BBA 46399

NITROGENASE-CATALYZED REACTIONS

J. C. HWANG* AND R. H. BURRIS

The Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

(Received May 30th, 1972)

SUMMARY

1. H_2 evolution, N_2 reduction and ATP hydrolysis, catalyzed by a particulate nitrogenase from *Azotobacter vinelandii*, showed similar dependence on the concentration of ATP. Higher concentrations of ATP were inhibitory.

2. Evolution of H_2 by nitrogenase under the conditions studied could not be completely stopped.

3. An average of 4.3 ATPs was required per two electrons transferred for the evolution of 1 H_2 or for the formation of 2/3 NH_3 from N_2 (corrected for H_2 evolution). 2.7 ATPs were required for two electron transfer needed for C_2H_2 reduction to C_2H_4 (no appreciable H_2 evolved); this lower requirement suggested that the mechanisms for H_2 evolution (or N_2 reduction) and C_2H_2 reduction might differ.

4. The minimal number of ATPs required per two electrons to support the formation of 2/3 NH_3 from 1/3 N_2, 1 NH_3 from N_3^-, 1 C_2H_4 from C_2H_2, 1/3 CH_4 from 1/3 HCN and 1/3 CH_4 from 1/3 CH_3NC were found to be 7, 10, 2.7, 23 and 19 (uncorrected for H_2 evolution), respectively. The higher numbers among these indicate that more H_2 was evolved in the system.

5. Optimal concentrations of electron acceptors were 0.4–1.0 atm N_2, 5–20 mM NaN_3, 0.2 atm C_2H_2, 3 mM KCN and 6–12 mM CH_3NC. Nitrogenase-catalyzed ATP hydrolysis was enhanced by the presence of N_2, NaN_3, KCN or CH_3NC. 60% enhancement was observed with 3 mM KCN and with 10 mM CH_3NC; this enhancement was decreased by higher concentrations of KCN and CH_3NC.

6. K_m values for N_2, NaN_3, C_2H_2, KCN and CH_3NC were 0.122 atm, 1.15 mM, 0.015 atm, 1.28 mM and 2 mM, respectively.

INTRODUCTION

Nitrogenase is a complex of two protein fractions, an Mo–Fe protein and an Fe protein[1,2]. In addition to the fixation of N_2, nitrogenase has reductant-dependent ATP-hydrolyzing activity[3,4], ATP-dependent H_2-evolving activity[5], and supports an exchange reaction between $^2H_2$ and endogenous hydrogen donors to yield H^2H[6–8]. Nitrogenase also reduces a variety of compounds structurally similar to N_2, such as N_2O[9], azide[10,11], acetylene[10,12,13], cyanide and its related compounds[14], and isocyanide

* Present address: Corporate Research Laboratory, Allied Chemical Corporation, Morristown, N.J., U.S.A.

and its analogues[15]. All of these reductions have the same requirements as does N_2 fixation: ATP as an energy source, a low potential electron donor, a divalent ion and an electron acceptor.

In this paper nitrogenase-catalyzed reactions by a purified particulate fraction are discussed.

MATERIALS AND METHODS

Chemicals

N_2, H_2 and argon were obtained as high purity cylinder gases from commercial sources. Acetylene, purified grade, was purchased from Matheson Co. and was used after removal of acetone with a solid CO_2–acetone freezing trap. ATP and creatine kinase (EC 2.7.3.2) were purchased from Sigma Chemical Co. All other chemicals were reagent grade except for disodium creatine phosphate (synthesized[16]; 99.4% pure) and methyl isocyanide prepared by the method of Schuster *et al.*[17].

Growth of bacteria and preparation of extracts

Cultures of *Azotobacter vinelandii* O strain or OP strain grown on an inorganic salts *plus* sucrose medium in aerated culture were harvested by centrifugation, and stored at −20 °C as a frozen paste[16]. They were disrupted with a French pressure cell, and the extract obtained by centrifugation was dialyzed against water for 3 h and was heated anaerobically at 60 °C for 10 min. The heated supernatant was treated with 0.45 mg streptomycin sulfate per mg of protein. The resultant supernatant was centrifuged at 144000 × *g* for 1 h, and the dark brown precipitate obtained was resuspended in buffer and was designated as P_{144-1}. These suspensions had specific activities of 60–100 nmoles N_2 fixed per mg of protein per min. The particulate nitrogenase from *A. vinelandii* is convenient for general studies of N_2 fixation, because it is readily prepared, is less labile to O_2 than the highly purified Fe protein and Mo–Fe protein of nitrogenase, appears to contain no components which interfere with any of its nitrogenase functions, has no measurable activity without suitable supplementation with a reductant and ATP-generating system, has reasonably high specific activity and can be stored conveniently in liquid N_2 or for several days at 4 °C.

Analytical

N_2 fixation was measured[3] by the formation of NH_3 from N_2. A typical reaction mixture in a total volume of 1 ml was 50 μmoles creatine phosphate, 5 μmoles $MgCl_2$, 5 μmoles ATP, 15 units creatine kinase, 50 μmoles Tris–HCl buffer (pH 7.4), 15 μmoles $Na_2S_2O_4$, 0.6–1.0 mg of protein (purified particulate fraction P_{144-1}) and N_2 atmosphere for N_2 fixation or argon for control vessels. The incubation was 15 min at 30 °C and the reaction was terminated by adding saturated K_2CO_3. The NH_3 diffused[3] was measured by Nesslerization. Specific activity was defined as nmoles N_2 fixed per mg of protein per min. Azide reduction was assayed by measuring NH_3 formed in the reaction mixture containing azide under an atmosphere of argon. The azide concentration was routinely 10 mM and azide was added by injection after evacuation and filling of the bottles with argon; controls without azide were run. Specific activity was expressed as nmoles NH_3 formed per mg of protein per min. Acetylene reduction was measured by the formation of ethylene. The reaction mixture (same as for N_2

fixation) was placed in 20 ml or 6 ml serum bottles; the amount of acetylene added depended upon the purpose of the experiments, and the pressure was brought to one atmosphere with argon. The reactions were terminated by injection of 0.2 ml of 20 % trichloroacetic acid. A 0.2-ml gas sample was withdrawn from a bottle for ethylene analysis and was injected into an Aerograph 600D gas chromatograph equipped with a H_2 flame ionization detector and a 9-ft column of Porapak R of 1/8 inch inner diameter. The flow rate was 25 ml/min each for the N_2 carrier gas and the H_2. The quantity of ethylene was determined by measuring peak heights and comparing them with standards. Activity was expressed as nmoles ethylene formed per mg of protein per min.

Cyanide or methylisocyanide reduction was determined by measuring methane formation (same gas chromatographic apparatus), and specific activity was expressed as nmoles of methane formed per mg of protein per min. KCN or CH_3NC was injected into the argon-filled bottles.

Activity of the ATP-dependent H_2-evolving system was determined in the presence of dithionite and an ATP-generating system and in the absence or presence of N_2. Evolution of H_2 was measured with a Gilson constant-pressure differential volumometer with all glass manometers. The main chamber contained the reaction mixture except for enzyme and $Na_2S_2O_4$. The flask was evacuated and gassed with argon, and enzyme and $Na_2S_2O_4$ were placed in one sidearm while a stream of argon was passed through the flask. After evacuating and gassing three times with argon (or with N_2 or N_2 *plus* argon in some experiments), the flasks were shaken in a 30 °C water bath for 20 min before the contents of the sidearm were tipped into the main chamber to initiate the reaction. H_2 evolution was measured for 15 min, and specific activity was expressed as nmoles H_2 evolved per mg of protein per min.

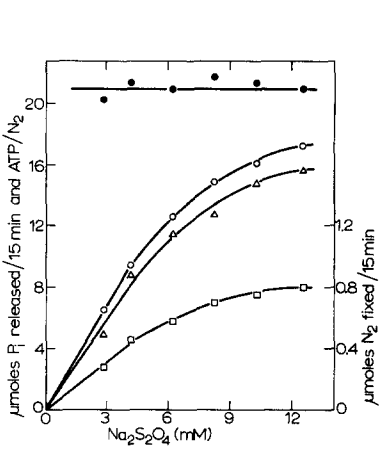
ATP hydrolysis catalyzed by nitrogenase was assayed by measuring creatine released from creatine phosphate by creatine kinase. The reaction mixtures were the same as described for N_2 fixation. Creatine was measured by the method of Eggleton *et al.*[18]. The difference in creatine analyses between reaction mixtures with $Na_2S_2O_4$ and without $Na_2S_2O_4$ represented the amount of ATP hydrolysis caused by nitrogenase; ATP hydrolysis in the absence of $Na_2S_2O_4$ was attributed to classical ATPase. ATP hydrolyzing activity was expressed as nmoles of P_i (inorganic phosphate) released per mg of protein per min. Protein concentrations were determined colorimetrically by the biuret method of Gornall *et al.*[19] with serum albumin as a standard.

RESULTS AND DISCUSSION

Effect of $Na_2S_2O_4$

A low potential electron donor is required for the functioning of nitrogenase. $Na_2S_2O_4$ is convenient and is effective with all nitrogenase systems tested. Other electron donors such as $K_2S_2O_5$, Na_2SO_3, Na_2S, $Na_2S_2O_3$, thioglycollate and mercaptoethanol were tried but did not support N_2 fixation by the *A. vinelandii* P_{144-1} fraction. Fig. 1 shows the effect of $Na_2S_2O_4$ concentration on nitrogenase-catalyzed ATP hydrolysis. These activities of reductant-dependent ATP hydrolysis were obtained by subtracting creatine released by classical ATPase (reductant-independent ATP hydrolysis) from the total creatine released in this system. Nitrogenase *per se* hydrolyzes ATP 6–10 % as fast[20] in the absence as in the presence of a reductant such

as $Na_2S_2O_4$. N_2 fixation and ATP hydrolysis increased with increasing concentrations of $Na_2S_2O_4$. There was an increase of about 10% in ATP hydrolysis in 1 atm N_2 compared to 1 atm argon. The ATP/N_2 molar ratios (total P_i released divided by total N_2 fixed) were relatively constant with a ratio of 21. Double reciprocal plots of data from Fig. 1 gave K_m values of $8 \cdot 10^{-3}$ M $Na_2S_2O_4$ for P_i released under N_2 or Ar and for N_2 fixation. Burns[21] reported a K_m of $9 \cdot 10^{-3}$ M for $Na_2S_2O_4$ in support of N_2 fixation by preparations from *A. vinelandii*. Measurements with a continuous assay show a K_m well below $1 \cdot 10^{-5}$ M for dithionite oxidation[22] with preparations from *Clostridium pasteurianum*; this may reflect a difference between the enzymes from the two organisms.



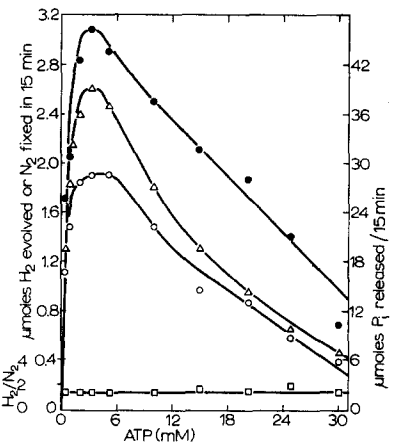


Fig. 1. Effect of $Na_2S_2O_4$ concentration on N_2 fixation and energy consumption under N_2 or argon. □—□, N_2 fixed; △—△, P_i released under argon; ○—○, P_i released under N_2; ●—●, ATP/N_2.

Fig. 2. Effect of ATP concentration in the ATP-generating system on N_2 fixation, H_2 evolution and energy consumption. The main chamber contained in 1.4 ml: 70 μmoles creatine phosphate, 10 μmoles $MgCl_2$, 100 μmoles Tris–HCl buffer (pH 7.4), 0.5 mg creatine kinase, and ATP as indicated. The side arm contained in 0.6 ml: 30 μmoles $Na_2S_2O_4$ and 1.8 mg P_{144-1} protein. The gas phase was N_2 or argon. The content of the side arm was dumped into the main chamber after 20 min equilibration in a 30 °C bath. □—□, H_2 evolved/N_2 fixed; ○—○, N_2 fixed; △—△, P_i released; ●—●, H_2 evolved.

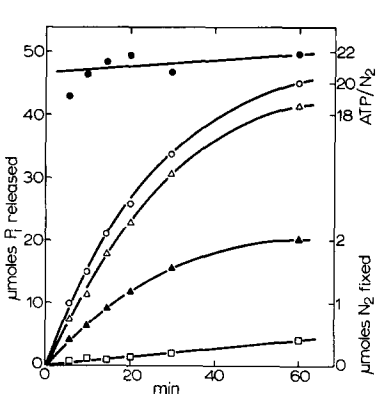
Effect of ATP

Fig. 2 shows the effect of ATP concentration on N_2 fixation, H_2 evolution and reductant-dependent ATP hydrolysis under 1 atm of N_2 and with 5 mM Mg^{2+}. Amounts of H_2 evolved and N_2 fixed were dependent upon the amounts of ATP utilized. The concentration of ATP (about 3 mM) optimal for $Na_2S_2O_4$-dependent ATP hydrolyzing activity also was optimal for N_2 fixation and H_2 evolution. Concentrations of ATP lower than 1.5 mM and higher than 5 mM decreased all three activities over the 15-min period of measurement. Because Mg^{2+} was 5 mM, the decrease of activity by ATP concentrations higher than 5 mM might be caused by ATP not complexed with Mg^{2+}. The ratios for H_2 evolved to N_2 fixed averaged 1.6 under 1 atm of N_2 in the system described; about one-third of the energy was utilized to yield H_2, and the rest was channelled to reduce N_2. ATP did not seem to inhibit the N_2-reducing step or the H_2-evolving step differentially, because the H_2 evolved/N_2 reduced remained constant. The Lineweaver–Burk double reciprocal plots of data from Fig. 2

for N_2 fixed, H_2 evolved, P_i released and total electron flow *vs* ATP concentration showed similar K_m values[16] of $3 \cdot 10^{-4}$ M, $2.9 \cdot 10^{-4}$ M, $3.6 \cdot 10^{-4}$ M and $3 \cdot 10^{-4}$ M, respectively. The K_m for ATP supporting N_2 fixation was $2.6 \cdot 10^{-4}$ M with a *C. pasteurianum* extract which had been passed through a Sephadex G-25 column[23]. Burns[21] reported a K_m of $3 \cdot 10^{-4}$ M for preparation from *A. vinelandii*, and Biggins and Kelly[24] found a K_m of $1.2 \cdot 10^{-4}$ M for extracts from *Klebsiella pneumoniae*.

N_2 reduction

N_2 fixation and nitrogenase-related ATP hydrolysis under N_2 and argon were essentially linear up to 15 min (Fig. 3). Reductant-independent ATPase also was observed in the particulate fraction, P_{144-1}. $Na_2S_2O_4$-dependent ATP hydrolysis under N_2 consistently was about 10% more rapid than under argon[16]. The number of ATP molecules required for each N_2 fixed in the system described (Range 19–22, average 21 total P_i released divided by N_2 fixed uncorrected for H_2 evolution under N_2) was nearly constant, regardless of the amount of H_2 evolved in this system; water was not limiting and N_2 was held constant at 1 atm. Specific activities of the $Na_2S_2O_4$-dependent ATP-hydrolyzing system under N_2 and under argon were 1370 and 1250 nmoles P_i released per mg of protein per min, respectively. Reductant-independent ATPase activity was 80, less than 6% of the ATP-hydrolyzing activity of the nitrogenase.



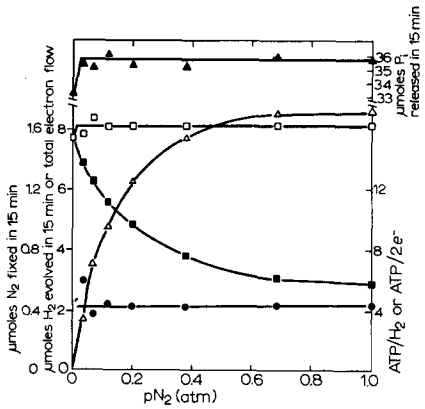


Fig. 3. Time course of N_2 fixation, nitrogenase-catalyzed ATP hydrolysis and classical ATPase activity. □—□, classical ATPase activity; ▲—▲, N_2 fixed; △—△, P_i released under argon; ○—○, P_i released under N_2; ●—●, ATP/N_2.

Fig. 4. Effects of the pN_2 on N_2 fixation, H_2 evolution, total electron flow, ATP hydrolysis by nitrogenase, and ATP/2 e^- for H_2 evolved or N_2 fixed. The conditions were the same as described in Fig. 2 except that 5 mM ATP was used. □—□, total electron flow; △—△, N_2 fixed; ■—■, H_2 evolved; ▲—▲, total P_i released by nitrogenase; ●—●, ATP/2 e^- for H_2 evolution or 1/3 N_2 fixation.

Fig. 4 records the influence of the pN_2 on nitrogenase as reflected in total P_i released, N_2 fixed, H_2 evolved, ATP/H_2 ratios (uncorrected for N_2 fixation), total electron flow (expressed as H_2 by calculating from 2 electrons/H_2 evolved and 6 electrons/N_2 fixed), and ATP required for 2 electrons channelled to each H_2 evolved or 1/3 N_2 fixed. H_2 evolution decreased as N_2 fixation increased in response to the

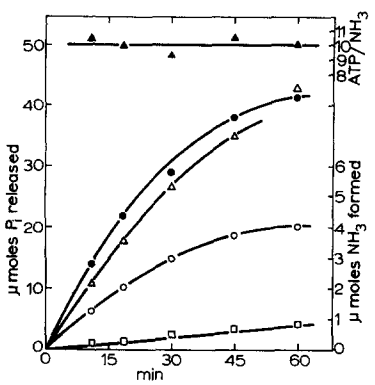
increasing pN_2. This indicated that H^+ and N_2 compete for electrons utilized in the formation of H_2 and NH_3. The total energy utilized and the total electron flow were constant except in the absence of N_2. The stoichiometry of ATP/2 e^- was nearly constant at 4.3 over the pN_2 range, regardless of whether it was used for H_2 evolution or for N_2 fixation. A ratio of 1.61 for H_2 evolved/N_2 fixed was obtained under 1 atm of N_2. Lineweaver–Burk plots of two separate experiments gave K_m values of 0.114–0.130 atm for N_2.

Azide reduction

A 10–15 min lag in azide reduction was observed with crude extracts from *Rhodospirillum rubrum*[25]. The time course of azide reduction by nitrogenase from *A. vinelandii* (Fig. 5) exhibits no lag. Similar curves for ATP hydrolysis under argon by nitrogenase were obtained in the presence or absence of azide. Azide reduction was proportional to ATP hydrolysis, but there was somewhat more ATP hydrolysis with than without azide. The ATP/NH_3 ratio of 10 (uncorrected for H_2 evolution) was nearly constant with time. The stoichiometry of azide reduction has been reported to be 1 mole of N_2 and 1 mole of NH_3 formed from 1 mole of azide[11,14]. To minimize reutilization of the N_2 formed, most of the experiments on azide reduction were run for less than 18 min in 20-ml serum bottles.

The effect of azide concentration on NH_3 formation and P_i release is shown in Fig. 6. Although Hardy and Knight[14] reported the inhibition of azide reduction by azide concentrations above 15 mM, no significant inhibition of azide reduction was observed up to 20 mM azide. The minimum ATP/NH_3 ratio was 10. Higher ratios at the lower concentrations of azide indicated increased formation of H_2 from the interaction of activated reductant and protons or water. 5 mM azide gaven early maximal NH_3 formation.

A Lineweaver–Burk plot of data from Fig. 6 gave a K_m of 1.15 mM NaN_3. Hardy and Knight[14] have reported a K_m for azide of 1.3 mM with a heated extract from *A. vinelandii*. A much lower K_m of 0.2 mM has been reported with *C. pasteurianum*[11].



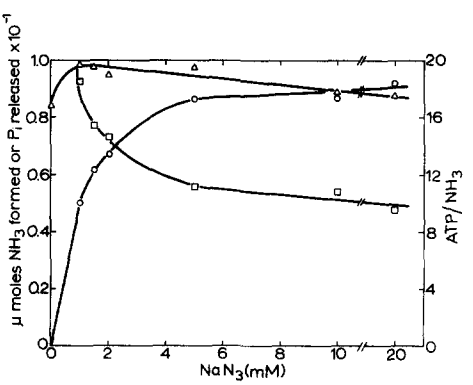
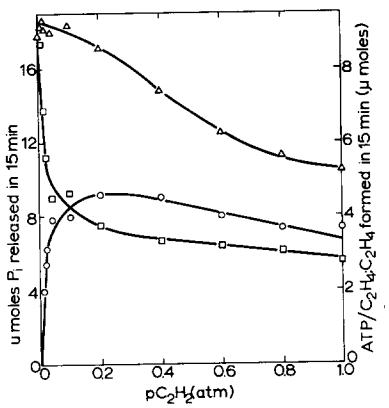


Fig. 5. Time course of azide reduction. □—□, classical ATPase; △—△, P_i released in the absence of azide; ○—○, NH_3 formed from azide; ▲—▲, ATP/NH_3 (uncorrected for H_2 evolution); ●—●, P_i released in the presence of azide.

Fig. 6. Effect of azide concentration on NH_3 formation and ATP hydrolysis by nitrogenase. □—□, ATP/NH_3; △—△, P_i released; ○—○, NH_3 formed.

Acetylene reduction

Fig. 7 shows the effect of the pC_2H_2 on C_2H_4 formation, the P_i released, and the ATP/C_2H_4 ratios. The optimal pressure of C_2H_2 for C_2H_4 formation was 0.2 atm. The partial pressures of C_2H_2 above 0.2 atm inhibited reductant-dependent ATP-hydrolyzing activity and above 0.4 atm inhibited C_2H_4 formation. 1 atm of C_2H_2 inhibited nitrogenase-catalyzed ATP hydrolysis by 44 % and C_2H_4 formation by 20 % as compared with these reactions at 0.2 atm of C_2H_2. The specific activity at 0.2 atm of C_2H_2 was 291 nmoles of C_2H_4 formed per mg of protein per min. The minimal ATP/C_2H_4 was about 2.7. The higher ratios at the lower pressures of C_2H_2 indicated the competing formation of H_2. No C_2H_6 was detected by gas chromatography. A Lineweaver–Burk plot gave a K_m of 0.015 atm of C_2H_2. The value fell within the reported values between 0.01 and 0.03 atm for crude extracts from *C. pasteurianum*[12,13] and whole soybean root nodules[26].



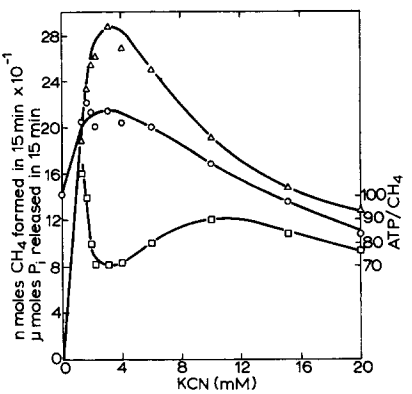


Fig. 7. Effect of pC_2H_2 on C_2H_4 formation and ATP hydrolysis by nitrogenase. □—□, ATP/C_2H_4; △—△, total P_i released; ○—○, C_2H_4 formation.

Fig. 8. Effect of cyanide concentration on CH_4 formation and ATP hydrolysis by nitrogenase. □—□, ATP/CH_4; △—△, CH_4 formation; ○—○, P_i released.

Cyanide reduction

The optimal concentration of cyanide for cyanide reduction and ATP hydrolysis was found to be 2–4 mM (Fig. 8). Cyanide concentrations below 2 and above 5 mM greatly decreased CH_4 formation. 20 mM cyanide inhibited over 50 % relative to the maximal rate of CH_4 formation. ATP hydrolysis by nitrogenase was enhanced by cyanide by as much as 60 % (3 mM cyanide). Unlike the curves for substrate concentration *vs* ATP/N_2 fixed, ATP/N_3^- reduced and ATP/C_2H_4 formed, the curve for ATP/CH_4 *vs* cyanide was S-shaped. This suggests that cyanide concentration not only affects CH_4 formation and H_2 evolution but also influences the formation of other products, such as CH_3NH_2, C_2H_4 and C_2H_6. Formation of these products has been reported by Hardy and Knight[14] and Kelly *et al.*[15]. The minimal ATP/CH_4 from cyanide was 70 at 3 mM cyanide. A Lineweaver–Burk plot gave a K_m of 1.28 mM for cyanide. K_m values of 1.4 mM and 4 mM were reported for preparations from *Azotobacter chroococcum*[27] and *A. vinelandii*[14].

Methylisocyanide reduction

Fig. 9 shows the effect of CH_3NC concentration on CH_4 formation and ATP hydrolysis by nitrogenase. Like cyanide, CH_3NC also enhanced ATP hydrolysis; a maximum increase of 62 % was observed at 6–10 mM CH_3NC. CH_4 formation also was optimal between 6 and 10 mM CH_3NC. The ATP/CH_4 curve indicates that CH_3NC concentration, like cyanide concentration, affects not only CH_4 formation but apparently other reactions as well such as H_2 evolution and formation of CH_3NH_2, C_2H_4 and C_2H_6. The minimal ATP/CH_4 was 56. A K_m of 1.96 mM CH_3NC was observed. A K_m of 0.18 mM was reported for a preparation from *A. chroococcum*[27].

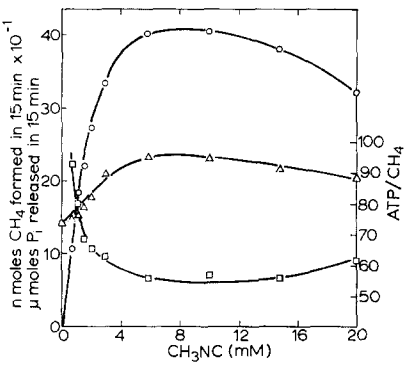


Fig. 9. Effect of CH_3NC on CH_4 formation and ATP hydrolysis by nitrogenase. □—□, ATP/CH_4; △—△, P_i released; ○—○, CH_4 formation.

ATP is an absolute requirement for nitrogenase-catalyzed reactions[28]. Nitrogenase-catalyzed ATP hydrolysis was enhanced in the presence of electron acceptors such as N_2, N_3^-, CN^- and CH_3NC relative to controls under argon. Enhancement under N_2 was about 10 %, and by 3 mM cyanide or 10 mM methylisocyanide was 60 %. No concentration of acetylene enhanced ATP hydrolysis, and concentrations higher than 0.2 atm greatly inhibited ATP hydrolysis.

Reports on the stoichiometry of ATP consumed to H_2 evolved or to 1/3 N_2 fixed vary from 2 to 20[1,2,20,29–33]. A value of 4.9 ATPs per 2 e^- in the reduction of C_2H_2 to C_2H_4 was reported with *A. vinelandii* preparations[31]. An examination of this relationship with a purified particulate fraction (P_{144-1}) from *A. vinelandii* gave an almost constant ATP/2 e^- ratio of 4.3 over most of the pN_2 range tested. The lack of agreement about the ATP/2 e^- values may reflect the differences in enzyme preparations and techniques employed among investigators.

Table I summarizes the minimal amount of ATP required for reduction of an electron acceptor or for formation of a product; specific activities were obtained at the concentrations of electron acceptors indicated in parentheses. Although H_2 evolution catalyzed by nitrogenase occurred even in the presence of electron acceptors, the amount of ATP required (Table I) was not corrected for H_2 evolution except when H^+ was the electron acceptor under argon. An average of 4.3 ATPs were required for each two electron transfer in H_2 evolution and N_2 reduction (N_2 reduction data corrected for H_2 evolution), whereas only 2.7 ATPs were needed for two electrons channelled to reduce C_2H_2 to C_2H_4 (no appreciable H_2 evolved). The ATP requirement with ideal coupling may actually be lower than 2.7. The results raise the question,

TABLE I

COMPARISON OF AMOUNT OF ATP REQUIRED AND SPECIFIC ACTIVITY ON VARIOUS SUBSTRATES

The reported values were obtained in experiments with the P_{144-1} fraction. Values were obtained at the concentrations indicated. The specific activity of N_2 fixation was expressed as nmoles N_2 fixed/mg of protein × min and all the others were recorded as nmoles of product formed/mg of protein × min.

Substrate	*Reaction*	*Amount of ATP required (condition)* *	*ATP/2 e^-* *	*Specific activity (condition)*	*Specific activity/2 e^-*
N_2	$N_2 + 6\,H^+ + 6\,e^- \longrightarrow 2\,NH_3$	ATP/N_2: 21 (N_2: 1 atm)	7	78 (N_2: 1 atm)	234
N_3^-	$HN_3 + 2\,e^- + 2\,H^+ \longrightarrow N_2 + NH_3$	ATP/NH_3: 10 (N_3^-: 10 mM)	10	104 (N_3^-: 10 mM)	104
C_2H_2	$C_2H_2 + 2\,H^+ + 2\,e^- \longrightarrow C_2H_4$	ATP/C_2H_4: 2.7 (C_2H_2: 1 atm)	2.7	300 (C_2H_2: 0.21 atm)	300
HCN	$HCN + 6\,H^+ + 6\,e^- \longrightarrow CH_4 + NH_3$**	ATP/CH_4: 70 (HCN: 3 mM)	23	25 (HCN: 3 mM)	75
CH_3NC	$CH_3NC + 6\,H^+ + 6\,e^- \longrightarrow CH_3NH_2 + CH_4$**	ATP/CH_4: 56 (CH_3NC: 15 mM)	19	30 (CH_3NC: 10 mM)	90
H^+***	$2\,H^+ + 2\,e^- \longrightarrow H_2$	ATP/2e: 4.3 (Ar: 1 atm)	4.3	290 (Ar: 1 atm)	290

* H_2 evolution is neglected.

** Other reactions not listed are not quantitatively significant compared to the one described.

*** Experiments were performed with a manometric technique.

whether or not there is a basic difference between the mechanism for ATP-supported reduction of acetylene and the mechanisms for N_2 and H^+ reduction.

Neglecting corrections for H_2 evolved and based on transfer of two electrons, the most efficient reactions are C_2H_2 reduction and H_2 evolution under argon, followed by reduction of N_2, azide, CH_3NC and HCN in order. When C_2H_2 reduction was measured at 0.21 atm of C_2H_2, and H_2 evolution was measured under argon, they had similar specific activities. Measurements of ATP hydrolysis and H_2 evolution (no electron acceptors except protons) catalyzed by nitrogenase are convenient indicators of nitrogenase activity, because H_2 evolution complicates quantitation of other electron acceptor systems. However, the ratio of ATP/2 e^- varies with the balance between the Mo–Fe and Fe proteins[20]. In *A. vinelandii* there is no interfering H_2 evolution by classical hydrogenase and no significant classical ATPase to confuse with nitrogenase-catalyzed ATP hydrolysis[16]. Measurements of NH_3 formed from N_2 and N_3^-, CH_4 from HCN and CH_3NC, and C_2H_4 from C_2H_2 may be less accurate indices of nitrogenase activity, because H_2 evolution catalyzed by nitrogenase occurs during the reactions and these activities are dependent upon the concentration of the electron acceptor. Measurement of $Na_2S_2O_4$ oxidation[22] clearly indicates the total electrons donated by the reductant without designating the final acceptor.

On the basis of the electron requirements for the reduction of C_2H_2, N_3^- and N_2, it has been predicted that the reduction of C_2H_2 to C_2H_4 and N_3^- to $NH_3 + N_2$ should proceed three times as fast as the reduction of N_2 to NH_3. Variable ratios for the production rates of C_2H_4 from C_2H_2 or NH_3 from N_3^- as compared to NH_3 from N_2 have been reported[11,12,34–36]. Kinetic studies have indicated that the ratios vary with the concentration of the electron acceptor used. Even under optimal conditions, H_2 evolution continues, so some of the electron pool and energy from ATP is used to reduce H^+ rather than the normal substrate. Unless all the energy is utilized for reduction of the electron acceptor under study, one cannot expect to obtain an equivalence among the rates for the various nitrogenase-catalyzed reductions.

A comparison of data on the K_{N_2} values for N_2 fixation by intact cells and cell-free preparations of N_2-fixing organisms has been given by Strandberg and Wilson[37]. Hadfield and Bulen[31] subsequently reported a K_{N_2} of 0.18–0.25 atm for the cell-free system from *A. vinelandii*, and our experiments have indicated a K_{N_2} of 0.12 atm. K_{N_2} values for growing cells generally are lower than those for cell-free preparations. The lower pressure of N_2 needed for half saturation of nitrogenase in intact cells is advantageous, because the pN_2 inside may be much lower than outside the cells. The difference in K_{N_2} values between the cell-free preparations and whole cells is particularly marked in *A. vinelandii*. The K_{N_2} values for growing *A. vinelandii* cells[37] generally range from 0.01 to 0.02 atm N_2. The K_{N_2} observed has varied with the experimental conditions. A K_{N_2} of 0.01 atm for *A. vinelandii* cells at a pO_2 of 0.1 atm rose to 0.023 atm N_2 when a pO_2 of 0.2 atm was used[38].

The presence of an inhibitor such as H_2 or O_2 gives a higher apparent K_{N_2} (Dilworth *et al.*[3] calculated that, corrected for the influence of H_2, the K_{N_2} for an extract from *C. pasteurianum* was 0.037 atm), however, this does not explain the difference in K_{N_2} values between cell-free extracts and intact cells of *A. vinelandii*.

The Michaelis constant is not the dissociation constant of the enzyme–substrate complex, and therefore $1/K_m$ is not the true affinity constant of the enzyme–substrate complex. However, the Michaelis constant usually is within an order of magnitude of

the dissociation constant for the enzyme–substrate complex. Approximations of the affinities were calculated from the K_m values, and nitrogenase-substrate affinities are compared in Table II. The K_{N_2} of 0.122 atm is about 8 times the $K_{C_2H_2}$ of 0.015 atm. However, acetylene is about 63 times more soluble than N_2, so based on the concentrations of dissolved gases, nitrogenase has about 8 times greater affinity for N_2 than for acetylene.

TABLE II

APPARENT AFFINITIES OF SUBSTRATES FOR NITROGENASE

Electron acceptor	K_m *(atm)*	*Solubility of gas at 1 atm (M)*	K_m *(M)*	*Affinity* $(1/K_m)$
N_2	0.122	$5.7 \cdot 10^{-4}$	$7.0 \cdot 10^{-5}$	$1.43 \cdot 10^{4}$
NaN_3			$1.15 \cdot 10^{-3}$	$8.7 \cdot 10^{2}$
C_2H_2	0.015	$3.61 \cdot 10^{-2}$	$5.42 \cdot 10^{-4}$	$1.85 \cdot 10^{3}$
KCN			$1.28 \cdot 10^{-3}$	$7.81 \cdot 10^{2}$
CH_3NC			$1.96 \cdot 10^{-3}$	$5.1 \cdot 10^{2}$
ATP			$3.0 \cdot 10^{-4}$	$3.33 \cdot 10^{3}$

ACKNOWLEDGEMENTS

This investigation was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison; by Public Health Service Grant AI-00848 from the National Institute of Allergy and Infectious Diseases, and by National Science Foundation Grant GB-483.

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