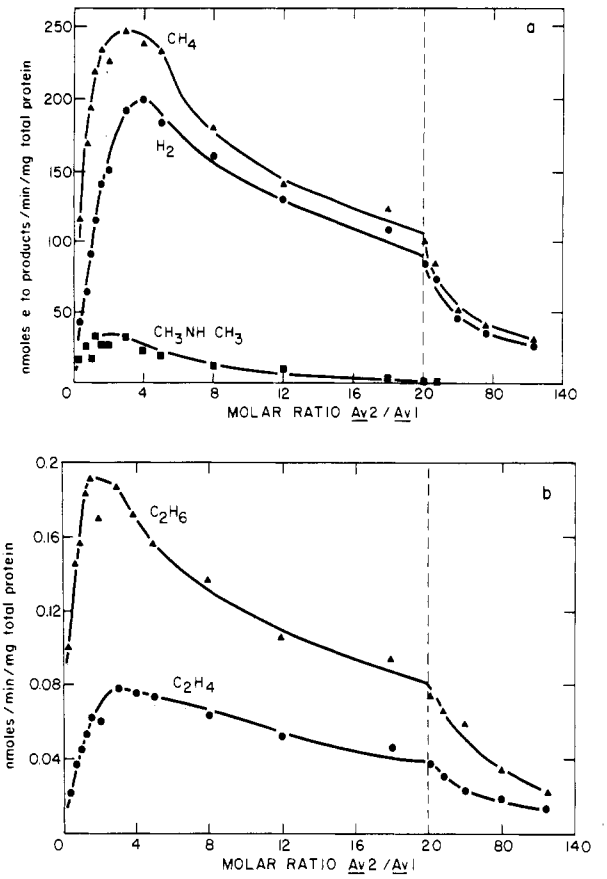

FIGURE 8: Plot of nmol of electrons to products min^{-1} (mg of total protein)$^{-1}$ vs. the molar component ratio *Av*2/*Av*1. (a) (▲) 6 CH_4, (●) 2 H_2, and (■) 4 CH_3NHCH_3. (b) (▲) C_2H_6 and (●) C_2H_4 as nmol of products. All assays and calculations are as described under Materials and Methods. Assay contained 1 mM CH_3NC.

*Av*2/*Av*1 ratio in the range 1–50.

Total electron flow shows the same smooth variation with component ratio (Figure 2b of supplemental material) as has been seen in all previous experiments (Wherland et al., 1981; Li et al., 1982). If the same data are normalized to *Av*2 and *Av*1 protein (Figure 2c of supplemental material), the general shape of these curves is very similar to that from previously reported titrations (Wherland et al., 1981; Li et al., 1982). With CH_3NC (1 mM) present, total electron flow is decreased, and the specific activities of both component proteins are concomitantly decreased. *Av*2 specific activity is only 800 [(nmol of electrons)/2] min^{-1} (mg of *Av*2)$^{-1}$ (vs. 1950 with no CH_3NC at *Av*2/*Av*1 = 0.7), and *Av*1 is only 750 [nmol of electron)/2] min^{-1} (mg of *Av*1)$^{-1}$ (vs. 2900 with no CH_3NC at *Av*2/*Av*1 = 30).

Effects of Other Substrates and Inhibitors on CH_3NC Reduction. H_2 is known to be an inhibitor of N_2 fixation. As shown in Table II, the addition of H_2 to a CH_3NC reduction system has no effect on total electron flow or on the reduction of CH_3NC to CH_4 + CH_3NH_2 (Table III). As expected, CO inhibits CH_3NC reduction with a K_i of 0.00025 ± 0.00002 atm.

Trying to determine the effect of N_2, azide, N_2O, and C_2H_2 on CH_3NC reduction is complicated by the fact that all four substrates partially relieve CH_3NC inhibition of total electron flow. Table III clearly shows that N_2, azide, and C_2H_2 inhibit CH_4 formation. These inhibitions are in fact much more dramatic if changes in total electron flow are considered and the percentages of total electron flow being used to reduce CH_3NC in the presence of N_2, azide, or C_2H_2 are compared to the percentage under Ar (Table III). Similarly, N_2O, which

Table III: Effect of Various Substrates and Inhibitors on CH_3NC Reduction (0.4 mM CH_3NC)

gas phase[a]	nmol of CH_4 min^{-1} (mg of total protein)$^{-1}$	% electrons to CH_3NC reduction
Ar	24.72 ± 1.19	38
H_2	27.17 ± 1.77	40.8
N_2O	27.98 ± 1.27	26.3
N_2	19.19 ± 0.76	16.3
Ar + 10 mM NaN_3	18.80 ± 2.10	16.2
C_2H_2	5.96 ± 0.22	4.1
CO	0.89 ± 0.06	0.4

[a] All at 1 atm except C_2H_2 (0.5 atm) with 0.4 mM CH_3NC, sextuplicate.

does not have a noticeable effect on CH_4 formation, does appear to inhibit CH_3NC reduction when N_2O relief of CH_3NC inhibition of total electron flow is considered (Table III). For the experiment in the presence of N_2, electron balance was achieved by NH_3 formed from N_2 reduction. For C_2H_2, electron balance was similarly achieved by C_2H_4 formation from C_2H_2 reduction. For azide, electron balance was achieved by N_2, N_2H_4, and NH_3 formation from azide reduction. N_2O reduction to N_2 was not directly measured but is needed to account for missing electrons.

The effects of N_2 on CH_3NC reduction were studied further by performing a CH_3NC concentration dependence (0–1.6 mM) experiment under 1 atm N_2. The presence of N_2 not only inhibited the rate of CH_4 formation but also greatly stimulated the formation of excess CH_3NH_2 (Figure 3 of supplementary material). It should be noted that more than one H_2 was evolved per N_2 fixed at all CH_3NC concentrations tested.

Discussion

One of the general features of the enzyme nitrogenase is that its turnover rate is essentially independent of the substrate being reduced. This has been demonstrated for the reductions of H^+, C_2H_2 and N_2 (Watt & Burns, 1977), azide (Dilworth & Thorneley, 1981), and HCN (Li et al., 1982) and interpreted as strong evidence that the rate-limiting step for nitrogenase turnover occurs prior to substrate reduction. The data presented here show definitively that nitrogenase turnover is *not* strictly independent of the substrate being reduced.

These data demonstrate that one substrate, CH_3NC, is a potent (K_i = 158 μM) reversible inhibitor of total electron flow through nitrogenase and, at infinite CH_3NC concentrations, all electron flow through the enzyme should cease. CH_3NC seems to be reversibly bound to nitrogenase at an inhibitory site or in an inhibitory mode [called CH_3NC-(i)] and in rapid equilibrium between free and bound forms. When CH_3NC-(i) is bound to nitrogenase, *all* substrate reductions cease. When it is not bound, total electron flow continues and electrons are distributed normally to whatever substrates are available. Previous results showing an inhibition of CH_4 formation by high concentrations of CH_3NC (Kelly, 1968; Hwang & Burris, 1972) can now be explained in terms of CH_3NC inhibition of total electron flow rather than as substrate self-inhibition.

To which protein does CH_3NC-(i) bind? CO, C_2H_2, N_2, azide, and N_2O can at least partially relieve CH_3NC inhibition of total electron flow. There is some physical evidence for CO binding to the MoFe protein (Orme-Johnson et al., 1977; Davis, et al., 1979). In addition, the MoFe protein is believed to contain the site(s) of substrate (N_2, C_2H_2, azide, and N_2O) binding and reduction (Shah et al., 1973; Hageman & Burris, 1979). Our results would therefore strongly suggest that CH_3NC-(i) exerts its inhibitory effect by binding to the MoFe protein.

CH_3NC-(i) not only inhibits total electron flow through nitrogenase but it also makes the enzyme less efficient by uncoupling MgATP hydrolysis and electron transfer to substrate. CO relieves both effects. The simplest explanation for this observation is that CH_3NC-(i) causes futile cycling of electrons between the two component proteins (Orme-Johnson et al., 1977). This would explain why CH_3NC-(i) has no apparent differential effect on the specific activities of the two component proteins. Although the inhibitory effects of CH_3NC-(i) described here and of CN^- described elsewhere (Li et al., 1982) are quite similar, there are two differences worth noting. The first is that the rate of MgATP hydrolysis is stimulated by CN^- (Li et al., 1982) while the rate of MgATP hydrolysis appears to be unaffected by CH_3NC-(i). The second is that when the other substrates, N_2, azide, N_2O, and C_2H_2, are added to nitrogenase in the presence of CN^- (~2.5 K_i), only azide partially relieves the inhibition (Li et al., 1982). With CH_3NC-(i) (~2.5 K_i), all substrates partially relieve the inhibition. It is therefore possible that, although the effects of CN^- and CH_3NC-(i) are quite similar, they exert their effects on binding to different sites. CO relieves inhibition caused by both, and there is evidence for two CO binding sites on nitrogenase (Davis et al., 1979). Azide, which is present as two species in solution (HN_3 and N_3^-) also relieves both inhibitions and thus may bind to more than one site on nitrogenase.

Mechanism of CH_3NC Reduction. In addition to being an inhibitor of nitrogenase, CH_3NC is also a substrate [called CH_3NC-(s)]. We demonstrate that nitrogenase catalyzes the following reactions:

$$CH_3NC\text{-(s)} + 4e^- + 4H^+ \rightarrow CH_3NHCH_3 \quad (2)$$

$$CH_3NC\text{-(s)} + 6e^- + 6H^+ \rightarrow CH_3NH_2 + CH_4 \quad (3)$$

At an $Av2/Av1$ ratio of 8, the stoichiometry of (2) to (3) is 0.16:1. The fact that this stoichiometry is independent of CH_3NC concentration suggests that (2) and (3) occur in a common pathway at the same site. Changing the $Av2/Av1$ ratio should influence the rate at which the next two electrons become available, and this should change the distribution of (2) to (3). The effect is, indeed, observed, and it occurs in the expected direction with pathway 2 being favored at low $Av2/Av1$ ratios and pathway 3 being highly favored at high ratios.

Our data show that at high concentrations of CH_3NC-(s), the ratio of CH_3NH_2 is greater than the anticipated 1:1. One explanation that is consistent with our electron-balance studies is that the excess CH_3NH_2 is formed by

$$CH_3NC\text{-(s)} + 2e^- + 2H^+ \rightarrow CH_3NHCH \xrightarrow{H_2O} HCHO + CH_3NH_2 \quad (4)$$

This explanation is offered by analogy to the HCN system (Li et al., 1982) although, unlike that system, further support for it was not found in our component ratio titration experiments. If this explanation is correct, then CH_3NC or N_2 (other substrates were not tested) must be able to displace the proposed two-electron-reduced intermediate, causing its rate of hydrolysis to be accelerated with increasing CH_3NC or N_2. This would further mean that the active site must be large enough to accommodate a bound two-electron intermediate and a free, incoming CH_3NC (or N_2). A second possibility for the excess CH_3NH_2 is a two-step hydrolysis reaction whereby CH_3NC is first hydrolyzed to *N*-methylformamide and then to CH_3NH_2 and HCO_2H. It is difficult to explain,

however, why such a two-step hydrolysis reaction occurs *only* when the complete turning-over nitrogenase system is present and why it would be stimulated by increasing CH_3NC or N_2.

Previous studies (Kelly, 1968) have demonstrated that nitrogenase also catalyzes the following reactions:

$$2CH_3NC^* + 8e^- + 8H^+ \rightarrow **C_2H_4 + 2CH_3NH_2$$

$$2CH_3NC^* + 10e^- + 10H^+ \rightarrow **C_2H_6 + 2CH_3NH_2$$

Our studies of the rates of formation of CH_4, C_2H_4, and C_2H_6 strongly support the reduction followed by an insertion mechanism proposed by Hardy (1979) and are inconsistent with a mechanism whereby two bound C_1 radicals react to form the C_2 products (Kelly et al., 1967). This mechanism (Hardy, 1979) predicts that, during the normal course of the reduction of CH_3NC-(s) by six electrons, the product CH_3NH_2 should be released prior to the product CH_4. It would be of interest to test this prediction by studying pre-steady-state kinetics of the system. The reduction followed by an insertion mechanism also predicts that the active site is large enough to accommodate a bound C_1 radical and an incoming CH_3NC and the insertion product. Our results show that CO decreases the rates of CH_4, C_2H_4, and C_2H_6 formation concomitantly and are inconsistent with a CO insertion mechanism (Hardy, 1979). Previous results showing CO stimulation of C_2 product formation (Kelly, 1967 and 1969a) might be attributed to failure to account accurately for contaminating C_2 impurities in CO. We were also unable to confirm the reported formation of C_3 products (Kelly, 1968).

Redox States. It has been suggested that at low ratios of Fe protein to MoFe protein, when electron flux is decreased, the reduction of two-electron substrates is favored over the reduction of six-electron substrates (Silverstein & Bulen, 1970; Davis et al., 1975). Although this is certainly true for competition between N_2 fixation and H_2 evolution [e.g., Wherland et al. (1981)], it does not appear to be true for the competition of other six-electron substrates with H_2 evolution. Our data show that CH_3NC reduction is maximally favored over H_2 evolution at low $Av2/Av1$ ratios as has previously been seen for HCN reduction (Li et al., 1982). This phenomenon also appears to be true for the six-electron reduction of azide to N_2H_4 and NH_3 by *Kp* nitrogenase (Dilworth & Thorneley, 1981). These observations support a mechanism whereby CH_3NC-(s) is reduced at a redox state of nitrogenase more oxidized than that reactive toward H_2 evolution or N_2 reduction. A recently proposed mechanism (Thorneley & Lowe, 1982) allows for the interaction of various substrates with different redox states of nitrogenase. It will be of interest to fit the CH_3NC component ratio titration data shown here to that mechanism.

There appear to be two ways in which the six-electron reduction of N_2 differs significantly from the six-electron reductions of other substrates (e.g., CH_3NC, HCN, and azide). One, described above, is the effects of changing the component protein ratio on the distribution of electrons between H_2 evolution and the six-electron reduction. The second is that at infinite concentrations of CH_3NC-(s), HCN (Li et al., 1982), and azide (Dilworth & Thorneley, 1981), H_2 evolution can be eliminated while at infinite concentrations of N_2, H_2 evolution continues [e.g., Rivera-Ortiz & Burris (1975)]. It is likely that these two observations are related.

Relationship of Binding Sites. The finding that CH_3NC can serve as both a substrate and an inhibitor of nitrogenase means either that there are two binding sites for CH_3NC on the enzyme or that CH_3NC can bind in both a productive (substrate) and nonproductive (inhibitor) mode to the same site. Our observation is that a number of nitrogenase substrates ($C_2H_2 > N_2 \simeq$ azide $> N_2O$) can all both relieve CH_3NC-(i) inhibition and inhibit CH_3NC-(s) reduction and in the same order. Because there is no evidence for multiple binding sites for these substrates, we prefer a mechanism whereby CH_3NC serves as either substrate or inhibitor on binding to the same site of nitrogenase. It should be noted that the K_i for CH_3NC-(i) is about 4-fold lower than the K_m for CH_3NC-(s). It is hoped that biophysical techniques may soon provide some direct evidence concerning the sites of interaction of CH_3NC and other substrates and inhibitors on nitrogenase.

Summary

In summary, our studies have shown the following: (1) CH_3NC-(i) is a potent reversible inhibitor (K_i = 158 μM) of total electron flow and appears to uncouple MgATP hydrolysis and electron transfer to substrate; (2) the previously reported self-inhibition by CH_3NC is artifactual; (3) CH_3NC-(s) is a substrate that is reduced (six electrons) to $CH_4 + CH_3NH_2$, (four electrons) to CH_3NHCH_3, and to more CH_3NH_2 possibly via hydrolysis of a two-electron intermediate; (4) at infinite concentrations, CH_3NC-(s) can eliminate H_2 evolution; (5) CH_3NC-(s) appears to be reduced at a state of nitrogenase that is more oxidized than the state responsible for H_2 evolution or N_2 fixation; (6) C_2H_4 and C_2H_6 appear to be formed via a reduction followed by an insertion mechanism; (7) $C_2H_2 > N_2 \simeq$ azide $> N_2O$ can all both relieve CH_3NC-(i) inhibition and inhibit CH_3NC-(s) reduction and in the same order, implying productive and nonproductive modes of binding of CH_3NC to one site.

Acknowledgments

We are pleased to acknowledge the talents of Deloria Jacobs and Dorothy Lyons in cell growth and protein purification. We also thank Drs. John McDonald, Gary Watt, Franklin Schultz, and William Newton for helpful discussions.

Supplementary Material Available

Seven tables showing all results in terms of number of replicates, means, and standard deviations and three figures (11 pages). Ordering information is given on any current masthead page.

Registry No. CH_3NC, 593-75-9; N_2O, 10024-97-2; N_2, 7727-37-9; NaN_3, 26628-22-8; C_2H_2, 74-86-2; CO, 630-08-0; CH_4, 74-82-8; CH_3NH_2, 74-89-5; CH_3NHCH_3, 124-40-3; H_2, 1333-74-0; nitrogenase, 9013-04-1.

References

Biggins, D. R., & Postgate, J. R. (1969) *J. Gen. Microbiol. 56*, 181–193.

Blair, D., & Diehl, H. (1961) *Talanta 7*, 163–174.

Braaksma, A., Haaker, H., Grande, H. J., & Veeger, C. (1982) *Eur. J. Biochem. 121*, 483–491.

Bulen, W. A., & LeComte, J. R. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 979–986.

Burgess, B. K., Jacobs, D. B., & Stiefel, E. I. (1980) *Biochim. Biophys. Acta 614*, 196–209.

Burgess, B. K., Wherland, S., Stiefel, E. I., & Newton, W. E. (1981) *Biochemistry 20*, 5140–5146.

Corbin, J. L. (1978) *Anal. Biochem. 84*, 340–342.

Davis, L. C., Shah, V. K., & Brill, W. J. (1975) *Biochim. Biophys. Acta 403*, 67–78.

Davis, L. C., Henzl, M. T., Burris, R. H., & Orme-Johnson, W. H. (1979) *Biochemistry 18*, 4860–4869.

Dilworth, M. J., & Thorneley, R. N. F. (1981) *Biochem. J. 193*, 971–983.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 5103–5108.
Hageman, R. V., & Burris, R. H. (1978a) *Biochemistry 17*, 4117–4124.
Hageman, R. V., & Burris, R. H. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2699–2702.
Hageman, R. V., & Burris, R. H. (1979) *J. Biol. Chem. 254*, 11189–11192.
Hardy, R. W. F. (1979) in *A Treatise on Dinitrogen Fixation* (Hardy, R. W. F., Bottomley, F., & Burns, E. C., Eds.) pp 515–568, Wiley, New York.
Hardy, R. W. F., & Jackson, E. K. (1967) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 26*, 725.
Hwang, J. C., & Burris, R. H. (1972) *Biochim. Biophys. Acta 283*, 339–350.
Hwang, J. C., Chen, C. H., & Burris, R. H. (1973) *Biochim. Biophys. Acta 292*, 256–270.
Karwelk, D. H., & Meyers, C. H. (1979) *Anal. Chem. 51*, 319–320.
Kelly, M. (1968) *Biochem. J. 107*, 1–6.
Kelly, M. (1969a) *Biochim. Biophys. Acta 171*, 9–22.
Kelly, M. (1969b) *Biochim. Biophys. Acta 191*, 527–540.
Kelly, M., Postgate, J. R., & Richards, R. L. (1967) *Biochem. J. 102*, 1c–3c.
Li, J.-G., Burgess, B. K., & Corbin, J. L. (1982) *Biochemistry 21*, 4393–4402.
Lindroth, P., & Mopper, K. (1979) *Anal. Chem. 51*, 1667–1674.
Ljones, T., & Burris, R. H. (1978a) *Biochem. Biophys. Res. Commun. 80*, 22–25.
Ljones, T., & Burris, R. H. (1978b) *Biochemistry 17*, 1866–1872.
Mortenson, L. E., & Thorneley, R. N. F. (1979) *Annu. Rev. Biochem. 48*, 387–418.
Munson, T. O., & Burris, R. H. (1969) *J. Bacteriol. 97*, 1093–1098.
Orme-Johnson, W. H., Davis, L. C., Henzl, M. T., Averill, B. A., Orme-Johnson, N. R., Münck, E., & Zimmerman, R. (1977) in *Recent Development in Nitrogen Fixation* (Newton, W. E., Postgate, J. R., & Rodriguez-Barrueco, C., Eds.) pp 131–178, Academic Press, London.
Reisberg, P. I., & Olson, J. S. (1980) *J. Biol. Chem. 255*, 4144–4150.
Rivera-Ortiz, J. M., & Burris, R. H. (1975) *J. Bacteriol. 123*, 537–545.
Roth, M. (1971) *Anal. Chem. 43*, 880–882.
Segel, I. H. (1975) *Enzyme Kinetics*, p 46, Wiley-Interscience, New York.
Seiler, N. (1970) *Methods Biochem. Anal. 18*, 318.
Shah, V. K., Davis, L. C., Gordon, J. K., Orme-Johnson, W. H., & Brill, W. J. (1973) *Biochim. Biophys. Acta 292*, 246–255.
Sidgwick, N. V. (1937) *The Organic Chemistry of Nitrogen*, p 318, Oxford University Press, London.
Silverstein, R., & Bulen, W. A. (1970) *Biochemistry 9*, 3809–3815.
Smith, B. E. (1983) *in Nitrogen Fixation: The Chemical-Biochemical-Genetic Interface* (Müller, A., & Newton, W. E., Eds.) pp 23–62, Plenum Press, New York.
Taylor, S., Ninjor, V., Dowd, D. M., & Tappel, A. L. (1974) *Anal. Biochem. 60*, 153–162.
Thorneley, R. N. F., & Lowe, D. J. (1982) *Isr. J. Bot. 31*, 1–11.
Van Hoof, F., & Heyndrickx, A. (1974) *Anal. Chem. 46*, 286–288.
Watt, G. D., & Burns, A. (1977) *Biochemistry 16*, 264–270.
Wherland, S., Burgess, B. K., Stiefel, E. I., & Newton, W. E. (1981) *Biochemistry 20*, 5132–5140.