N_2O as a Substrate and as a Competitive Inhibitor of Nitrogenase†

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ABSTRACT: We have investigated the inhibitory effect of N_2O on NH_3 formation by purified component proteins from *Klebsiella pneumoniae* and have confirmed that the inhibition is competitive with respect to N_2 and that N_2O is reduced to N_2, which in turn is further reduced to NH_3. In addition, we have shown that N_2O is unable to support HD formation from D_2 and H_2O. N_2-supported HD formation from D_2 and H_2O was found to be inhibited by N_2O. In contrast to N_2, N_2O was found to suppress nitrogenase-mediated H_2 evolution completely at infinitely high pN_2O. H_2 was found to inhibit N_2O-supported NH_3 production but not N_2O-supported N_2 production. The steady-state kinetics of N_2O reduction showed a good fit to Michaelis–Menten kinetics with a K_m for N_2O of 5 mM at 30 °C, corresponding to 24 kPa of N_2O. A model is proposed that fits the observed results.

Nitrogenase, consisting of dinitrogenase (Mo–Fe protein, component 1) and dinitrogenase reductase (Fe protein, component 2), reduces a variety of substrates in addition to N_2. The reactions require MgATP (McNary & Burris, 1962) and a reductant (Mortenson, 1964). The proteins are similar from various prokaryotes (Emerich & Burris, 1978), and their properties have been reviewed by Mortenson and Thorneley (1979). Dinitrogenase reductase is a one-electron donor (Ljones & Burris, 1978) specific for dinitrogenase, and dinitrogenase after reduction by dinitrogenase reductase effects the reduction of N_2 and other substrates.

Mozen and Burris (1954) showed that cultures of *Azotobacter vinelandii* and soybean nodules assimilated ^{15}N when incubated with $^{15}N_2O$, an observation that constituted the first evidence that any compound other than N_2 could be reduced by nitrogenase. Hoch et al. (1960) showed that N_2O was reduced to N_2, which in turn was reduced further before assimilation. Formation of N_2 from N_2O was confirmed later by Hardy and Knight (1966), and they suggested that N_2O was reduced to N_2 and H_2O. N_2O is the only substrate for nitrogenase (unless one considers H_2 a substrate in the HD reaction) that has been shown to be a competitive inhibitor of N_2 reduction (Repaske & Wilson, 1952; Rivera-Ortiz & Burris, 1975). All other substrates (C_2H_2, HCN, HN_3, etc.) have been reported to inhibit N_2 reduction in a noncompetitive manner.

In contrast to N_2 reduction, which is inhibited by H_2 (Wilson & Umbreit, 1937), Hoch et al. (1960) reported that H_2 did not inhibit N_2O reduction by soybean root nodules. Also in contrast to N_2, N_2O has been reported to be unable to support nitrogenase-catalyzed HD formation from D_2 and H_2O (Hoch et al., 1960; Jackson et al., 1968), so in that respect N_2O resembles azide, acetylene, cyanide, methyl isocyanide (Jackson et al., 1968), and hydrazine (Newton et al., 1977), all of which also are substrates of nitrogenase.

In this paper we will describe recent studies on the reduction of N_2O by purified component proteins of nitrogenase from *Klebsiella pneumoniae*.

Materials and Methods

Growth of Bacteria and Purification of Dinitrogenase. *K. pneumoniae* M5aL was grown at 30 °C in 300-L nitrogen-sparged batches in a fermentor on the medium described by Eady et al. (1972). The cells were harvested in the exponential phase by continuous centrifugation, frozen, and stored in liquid nitrogen. Nitrogenase was prepared from frozen cell paste as described by Li & Burris (1983). The specific activity of Kp1[1] was 1600–2000 nmol of C_2H_4 formed min^{-1} (mg of Kp1)$^{-1}$. The specific activity of Kp2 was 1100–1400 nmol of C_2H_4 min^{-1} (mg of Kp2)$^{-1}$. No hydrogenase activity (measured as ATP-independent H_2 evolution from dithionite and methylviologen) could be detected in the purified *K. pneumoniae* nitrogenase preparations.

Unless otherwise stated, all experiments were performed with a 4-fold molar excess of dinitrogenase reductase (Fe protein, Kp2) to dinitrogenase (Mo–Fe protein, Kp1). We assumed the molecular weights of Kp1 (210 000) and Kp2 (66 800) given by Eady (1980). Protein was measured by the microbiuret method as described by Goa (1953).

Nitrogenase Assays. Unless otherwise stated, all reactions were carried out at 30 °C in 9-mL vials fitted with vaccine stoppers. The volume of the reaction mixture was 1 mL containing 5 μmol of ATP, 10 μmol of $MgCl_2$, 40 μmol of creatine phosphate, 0.1 mg of creatine phosphokinase (EC 2.7.3.2), 20 μmol of $Na_2S_2O_4$, 50 μmol of MOPS, adjusted to pH 7.0 with KOH, and nitrogenase proteins as indicated.

C_2H_2 Reduction. C_2H_2 reduction assays were performed in either 9- or 23-mL bottles fitted with serum and vaccine stoppers, respectively. The gas phase was 10% C_2H_2 in argon. C_2H_2 was generated by addition of CaC_2 to H_2O in a gas-generator bottle (Burris, 1974). Assays were initiated by addition of enzyme and terminated with 0.3 mL of 25% (w/v) trichloroacetic acid. Gas samples (0.5 mL) were removed with 1.0-mL plastic syringes, and ethylene was measured on a

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[1] Abbreviations: Kp1 and Kp2, dinitrogenase and dinitrogenase reductase from *K. pneumoniae*; MOPS, 4-morpholinepropanesulfonic acid.

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Varian 600D gas chromatographic unit equipped with a flame ionization detector and a column of Porapak N, operated at 50 °C with N_2 as carrier gas.

HD Determinations. In our experiments, four products were formed: N_2, H_2, HD, and NH_3, of which H_2, HD, and NH_3 were measured in each reaction vessel and N_2 was measured when pertinent. The volume of the gas phase was 8.2 mL. Ar was added through a glass manifold fitted with stopcocks and 22-gauge needles; N_2O (Matheson Gas Products, CP grade, freed of O_2 by bubbling the gas through a buffer solution containing 1 mM $Na_2S_2O_4$), N_2, and D_2 were removed from storage vessels over mercury and were added to the serum bottles with plastic syringes. The pN_2, pN_2O, and pD_2 in kilopascals (kPa; 100 kPa is equivalent to 750 torr) were calculated as 100 times the ratio of added volume to 8.2 mL, multiplied by the ratio of atmospheric pressure to 750 torr and multiplied by the ratio of 303 K to room temperature. pAr was calculated by applying these pressure and temperature corrections to the pAr as measured by a Hg manometer on the manifold. After addition of gases, $Na_2S_2O_4$ was added, and the bottles were incubated at 30 °C on a shaker for 5–10 min to equilibrate the temperature and to scavenge traces of O_2 from the gas phase. Reactions were initiated by addition of enzyme mixture and terminated by addition of 0.3 mL of 25% (w/v) trichloroacetic acid. Unless otherwise stated, the reaction time was 30 min; reactions were linear for this period.

If D_2 was not present in the gas phase, H_2 was determined in a 0.5-mL gas sample by gas chromatography with a thermal conductivity detector and a column of molecular sieve 5A. Ar was used as carrier gas, and column temperature was 50 °C. The gas chromatograph was calibrated with cylinder H_2 diluted into argon. If D_2 was present, H_2, HD, and D_2 were determined by injection of 0.1–0.3 mL of gas into a Finnigan MAT 250 isotope ratio mass spectrometer, and the gases were analyzed and calculated as described by Guth & Burris (1983). Then NH_3 was determined in each reaction vessel as described below.

Ammonia Determination. One milliliter of 4.5 M K_2CO_3 was added to the reaction mixtures to initiate the microdiffusion of NH_3 to glass rods previously dipped in 1 M H_2SO_4 (Burris, 1972). After overnight microdiffusion, NH_3 was assayed by the indophenol method of Chaykin (1969). A_{625} was measured after incubation with the indophenol reagent at room temperature for 1 h. NH_4Cl was used as standard.

N_2 Determinations. N_2 was determined in a 0.5-mL gas sample by gas chromatography with a thermal conductivity detector and a column of molecular sieve 5A (limit of detection was 0.1% N_2 in N_2O). Argon was used as carrier gas, and the column temperature was 50 °C. The gas chromatograph was calibrated with air diluted into argon.

Results

Effect of pN_2O on H_2 Evolution, HD Formation, and NH_3 Production. Figure 1 shows an experiment in which the rates of H_2 evolution, HD formation, and NH_3 production by *K. pneumoniae* nitrogenase were measured as influenced by the pN_2O at a fixed value of pD_2 (15 kPa). Increasing the pN_2O caused the rate of H_2 evolution to decrease, but this decrease in H_2 production was not matched by a complementary increase in either HD formation or NH_3 production. A similar experiment to that shown in Figure 1 was made with a fixed value of 50 rather than 15 kPa of D_2. The HD and NH_3 production rates from that experiment together with the results from Figure 1 are shown in Figure 2 (y axis expanded). Both NH_3 production and HD formation increased with increasing pN_2O, reached a peak, and then decreased again with further

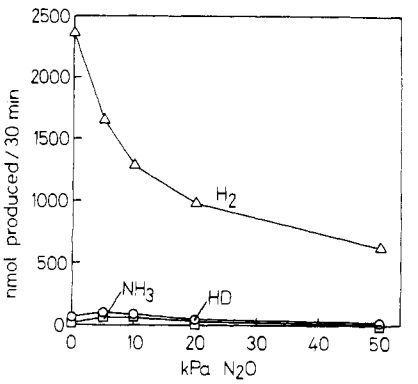


FIGURE 1: Effect of pN_2O on H_2 evolution, HD formation, and NH_3 production at 15 kPa of D_2 by nitrogenase from *K. pneumoniae*. Reactions were performed in 9.5-mL serum bottles at 30 °C as described under Materials and Methods. Argon was used as diluent gas. Reactions were begun by addition of 75 μg each of Kp1 and Kp2 and terminated after 30 min by addition of trichloroacetic acid.

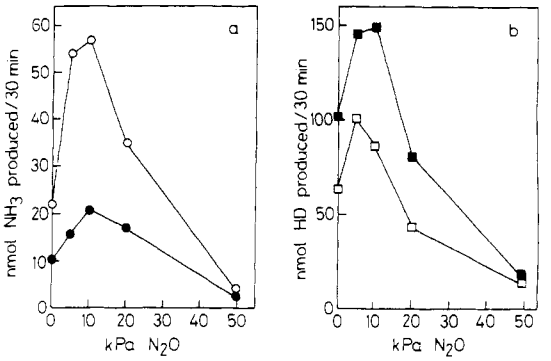


FIGURE 2: Effect of pN_2O on NH_2 production (a) and on HD evolution (b) at two fixed pressures of pD_2 (15 kPa, open symbols; 50 kPa, closed symbols). Conditions were as described in the legend to Figure 1 and under Materials and Methods.

increase in the pN_2O. Substantial HD formation and NH_3 production were detected in the samples without any N_2O. Li & Burris (1983) showed that HD formation could be reduced to less than 1% of the total electron flux if all gases were scrubbed free of N_2 by passage over molecular sieve at liquid N_2 temperature and if experiments were performed in an all-glass system. Using this method, we consistently obtained HD formation rates as low as 1% of the total electron flux under 100% D_2. When using rubber-stoppered serum bottles, however, we observed 5–10% of the electron flux as HD, even with scrubbed gases. We therefore consider the HD formation and NH_3 production detected in the samples without N_2O in Figure 2 to be N_2-dependent and supported by N_2, which is a contaminant of cylinder gases and which leaks into the serum bottles via the rubber stoppers.

Figure 2 also shows that the N_2O-dependent NH_3 production and HD evolution differ with respect to their dependence on D_2. Whereas the HD formation rate is highest at the high pD_2 (50 kPa), the NH_3 production rate is highest at the lower pD_2 (15 kPa).

We investigated further the N_2O dependence of H_2, NH_3, and HD formation with *K. pneumoniae* nitrogenase in an experiment in which fixed pressures of both D_2 (50 kPa) and N_2 (50 kPa) were present in addition to N_2O; our results are illustrated in Figure 3. Whereas only a small increase in pN_2O caused a decrease in the HD and NH_3 production rate, the H_2 production rate was constant up to 20 kPa of N_2O and then started to decrease on further increase in the pN_2O.

CO had been shown to inhibit formation of HD and NH_3 by nitrogenase from *K. pneumoniae* identically and essentially

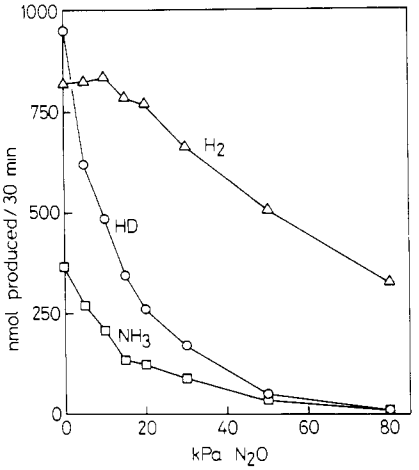


FIGURE 3: Effect of pN_2O on H_2, NH_3, and HD formation by nitrogenase from *K. pneumoniae* in the presence of fixed pressures of both D_2 (50 kPa) and N_2 (50 kPa). Conditions were as described in the legend to Figure 1 and under Materials and Methods.

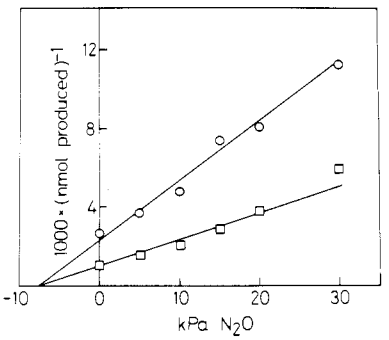


FIGURE 4: Dixon plot of the HD and NH_3 production rates obtained in the experiment shown in Figure 3. Symbols are as in Figure 3.

completely (Guth & Burris, 1983). A similar plot of our results (not shown) indicated that N_2O inhibited formation of both HD and NH_3 completely, although it was not clear whether the inhibitions were identical in nature. A replot of the results as a Dixon plot is shown in Figure 4, and it indicates that formation of HD and NH_3 are inhibited in the same way [$K_i(N_2O)$ values were identical]. The way in which HD and NH_3 production are in fact inhibited by N_2O was tested in two series of experiments. In the first type of experiment the NH_3 production rate was measured as a function of pN_2 at four fixed values of pN_2O (0, 5, 10, and 20 kPa). The results from these experiments are plotted as a double-reciprocal (Lineweaver–Burk) plot in Figure 5.

In the second set of experiments HD formation was measured as a function of pD_2 in the presence of 30 kPa of N_2 and four different fixed values of pN_2O (0, 5, 10, and 20 kPa). The results from these experiments are plotted as a Lineweaver–Burk plot in Figure 6.

Figure 5a confirms that N_2O is a competitive inhibitor of N_2 reduction. A plot of the slope of each reciprocal plot vs. the inhibitor concentration at which it was obtained gave a straight line (Figure 5b), from which a $K_m(N_2)$ for nitrogen fixation of 9 kPa of N_2 and a $K_i(N_2O)$ for nitrogen fixation of 12 kPa of N_2O were obtained.

The double-reciprocal plot of the results from the HD experiments (Figure 6) did not give a clear answer as to whether HD formation is inhibited competitively or noncompetitively by N_2O (the $1/v$ vs. $1/S$ plot obtained is characteristic for mixed-type inhibition), although noncompetitive inhibition appears dominant. All our experiments were carried out in

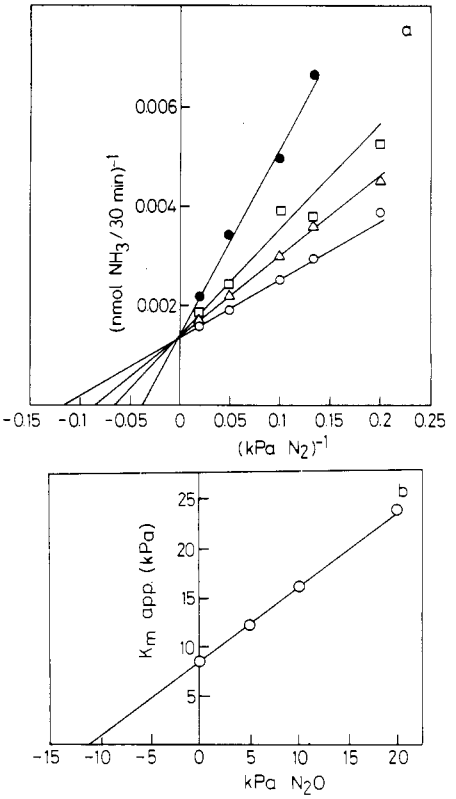


FIGURE 5: (a) Double-reciprocal (Lineweaver–Burk) plot of $1/v$ vs. $1/[N_2]$ for NH_3 production by nitrogenase from *K. pneumoniae* in the presence of different fixed values of pN_2O (○, 0 kPa of N_2O; △, 5 kPa of N_2O; □, 10 kPa of N_2O; ●, 20 kPa of N_2O). Experimental details were as described in the legend to Figure 1 and under Materials and Methods. (b) Plot of the slope of each reciprocal plot from panel a vs. the inhibitor concentration at which it was obtained.

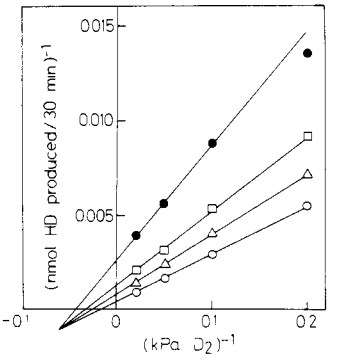


FIGURE 6: Double-reciprocal (Lineweaver–Burk) plot of $1/v$ vs. $1/pD_2$ for the HD production rate by nitrogenase from *K. pneumoniae* in the presence of different fixed concentrations of N_2O (○, 0 kPa of N_2O; △, 5 kPa of N_2O; □, 10 kPa of N_2O; ●, 20 kPa of N_2O). Experimental details were as described in the legend to Figure 1 and under Materials and Methods.

the presence of 30 kPa of N_2. We reported previously (Jensen & Burris, 1985) that HD formation is subject to competitive substrate inhibition by N_2. That means that 30 kPa of N_2 in fact inhibits HD formation at small D_2 concentrations, so one should not expect to get an unambiguous Lineweaver–Burk plot.

Can N_2O Completely Suppress H_2 Evolution by Nitrogenase? In the absence of other substrates nitrogenase reduces protons to H_2 (Bulen et al., 1965), and this H_2 evolution cannot be completely suppressed by N_2, although substrates such as C_2H_2 and HCN can completely suppress H_2 evolution at substrate concentrations extrapolated to infinity (Rivera-Ortiz & Burris, 1975; Simpson & Burris, 1984).

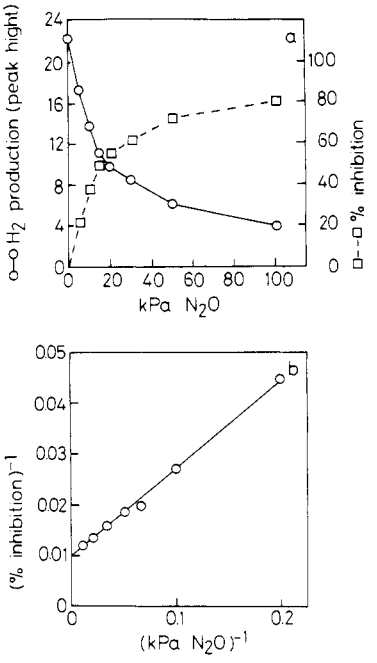


FIGURE 7: (a) Inhibition by N_2O of H_2 evolution by nitrogenase from *K. pneumoniae*. Experimental details are given in the legend to Figure 1, and the nature of the dashed curve is described in the text. (b) Double-reciprocal plot of the derived curve (percent inhibition curve) from panel a (see text for further details).

We next ran the experiment shown in Figure 7 to test whether N_2O is able to suppress H_2 evolution from nitrogenase completely. This figure shows that H_2 evolution continues at about 20% of its maximal rate even in the presence of 100 kPa of N_2O. Replotting the data as the difference between the maximum H_2 production rate (without N_2O) and the rate obtained at the different pN_2O's vs. the pN_2O at which they were obtained gave a curve corresponding to the mirror image of the solid curve shown. This curve appears like a substrate concentration vs. rate curve (dashed curve in Figure 7a). Analysis of this derived curve by a $1/v$ vs. $1/pN_2O$ plot (Figure 7b) defines the velocity (y intercept) in relative terms at infinite pN_2O. As can be seen, the intercept on the (% inhibition)$^{-1}$ axis is 0.01, indicating a 100% inhibition of H_2 evolution at infinitely high pN_2O.

Effect of H_2 on Nitrogenase-Catalyzed N_2 Release and NH_3 Production from N_2O. H_2 is a specific inhibitor of N_2 reduction by nitrogenase. In the experiment shown in Figure 8 we have investigated the effect of H_2 on N_2O reduction by nitrogenase. A series of experiments were made in which the production of N_2 and NH_3 were measured as functions of pH_2 at two fixed values of pN_2O (20 and 50 kPa). The N_2 release rate of both 20 and 50 kPa of N_2O is independent of H_2, but the NH_3 production rate clearly is inhibited by H_2. Figure 8 also shows that N_2O-dependent N_2 release and NH_3 production differ with respect to their dependence on pN_2O. Whereas the N_2 release rate is highest at the high pN_2O (50 kPa), the NH_3 production rate is highest at the lower pN_2O (20 kPa).

In interpreting these data, one must bear in mind that (a) N_2O is a source of N_2 as well as a specific, competitive inhibitor of N_2 reduction and (b) H_2 is a specific, competitive inhibitor of N_2 reduction. In Figure 8a the N_2O is serving its role as a source of N_2, and this reaction is not inhibited by H_2; more N_2 is produced at 50 kPa of N_2O than at 20 kPa N_2O. In Figure 8b the role of N_2O as a specific inhibitor of N_2 reduction is dominant. Note that at 0 pH_2 the inhibition of NH_3 formation is greater at 50 kPa of N_2O than at 20 kPa

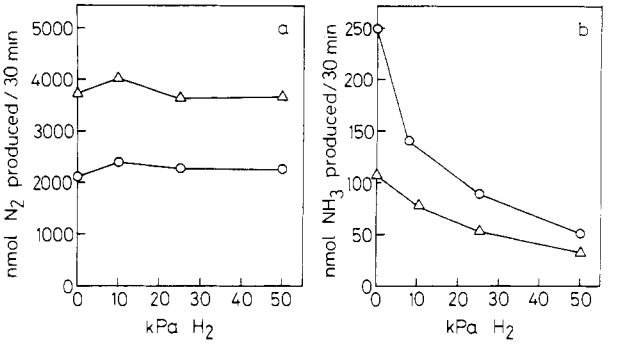


FIGURE 8: Inhibition by H_2 of N_2O reduction by nitrogenase from *K. pneumoniae*. (a) Effect of H_2 on N_2 production from N_2O at two fixed values of pN_2O (o, 20 kPa of N_2O; Δ, 50 kPa of N_2O). (b) Effect of H_2 on NH_3 production from N_2O at two fixed values of pN_2O (o, 20 kPa of N_2O; Δ, 50 kPa of N_2O). Experimental details are described under Materials and Methods and in the legend to Figure 1, except that 200 μg each of Kp1 and Kp2 was used.

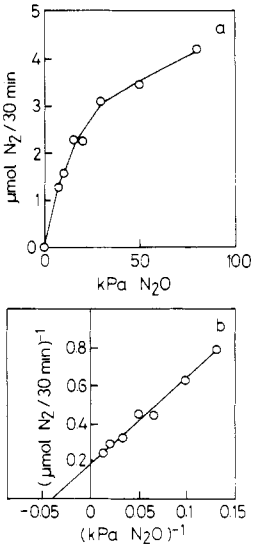


FIGURE 9: Steady-state kinetics of N_2O reduction by nitrogenase from *K. pneumoniae*. A total of 100 μg of Kp1 and 200 μg of Kp2 was used. (a) Influence of the pN_2O on the rate of nitrogenase-catalyzed release of N_2 from N_2O. (b) Double-reciprocal (Lineweaver–Burk) plot of the data from panel a.

of N_2O; i.e., the role of N_2O as inhibitor dominates its role as a producer of N_2 as a substrate. The effect of H_2 as an inhibitor of N_2 fixation (Figure 8b) is superimposed on the inhibition by N_2O, and the percentage effect of H_2 is greater at 20 kPa of N_2O than at 50 kPa of N_2O, because N_2 and H_2 are competitive and less N_2 is produced at 20 kPa of N_2O than at 50 kPa of N_2O.

Steady-State Kinetics of N_2O Reduction by Nitrogenase. Figure 9a shows the rate of nitrogenase-catalyzed N_2 release from N_2O as a function of pN_2O. In Figure 9b the results are replotted as a double-reciprocal (Lineweaver–Burk) plot. The results show a good fit to the straight line with values of K_m of 24 kPa of N_2O and V_{max} of about 1700 nmol of N_2 min^{-1} (mg of Kp1)$^{-1}$. (Note that for the data of Figure 9b, 100 μg of Kp1 was used.)

Effect of pN_2O on the Distribution of Electrons among the Various Products of Nitrogenase. Figure 10 illustrates how the formation of N_2, H_2, and NH_3 and the total electron flux depend on the pN_2O. N_2O had almost no effect on the total electron flux (the weak decrease is not significant), but N_2O diverted nitrogenase from production of H_2 to formation of N_2 and NH_3. The assumption is made in our calculation of the total electron flux that production of one molecule of N_2

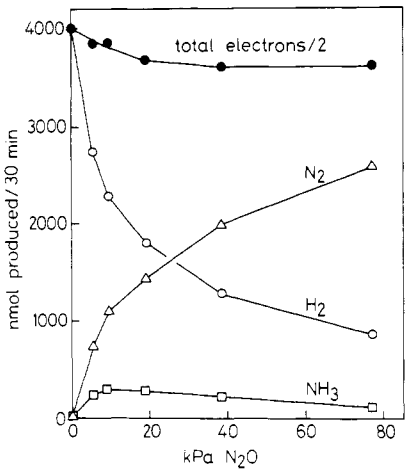


FIGURE 10: Effect of pN_2O on H_2 evolution, N_2 release, NH_3 production, and total electron flux from reactions catalyzed by nitrogenase from *K. pneumoniae*. Total electron flux is expressed as electron pairs and was calculated as $H_2 + 3NH_3/2 + N_2$. Experimental details are described in the legend to Figure 1 and under Materials and Methods. A total of 90 μg of Kp1 and 150 μg of Kp2 was used.

(from N_2O) and H_2 (from 2 H^+) requires two electrons each, whereas one molecule of NH_3 (from $^1/_2$ N_2) requires three electrons. Again, NH_3 production increases with increasing pN_2O, reaches a peak, and then decreases again during further increase in the pN_2O.

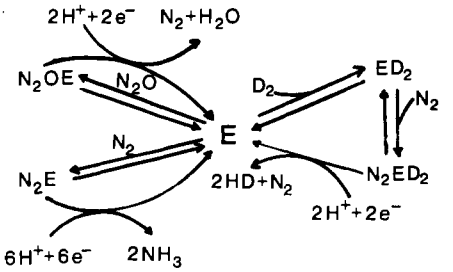
Discussion

Studies with alternative substrates and inhibitors of nitrogenase-catalyzed reductions led Hwang et al. (1973) to suggest that five modified substrate binding sites might exist on the nitrogenase complex. Since then, much work has been done with purified component proteins from nitrogenases (Thorneley & Lowe, 1984a,b; Lowe & Thorneley, 1984b,c), and all of this work indicates that only one substrate binding site exists on the nitrogenase complex. This binding site generally is associated with the iron–molybdenum cofactor (Fe–Mo cofactor) of dinitrogenase protein, but the problem of the complexity of the interactions among the various reducible substrates of nitrogenase remains.

H_2 is a competitive inhibitor, but N_2O is the only alternative substrate for nitrogenase that has been shown to inhibit nitrogen fixation competitively. N_2O has been demonstrated to be a competitive inhibitor both in intact cells (Repaske & Wilson, 1952) and in crude extracts from *A. vinelandii* (Rivera-Ortiz & Burris, 1975). In this paper we have shown that N_2O also is a competitive inhibitor of N_2 fixation when fixation is measured under optimal conditions with purified component proteins from *K. pneumoniae*. Our results confirm the finding of Hoch et al. (1960) that nitrogenase reduces N_2O to N_2, which in turn is reduced further to NH_3. As previously found for H^+, C_2H_2, and N_2 (Watt & Burns, 1977), azide (Dilworth & Thorneley, 1981), and HCN (Li et al., 1982), N_2O did not decrease the total electron flow through nitrogenase (Figure 10).

Our estimated value of 12 kPa of N_2O for the K_i of N_2O against N_2 fixation is very similar to the value of 11 kPa of N_2O reported by Rivera-Ortiz and Burris (1975). The K_m for N_2O reduction by nitrogenase does not appear to have been reported before. Our estimated value of 24 kPa (about 5 mM at 30 °C) of N_2O indicates a rather low affinity of nitrogenase for N_2O, especially if one takes into account the high solubility of N_2O in water. The solubility of nitrous oxide in water is 21.2 mM at 1 atm of N_2O at 30 °C (Wilhelm et al., 1977), or about 35 times greater than the solubility of N_2, which is about 0.6 mM at 1.0 atm and 30 °C. So on a molar basis the affinity of nitrogenase for nitrogen [K_m around 67 μM; 11.7 kPa (Guth & Burris, 1983); 11 kPa (Jensen Burris, 1985)] is much higher than the affinity for nitrous oxide (K_m around 5 mM).

Scheme I[a]



[a] Model that shows how the results with N_2O obtained in this investigation can be incorporated into the mechanism of nitrogenase-catalyzed reductions (Guth & Burris, 1983).

Compared to the affinity of other substrates for nitrogenase, the affinity for N_2O (K_m about 5 mM) is in the range of affinities for HCN (K_m = 4.5 mM; Li et al., 1982) and azide (K_m = 3.3 mM and 1.3 mM for N_2H_4 and NH_3 formation, respectively; Dilworth & Thorneley, 1981). Nitrogenase seems to have a somewhat higher affinity for CH_3NC (K_m = 0.7 mM; Rubinson et al., 1983) and for acetylene (K_m = 0.4 mM; Jensen & Cox, 1983). Not surprisingly, the highest affinity found is for the natural substrate N_2 (K_m about 67 μM; Guth & Burris, 1983; Jensen & Burris, 1985).

Several schemes have been proposed to explain the mechanism for nitrogenase-catalyzed reductions (Chatt, 1980; Li & Burris, 1983; Guth & Burris, 1983; Lowe & Thorneley, 1984a; Jensen & Burris, 1985). Scheme I in this paper shows how our results on N_2O reduction can be fitted into Guth's model and account for many of the observations obtained: (1) N_2O reduction is competitive with respect to dinitrogen reduction (i.e., N_2O and N_2 compete for the same enzyme site); (2) H_2 does not inhibit reduction of N_2O to N_2; (3) NH_3 production from N_2O is inhibited by H_2 (i.e., NH_3 is produced from N_2O via N_2); (4) N_2O at infinite pressure completely inhibits H_2 evolution; (5) production of NH_3 from N_2O shows "substrate" inhibition (i.e., as mentioned, NH_3 production from N_2O takes place via N_2, and because N_2 reduction is inhibited by N_2O, NH_3 production from N_2O shows substrate inhibition).

With respect to the reduction of C_2H_2 (and other substrates that inhibit N_2 reduction in a noncompetitive way), one can imagine that a less reduced state of dinitrogenase (Silverstein & Bulen, 1970) is necessary than is the case for the reduction of N_2. Such an explanation is in good agreement with the proposal by Chatt (1980) that the molybdenum site of dinitrogenase can exist in different reduction states depending on the electron flux through nitrogenase. So, instead of postulating the existence of five modified substrate binding sites as proposed by Hwang et al. (1973), it seems more reasonable to suppose that nitrogenase can occur in different redox states and that different substrates are able to bind to nitrogenase depending on how reduced the enzyme is. Only in a highly reduced state is nitrogenase able to react with and reduce its natural substrate N_2.

Summary

On the basis of the work presented in this paper one can conclude the following about N_2O as a substrate and as an inhibitor for nitrogenase-catalyzed reduction: (1) N_2O is a competitive inhibitor of dinitrogen reduction; (2) N_2O is unable

to support HD formation from H_2O and D_2; (3) N_2O at very high pressure completely blocks H_2 evolution by nitrogenase; (4) release of N_2 from N_2O is not inhibited by H_2; (5) NH_3 production from N_2O (via N_2) is inhibited by H_2; (6) production of N_2 from N_2O shows normal saturation kinetics; (7) production of NH_3 from N_2O (via N_2) shows substrate inhibition. These properties are compatible with the mechanism (Scheme I) for nitrogenase-catalyzed reductions proposed by Guth and modified to include N_2O.

Registry No. N_2O, 10024-97-2; H_2, 1333-74-0; HD, 13983-20-5; NH_3, 7664-41-7; N_2, 7727-37-9; nitrogenase, 9013-04-1.

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