Biochem. J. (1987) 247, 547–554 (Printed in Great Britain)

Klebsiella pneumoniae nitrogenase

Inhibition of hydrogen evolution by ethylene and the reduction of ethylene to ethane

Gillian A. ASHBY, Michael J. DILWORTH* and Roger N. F. THORNELEY†
A.F.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, U.K.

Ethylene (C_2H_4) inhibited H_2 evolution by the Mo-containing nitrogenase of *Klebsiella pneumoniae*. The extent of inhibition depended on the electron flux determined by the ratio of Fe protein (Kp2) to MoFe protein (Kp1) with $K_i^{C_2H_4} = 409$ kPa ([Kp2]/[Kp1] = 22:1) and $K_i^{C_2H_4} = 88$ kPa ([Kp1]/[Kp2] = 21:1) at 23 °C at pH 7.4. At [Kp2]/[Kp1] = 1:1, inhibition was minimal with C_2H_4 (101 kPa). Extrapolation of data obtained when C_2H_4 was varied from 60 to 290 kPa indicates that at infinite pressure of C_2H_4 total inhibition of H_2 evolution should occur. C_2H_4 inhibited concomitant $S_2O_4^{2-}$ oxidation to the same extent that it inhibited H_2 evolution. Although other inhibitors of total electron flux such as CN^- and CH_3NC uncouple MgATP hydrolysis from electron transfer, C_2H_4 did not affect the ATP/2e ratio. Inhibition of H_2 evolution by C_2H_4 was not relieved by CO. C_2H_4 was reduced to C_2H_6 at [Kp2]/[Kp1] ratios $\geqslant$ 5:1 in a reaction that accounted for no more than 1% of the total electron flux. These data are discussed in terms of the chemistry of alkyne and alkene reduction on transition-metal centres.

INTRODUCTION

The nitrogenase of *Klebsiella pneumoniae* comprises two metalloproteins, the MoFe protein (Kp1, M_r 218000) and the Fe protein (Kp2, M_r 67000). In the presence of MgATP and a reductant (flavodoxin *in vivo*, $Na_2S_2O_4$ *in vitro*), nitrogenase catalyses the reduction of N_2 to $2NH_3$. Under optimal conditions, the reduction of N_2 is accompanied by the stoichiometric reduction of $2H^+$ to H_2 and the hydrolysis of 16 MgATP to 16 MgADP + 16 P_i (for recent reviews on the structure and mechanism of nitrogenase see Burgess, 1985; Lowe *et al.*, 1985; Orme-Johnson, 1985; Stephens, 1985; Thorneley & Lowe, 1985).

Although a comprehensive mechanism for nitrogenase-catalysed H_2 evolution and N_2 reduction has been developed (Lowe & Thorneley, 1984*a*,*b*; Thorneley & Lowe, 1984*a*,*b*), it does not consider the mechanism of reduction of other substrates (for a review of these and their reduction products see Burgess, 1985). The most important of these is acetylene (C_2H_2), which is reduced to ethylene (C_2H_4) (Dilworth, 1966; Schöllhorn & Burris, 1967), and forms the basis for a widely used assay of nitrogenase activity both *in vivo* and *in vitro* (Koch & Evans, 1966; Hardy *et al.*, 1968). An understanding of the way C_2H_2 is reduced is therefore useful in defining the chemistry occurring at the active site of nitrogenase.

The relationships between H_2 evolution, C_2H_2 reduction and N_2 reduction under various conditions of electron flux through the enzyme are not well understood (Hageman & Burris, 1980; Wherland *et al.*, 1981). Before a detailed analysis can be made of pre-steady-state and steady-state kinetic data for C_2H_2 reduction to C_2H_4 and for concomitant H_2 evolution, it is necessary to determine the affinity of the MoFe protein not only for the substrate C_2H_2 but also for the potential product inhibitor C_2H_4.

A transient form of the MoFe protein with bound C_2H_4 has been detected at low concentrations by e.p.r. spectroscopy in assays run at 10 °C with low electron flux (Kp2/Kp1 molar ratio of 1:3) (Lowe *et al.*, 1978). The binding constants for C_2H_2 (15 μM and 13 mM) and C_2H_4 (1.3 mM) determined under these conditions at 10 °C are clearly inappropriate for computer simulations using the Lowe–Thorneley model, which uses rate constants determined at 23 °C. At 10 °C and low electron flux, C_2H_4 stimulated H_2 evolution by approx. 75% rather than inhibiting it (Lowe *et al.*, 1978), an observation that cannot be accommodated in the Lowe–Thorneley model in its present form. There is therefore evidence of C_2H_4 interacting chemically with the enzyme, but few data on the nature of that interaction.

The conventional Mo-containing nitrogenases reduce C_2H_2 to C_2H_4, but further reduction to ethane (C_2H_6) or methane (CH_4) has not been detected (Hardy *et al.*, 1968). However, the recently discovered vanadium-containing nitrogenase from *Azotobacter chroococcum* (Robson *et al.*, 1986) does reduce a significant proportion of C_2H_2 to C_2H_6 (Dilworth *et al.*, 1987).

A reduction product from nitrogenase that inhibits total electron flux and MgATP hydrolysis is potentially useful for preparing high concentrations of MoFe protein in a state that, during substrate reduction, is only present at a low concentration as a transient species. The spectroscopic characterization of such a complex could provide information about the site(s) of substrate and product binding and the level of reduction of the MoFe protein in the catalytic cycle when product is released. In the present paper we describe the conditions under which C_2H_4 can behave as such an inhibitor. We also show that C_2H_4 is slowly reduced to C_2H_6 at high electron flux.

MATERIALS AND METHODS

Nitrogenase

The nitrogenase component proteins from *Klebsiella pneumoniae* (*oxytoca*) N.C.I.B. 12204 were purified and

Abbreviations used: Kp1 and Kp2, MoFe protein and Fe protein components respectively of *Klebsiella pneumoniae* nitrogenase.

* Permanent address: School of Environmental and Life Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia.
† To whom correspondence should be addressed.

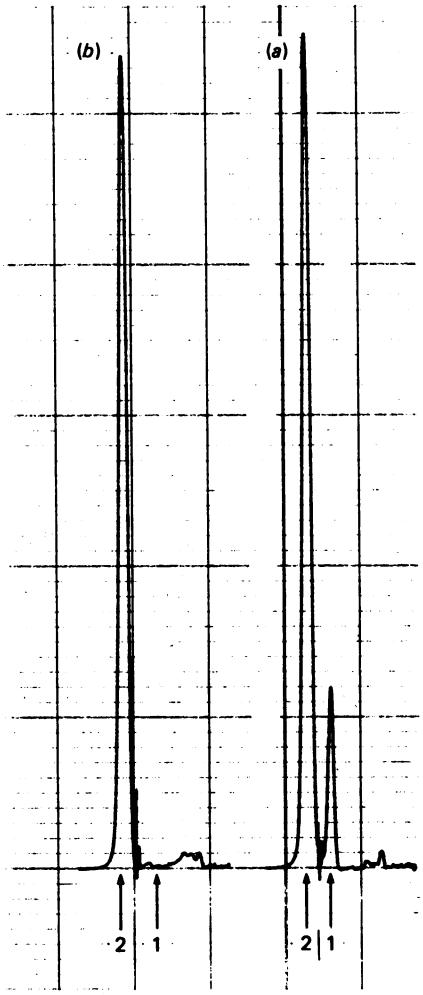


Fig. 1. Gas chromatograms of C_2H_4 before and after purification

C_2H_4 was purified as described in the Materials and methods section. Samples of cylinder and purified C_2H_4 were diluted 72-fold into Ar and 0.5 ml samples were chromatographed. (*a*) Cylinder C_2H_4. Peak 1 (C_2H_6), attenuation × 80; peak 2 (C_2H_4), attenuation × 32 × 10^3. (*b*) Purified C_2H_4. Peak 1 (C_2H_6), attenuation × 80 (< 0.005% C_2H_6); peak 2 (C_2H_4), attenuation × 32 × 10^3.

assayed as previously described (Thorneley & Lowe, 1983). Kp1 and Kp2 proteins had specific activities at 30 °C of 1800 and 1450 nmol of C_2H_4 produced/min per mg of protein respectively.

Gases

Cylinder argon, hydrogen and carbon monoxide (Air Products, Walton-on-Thames, Surrey, U.K.), methane (British Oxygen Co., London S.W.19, U.K.) and ethane (Messer Griesheim, Düsseldorf, W. Germany) were used without further purification. Cylinder ethylene (Air Products) was purified as described below. Acetylene was prepared by the action of water on CaC_2 (BDH Chemicals, Poole, Dorset, U.K.). Gases were added by back-filling evacuated serum vials fitted with Subaseal rubber closures (Ar and C_2H_4) or by injection with gas-tight syringes (C_2H_2, C_2H_4, H_2 and CO).

Ethylene purification

Ethylene was purified by selective absorption into acidic $Hg(ClO_4)_2$ solution, from which it was subsequently released by addition of LiCl (Young *et al.*, 1952). A 250 ml portion of 0.5 M-$Hg(ClO_4)_2$ in 2 M-$HClO_4$ was placed in a 2-litre round-bottomed flask with sockets for two stopcocks and a 250 ml separating funnel, and cooled to 0 °C in an ice bath. The flask was evacuated and refilled twice with argon, and then with cylinder ethylene containing 0.06% (v/v) ethane, and sealed. The absorption of ethylene was monitored manometrically as the solution was stirred, with absorption complete in 2 h. The flask was then evacuated and refilled twice with argon, and then fully evacuated and sealed. The ice bath was removed and a 4-fold molar excess of LiCl was added slowly from the separating funnel as a 4 M solution that had been sparged with argon. Gas evolution was rapid; the flask was left overnight to complete gas production. The resultant ethylene contained less than 0.0005% (v/v) ethane; no other C_2, C_3 or C_4 hydrocarbon was detectable in it. Fig. 1 shows gas chromatograms of ethylene before and after purification. Details of the chromatography and detection procedures are given below.

Assay procedure

Assays were performed in a shaking water bath at 23 °C and unless otherwise stated the assay mixture contained in a final volume of 1 ml 21.5 μmol of $MgCl_2$, 45 μmol of Hepes, pH 7.4, 16 μmol of phosphocreatine, 30 μmol of $Na_2S_2O_4$, 8 μmol of ATP and 66 μg of creatine kinase under various mixtures of Ar, C_2H_2, C_2H_4, H_2 and CO in the gas phase (6.3 ml). Assays were started by syringe addition of pre-mixed Kp1 and Kp2 (0.05 ml) to give the concentrations quoted in the Figure legends and text. Assays were stopped with 0.1 ml of 30% (w/v) trichloroacetic acid, except for those in which ATP hydrolysis was to be measured, when 0.3 ml of 0.5 M-EDTA, pH 7.4, was used.

Assays under hyberbaric C_2H_4 (202 and 303 kPa) required a modified procedure. The assays were prepared as described above under C_2H_4 (101 kPa) and equilibrated at 23 °C. Within 5 s after the injection of the protein mix, the requisite volumes of C_2H_4 to give total pressures of 202 (6.3 ml) and 303 kPa (12.6 ml) were injected. No leakages of gas occurred through the punctured rubber closure during the course of the assay terminated as described above. The recovery of an internal standard (0.1 ml of CH_4) added to the vial before the injection of hyperbaric C_2H_4 was used to verify that correction of the gaseous products recovered by a factor equal to the pressure in atmospheres was valid.

Water (0.5 ml) was injected into each vial containing a quenched assay, and 0.5 ml samples of gas were assayed for H_2 on a Pye Series 204 gas chromatograph fitted with a 1 m × 6 mm-diam. column of molecular sieve 5A (80–100 mesh). The column was operated at 100 °C with Ar (20 ml/min) as carrier gas and H_2 was detected with a katharometer.

CH_4, C_2H_6, C_2H_4 and C_2H_2 were measured by flame ionization detection following chromatography on a 1 m × 6 mm-diam. column of chromatographic alumina prepared as described by Smith & Dowdell (1973) and operated with a 45 ml/min flow of N_2 at 110 °C (Smith & Restall, 1971). C_2H_6 and C_2H_4 were completely resolved on this column: 60 pmol of C_2H_6 is easily measured.

Linear time courses for product formation were

obtained within the range 5–40 min under all experimental conditions, except in those experiments where $Na_2S_2O_4$ oxidation was monitored (see below).

ATP hydrolysis assay

ATP hydrolysis during the nitrogenase assay was determined by measuring creatine released from phosphocreatine. Ion-exchange resin (AG1-X8; Bio-Rad Laboratories, Richmond, CA, U.S.A.) was washed with 4 M-HCl and then exhaustively with water. The liquid content of EDTA-quenched assay vials after gas analysis were allowed to flow through individual columns (2 cm × 6 mm diam.) of AG1-X8 resin, followed by two washes of vials and columns with 0.5 ml portions of water. $S_2O_4^{2-}$ and its oxidation products, ATP, phosphocreatine, Hepes, EDTA and protein were adsorbed to the resin, leaving creatine quantitatively recoverable in the eluate. The resulting solutions were collected in weighed 30 ml Universal bottles and re-weighed to determine the sample volumes. Samples were then taken for creatine determination by the method of Ennor (1957).

Dithionite oxidation

Assays in which $S_2O_4^{2-}$ oxidation was to be determined were stopped with 0.1 ml of 36% (w/v) formaldehyde, and residual $S_2O_4^{2-}$ was measured by iodometric titration of the formaldehyde adduct (Li *et al.*, 1982). The initial $Na_2S_2O_4$ concentration in such assays was 7.5 mM rather than the normal 30 mM.

Reagents

All salts were purchased from BDH Chemicals, and biochemicals from Sigma Chemical Co., Poole, Dorset, U.K.

RESULTS AND DISCUSSION

Inhibition of H_2 evolution by C_2H_4

Fig. 2 shows the dependence of the rate of H_2 evolution under Ar (101 kPa) as a function of the [Kp2]/[Kp1] ratio. The limiting rate of H_2 evolution at high [Kp2]/[Kp1] ratio in this type of 'titration curve' is often used to calculate the specific activity of MoFe protein. The explanation given by Thorneley & Lowe (1984*b*) for the shape of similar curves is complicated and is based upon the kinetics of the partial reactions that comprise the catalytic cycle and the effects of inactive forms of Kp1 and Kp2 proteins. At high electron flux (high [Kp2]/[Kp1] ratio), H_2 evolution is considered to occur from the more reduced forms of Kp1 (E_3 and E_4 in the notation of Lowe & Thorneley, 1984*a*) and at low electron flux ([Kp2]/[Kp1] less than 1:1) from the less reduced forms (predominantly E_2). The effect of C_2H_4 (101 kPa) on H_2 evolution rates at various [Kp2]/[Kp1] ratios is also shown in Fig. 2, the inset being an expansion of the data at low [Kp2]/[Kp1] ratios. Significant inhibition of H_2 evolution by C_2H_4 (101 kPa) occurs at [Kp2]/[Kp1] ratios less than 1:1 and greater than 5:1, with little inhibition between these limits. This is more clearly shown in Fig. 3 (data derived from Fig. 2), which shows H_2 evolution rates under C_2H_4 (101 kPa) expressed as a percentage of the H_2 evolution rate under Ar (101 kPa) as a function of the [Kp2]/[Kp1] ratio. At [Kp2]/[Kp1] ratios less than 1:10 (low electron flux) and

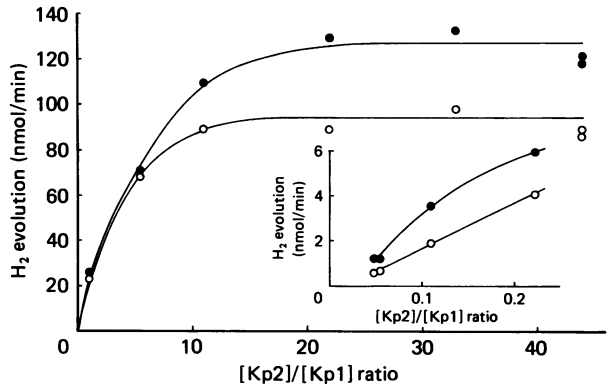


Fig. 2. Inhibition of H_2 evolution by C_2H_4 as a function of component protein ratio (electron flux) at 23 °C at pH 7.4

Assay conditions were as described in the text. At [Kp2]/[Kp1] ≥ 1:1, assay mixtures contained 0.1 mg of Kp1 and appropriate amounts of Kp2. At [Kp2]/[Kp1] < 1:1, assay mixtures contained 0.03 mg of Kp2 and appropriate amounts of Kp1. The inset shows data at [Kp2]/[Kp1] < 1:1 normalized to 0.1 mg of Kp1/assay. ○, C_2H_4 (101 kPa); ●, Ar (101 kPa).

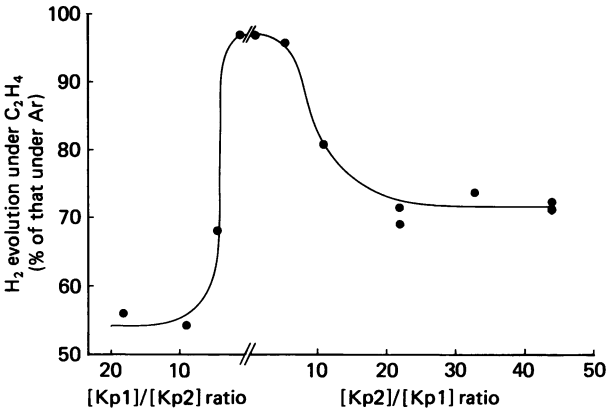


Fig. 3. Effect of the [Kp2]/[Kp1] ratio on the rate of H_2 evolution under C_2H_4 (101 kPa) expressed as a percentage of the rate of H_2 evolution under Ar (101 kPa) at 23 °C at pH 7.4

Data are re-plotted from Fig. 2. Data to the left of the break in the abscissa are plotted against [Kp2]/[Kp1] to expand the scale.

greater than 20:1 (high electron flux), H_2 evolution rates under C_2H_4 remain essentially constant at 55% and 70% respectively of the corresponding values under Ar. At a [Kp2]/[Kp1] ratio of 1:1, the H_2 evolution rate under C_2H_4 (101 kPa) was the same as that under Ar (101 kPa).

Fig. 4 shows two double-reciprocal plots obtained at two protein component ratios ([Kp2]/[Kp1] = 22:1 and 0.048:1) for C_2H_4 inhibition of H_2 evolution under various partial pressure of Ar and C_2H_4. The ratios were chosen because they are in the high- and low-electron-flux regions where inhibition by C_2H_4 is maximal (see Fig. 3). The data have been plotted in Fig. 4 as $10^2/\Delta H_2$ against $10^3/pC_2H_4$, where ΔH_2 is the rate of H_2 evolution (in nmol/min) under Ar minus the rate of H_2

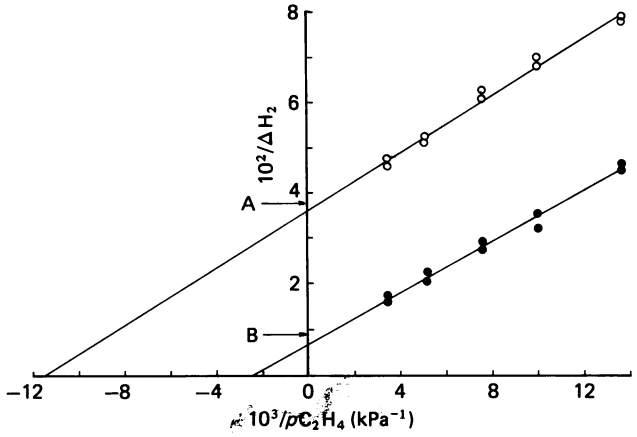


Fig. 4. Double-reciprocal plots of the extent of inhibition of H_2 evolution as a function of the pressure of C_2H_4 under high- and low-electron-flux conditions at 23 °C at pH 7.4

Assay conditions were as described in the text. ΔH_2 is the rate of H_2 evolution under Ar minus the rate of H_2 evolution under various partial pressures of C_2H_4. ●, High-electron-flux conditions, with [Kp2]/[Kp1] = 22:1 (0.1 mg of Kp1/assay) ($K_i^{C_2H_4}$ 409 kPa); ○, low-electron-flux conditions, with [Kp2]/[Kp1] = 1:21 (2 mg of Kp1/assay) ($K_i^{C_2H_4}$ 88 kPa). The intercepts A and B were calculated assuming complete inhibition of H_2 production at infinite pressure of C_2H_4. The lines are least-squares fits to the data points.

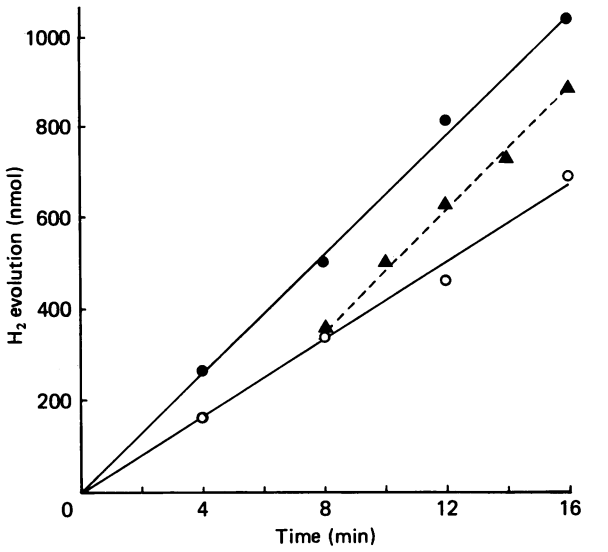


Fig. 5. Time courses for H_2 evolution demonstrating reversibility of C_2H_4 inhibition at 23 °C at pH 7.4

Assay conditions were as described in the text with [Kp2]/[Kp1] = 25:1 (0.05 mg of Kp1/assay). ●, Under Ar (101 kPa); ○, under C_2H_4 (101 kPa); ▲, incubated under C_2H_4 (101 kPa) for 8 min, at which time C_2H_4 replaced by Ar (101 kPa). The lines are least-squares fits to the data points, which are the means of duplicate determinations.

evolution under C_2H_4 and pC_2H_4 is the partial pressure of C_2H_4 (in kPa). The linear dependence provides no evidence for multiple binding sites for C_2H_4. The ordinate intercepts are within experimental error of points A and B, which assume that at infinite pC_2H_4 H_2 evolution is completely suppressed (i.e. ΔH_2 = rate of H_2 evolution under Ar when $pC_2H_4 = \infty$).

If Michaelis–Menten kinetics are assumed, with C_2H_4 a competitive inhibitor of protons (or whichever protic substrate, S, yields H_2) binding to Kp1, then the abscissa intercept in Fig. 4 is equal to $-K_m^S/K_i^{C_2H_4}([S]+K_m^S)$, where $K_i^{C_2H_4}$ is the apparent association constant for C_2H_4 binding to Kp1. The term $K_m^S/([S]+K_m^S)$ is constant at a given pH, but cannot be calculated, since the nature of the substrate that yields H_2 is uncertain and protic equilibria not directly associated with substrate binding preclude detailed analysis of pH-dependence of H_2 evolution rates.

Bearing in mind the limitations of simple steady-state kinetic analysis with an enzyme system as complicated as nitrogenase [see Lowe & Thorneley (1984*b*) for a detailed analysis of $K_m^{N_2}$ and $K_i^{H_2}$ dependencies on electron flux], Fig. 4 shows that C_2H_4 is a more potent inhibitor of H_2 evolution at low electron flux ($K_i^{C_2H_4} = 88$ kPa) than at high electron flux ($K_i^{C_2H_4} = 409$ kPa). The higher affinity for the less-reduced states of Kp1 (E_0 and E_1) is perhaps not surprising, since C_2H_4 is the product of C_2H_2 reduction and presumably dissociates from a relatively oxidized state of Kp1 in the catalytic cycle. The weak binding of C_2H_4 to Kp1 under conditions of both high and low electron flux is consistent with C_2H_4 not being a product inhibitor of C_2H_2 reduction under normal assay conditions where C_2H_4 formation is linear with time.

The reversible nature of C_2H_4 inhibition of H_2 evolution is shown by the data in Fig. 5. Linear time courses for H_2 evolution under Ar and C_2H_4 (both at 101 kPa) were obtained with the rate under C_2H_4 being 64% of that under Ar at a [Kp2]/[Kp1] ratio of 25:1. A third time course for H_2 evolution was obtained by allowing assays to run under C_2H_4 (101 kPa) for 8 min, at which time C_2H_4 was removed by three rapid partial evacuations and back-fillings with Ar (total time less than 1 min). Assays were then allowed to continue for a further 2, 4, 6 and 8 min under Ar (101 kPa) and the rate of H_2 evolution was determined. The rate following incubation under C_2H_4 was 1366 nmol/min per mg of Kp1, which compares with 1350 nmol/min per mg of Kp1 for assays under Ar without pre-exposure to C_2H_4. Besides showing the reversible nature of C_2H_4 inhibition, the time courses are linear with no evidence for a lag or burst phase before the onset of C_2H_4 inhibition.

Table 1 shows that CO at 9 kPa did not relieve the inhibition of H_2 evolution under either high- or low-electron flux conditions.

C_2H_4 inhibition of H_2 evolution could be due to an inhibition of total electron flux through the enzyme, or to redirection of electrons to yield a product other than H_2, such as methane or ethane. To explore these possibilities, we have measured $S_2O_4^{2-}$ oxidation and H_2 evolution under Ar (101 kPa) or C_2H_4 (101 kPa). In the experiment shown in Fig. 6, the correlation between H_2 evolution and $S_2O_4^{2-}$ oxidation was 0.999, and the data for assays under Ar or C_2H_4 lie on the same line. The slope of the composite line (1.04) indicates a maximum value for the excess of $S_2O_4^{2-}$ used over H_2 evolved of 4%. Since under the conditions of this experiment the inhibition of H_2 evolution by C_2H_4 was 34%, C_2H_4 must inhibit total electron flux rather than act as a significant alternative electron acceptor.

Preliminary evidence for C_2H_4 inhibition of H_2

Table 1. Effect of electron flux, CO, C_2H_2 and H_2 on H_2 evolution and C_2H_6 formation under C_2H_4

For details see the text.

Component ratio ...	Product formed (nmol/min per mg of Kp1)			
	[Kp2]/[Kp1] = 22:1		[Kp2]/[Kp1] = 1:21	
Product ...	H_2	C_2H_6	H_2	C_2H_6
Ar (101 kPa)	1127	–	13.4	–
C_2H_4 (101 kPa)	822	4.2	6.1	0
C_2H_4 (92 kPa)+CO (9 kPa)	828	0	6.9	0
C_2H_4 (100 kPa)+C_2H_2 (1 kPa)	393	1.1	–	–
C_2H_4 (101 kPa)+H_2 (101 kPa)	–	4.6	–	–

evolution was obtained with cylinder C_2H_4. Although it was possible that an inhibitor other than C_2H_4 itself was present, C_2H_4 purified as described above gave the same degree of inhibition as the cylinder C_2H_4.

The initial cylinder C_2H_4 contained readily detectable C_2H_6 (Fig. 1); this is not complexed by acidic $Hg(ClO_4)_2$, and 99% of it was lost during purification with no change in inhibition. No other C_2, C_3 or C_4 alkane or alkene was detectable on gas chromatography of the purified C_2H_4 on the alumina column described. C_2H_2, which is absorbed by acidic $Hg(ClO_4)_2$, was not detectable in cylinder C_2H_4 and is in any case not regenerated with LiCl. O_2 and N_2 would have been removed by the purification procedure. Accordingly, we believe that the possibility of an inhibitor other than C_2H_4 being responsible for inhibition of H_2 evolution can be virtually eliminated.

Electron transfer through the nitrogenase of *K. pneumoniae* to produce H_2 is accompanied by the hydrolysis of MgATP. Under our assay conditions and with a [Kp2]/[Kp1] ratio of 22:1, the ATP/2e ratio was 4.3 under Ar, in agreement with previously published values (Imam & Eady, 1980). Under C_2H_4 (101 kPa), where H_2 evolution was inhibited by 30%, the ATP/2e ratio was 4.4, indicating that C_2H_4, unlike CN^- (Li *et al.*, 1982) or CH_3NC (Rubinson *et al.*, 1983), did not uncouple ATP hydrolysis from electron transfer. Although CN^- and CH_3NC are similar to C_2H_4 in their ability to inhibit total electron flux, their effects on the ATP/2e ratio are clearly different.

Hardy *et al.* (1968) did not detect the reduction of C_2H_4 to C_2H_6 with a nitrogenase from *Azotobacter vinelandii*. Unfortunately, the precise conditions under which these experiments were done were not stated. Since the inhibition of H_2 evolution by C_2H_4 with nitrogenase from *K. pneumoniae* only occurs at extremes of high and low electron flux (Fig. 3) and reaches a maximum of about 35% at 101 kPa C_2H_4, we have re-examined the reduction of C_2H_4 to C_2H_6. The availability of C_2H_4 with a very low background of C_2H_6 made such experiments feasible (Fig. 1).

Ethane was identified as a product of C_2H_4 reduction since its retention time on the alumina column was identical with that of authentic C_2H_6 (45 s). At a [Kp2]/[Kp1] ratio greater than 5:1, C_2H_6 was produced from C_2H_4 (101 kPa) at 4.2 nmol/min per mg of Kp1. In a typical 15 min assay with 0.1 mg of Kp1, the C_2H_6 produced under C_2H_4 (101 kPa) was 6.1 nmol over a background of 1.0 nmol of C_2H_6 in the C_2H_4. At [Kp2]/[Kp1] ratios less than 5:1, the rate of C_2H_6 formation declined as electron flux decreased (Fig. 7). At ratios less than [Kp2]/[Kp1] = 1:5, C_2H_6 formation decreased to levels close to the analytical limit (see the legend to Fig. 1). A comparison between Figs. 7 and 2 shows that, whereas C_2H_6 formation from C_2H_4 was constant at [Kp2]/[Kp1] ratios above 5:1, H_2 evolution was still increasing with electron flux under either Ar or C_2H_4 at 101 kPa.

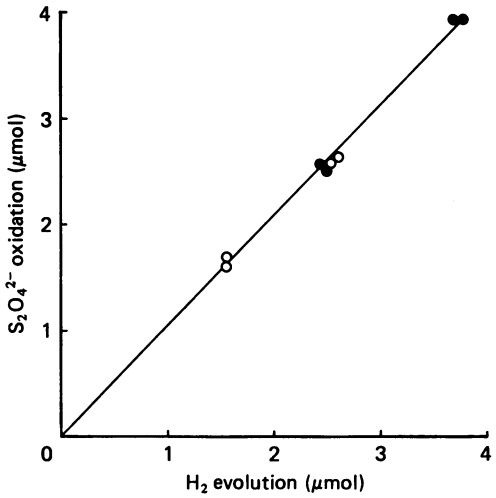


Fig. 6. Correlation between $S_2O_4^{2-}$ oxidation and H_2 evolution under Ar and C_2H_4 (101 kPa) under high-electron-flux conditions at 23 °C at pH 7.4

Assay conditions were as described in the text with [$Na_2S_2O_4$] = 7.5 mM and [Kp2]/[Kp1] = 25:1 (0.1 mg of Kp1/assay). Assays for dithionite determination were stopped with formaldehyde. Under C_2H_4 (101 kPa) H_2 evolution was inhibited by 34% and $S_2O_4^{2-}$ oxidation by 35%. ●, Under Ar (101 kPa); ○, under C_2H_4 (101 kPa).

C_2H_4 reduction accounted for at most 1% of the total electron flux through Kp1. Such a rate of C_2H_6 production cannot readily be detected by measuring differences in either $S_2O_4^{2-}$ oxidation or H_2 evolution.

We have also detected C_2H_4 reduction in the Mo nitrogenase of *Azotobacter chroococcum* at 5.3 nmol of C_2H_6/min per mg of Ac1 from C_2H_4 (101 kPa) with [Ac2]/[Ac1] = 12:1 at 30 °C. It is thus probable that this reaction will prove to be a general property of Mo

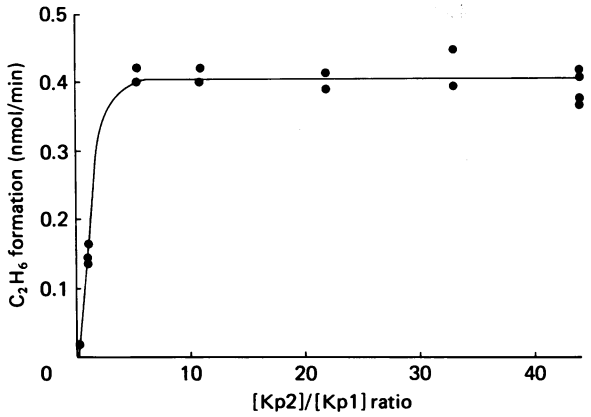


Fig. 7. Effect of component protein ratio on the rate of C_2H_6 formation from C_2H_4 (101 kPa) at 23 °C at pH 7.4

Assay conditions were as described in the text. At [Kp2]/[Kp1] $\geqslant$ 1:1 assay mixtures contained 0.1 mg of Kp1 and the appropriate amounts of Kp2. At [Kp2]/[Kp1] < 1:1, assay mixtures contained 0.03 mg of Kp2 and the requisite amounts of Kp1 (data normalized to 0.1 mg of Kp1/assay).

nitrogenases. Although evidence for the reduction of C_2H_4 to C_2H_6 was sought when the C_2H_2-reduction assay was first developed (Dilworth, 1966; Schöllhorn & Burris, 1967; Hardy *et al.*, 1968), probable reasons why it was not detected are (*a*) that it was masked by C_2H_6 contamination of their C_2H_4, (*b*) that their pC_2H_4 was insufficient, and (*c*) their nitrogenases were operating at too low electron flux. In addition, C_2H_6 formation from C_2H_4 is strongly inhibited by C_2H_2 (Table 1), so that C_2H_6 is not formed in C_2H_2-reduction assays with Mo nitrogenases. Finally, the column packings commonly used to separate C_2H_2 and C_2H_4 rapidly (Porapak N and Porapak T) do not resolve C_2H_4 and C_2H_6.

A double-reciprocal plot of the rates of C_2H_6 formation as a function of the partial pressure of C_2H_4 in the range between 62 and 295 kPa with a [Kp2]/[Kp1] = 22:1 yielded an apparent $K_m^{C_2H_4}$ of 130 kPa. This value is markedly different from the $K_i^{C_2H_4}$ of 409 kPa calculated from the H_2 evolution data under similar conditions (see Fig. 4 and above). Thus, with a C_2H_4 pressure of approx. 400 kPa, total electron flux and H_2 evolution would be inhibited by only *ca.* 50% whereas the rate of C_2H_6 would be almost maximal.

Table 1 shows that H_2 (101 kPa) did not inhibit nor significantly enhance C_2H_6 formation from C_2H_4 (101 kPa). In this respect C_2H_4 reduction to C_2H_6 resembles the reduction of other substrates by nitrogenase, with the exception of N_2, which is thought to bind by displacement of H_2 and for which H_2 is a competitive inhibitor (Lowe & Thorneley, 1984*a*).

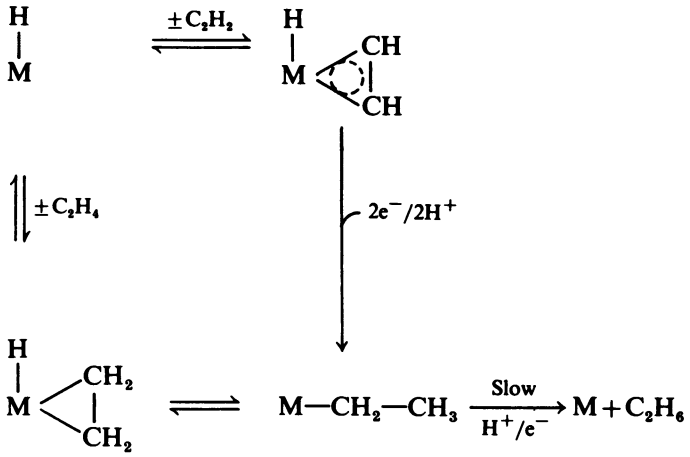
CO is not a substrate for wild-type nitrogenase, but inhibits the reduction of all substrates except H^+; the only other exception is the partial inhibition of H_2 formation by CO observed with the nitrogenase from *nifV* mutants of *K. pneumoniae* (McLean *et al.*, 1983). CO also relieves the inhibition of total electron flux induced by CN^- and CH_3NC (Li *et al.*, 1982; Rubinson *et al.*, 1983). CO did not relieve the inhibition of H_2 evolution induced by C_2H_4 under either high- or low-electron-flux conditions (Table 1). However, CO (9 kPa) completely inhibited C_2H_6 formation from C_2H_4 (Table 1).

The differential response to CO suggests that CO and C_2H_4 can both be bound at the same time. Lowe *et al.* (1978) obtained evidence for two C_2H_2-binding sites on Kp1 from the dependence of amplitudes of transient e.p.r. signals on the partial pressure of C_2H_2. Burgess (1985) has reviewed other evidence for two classes of site on the MoFe protein that bind reducible substrates and/or inhibitors such as CH_3NC, HCN and CN^-. We do not wish to speculate on the precise nature of the binding sites except to note that two co-ordination positions on the same metal atom could accommodate our observations.

Lowe *et al.* (1978) found no evidence for C_2H_4 binding to Kp1 in its resting state, i.e. no change in the e.p.r signal with features at $g = 4.3$, 3.7 and 2.01. However, they did detect a new e.p.r. signal ($g_{av.} = 2.042$) in the presence of C_2H_4 under turnover conditions when protons were reduced to H_2. This implies that C_2H_4 binds to a transient reduced state of Kp1. The coupling of electron transfer to protonation to yield metal hydride(s) at the active site is a key postulate in the Lowe–Thorneley model for nitrogenase action (Lowe & Thorneley, 1984*a*, and references cited therein). The transient presence of metal hydrides may be linked to the inhibition of H_2 evolution by C_2H_4 and the formation of C_2H_6 by reaction of the type shown in Scheme 1. This Scheme is not intended to be a comprehensive mechanism and does not include two binding sites for C_2H_2, C_2H_4 or CO binding to give a mixed CO plus C_2H_4 species that does not evolve H_2 or produce C_2H_6. It is based on known chemistry in that reversible formation of metal–alkyl complexes from hydride–alkene complexes of transition-metal ions by a β-elimination mechanism is well understood (Alt & Eicher, 1982). Further reduction of the co-ordinated alkyl can yield free alkane (Nakamura & Otsuka, 1972).

There is some indirect evidence for C_2H_4 binding to a monohydride form of Kp1 (E_1H in Scheme 2 of Lowe & Thorneley, 1984*a*). Pre-steady-state rapid-quench experiments on C_2H_2 reduction show that three electrons are transferred to Kp1 before C_2H_4 is released (D. J. Lowe & R. N. F. Thorneley, unpublished work). This is consistent with C_2H_2 reduction via a metal–alkyl intermediate that undergoes β-elimination to yield a hydride–ethylene complex (E_1H–C_2H_4) before dissociation of the product, C_2H_4. Evidence for this type of mechanism occurring with molybdenum–alkyne complexes has recently been obtained in these laboratories (R. L. Richards & N. Kashef, unpublished work) and has previously been discussed by Schrauzer *et al.* (1982). Restricted rotation about the carbon–carbon bond of the metal–alkyl intermediate due to steric constraints imposed by either the protein or other ligands at the metal site could account for the high proportion of *cis*-dideuteroethylene produced when C_2H_2 is reduced by nitrogenase in ^2H_2O (Dilworth, 1966; Hardy *et al.*, 1968; Kelly, 1969). However, reduction of propyne in ^2H_2O yields *cis*- and *trans*-dideuteropropene in the ratio approx. 2:1, indicating *cis*-addition to bound alkene is not obligatory (McKenna *et al.*, 1979).

The failure to detect C_2H_6 as a product of C_2H_2 reduction does not exclude a metal–alkyl structure for a common intermediate in C_2H_2 reduction to C_2H_4 and in C_2H_4 reduction to C_2H_6. Scheme 1 shows how this can



Scheme 1. Mechanism for C_2H_6 formation from C_2H_4 involving a metal–alkyl intermediate, and inhibition by C_2H_2, which is reduced only to C_2H_4

occur and also how C_2H_2 (1 kPa) could strongly inhibit C_2H_6 formation from C_2H_4 (100 kPa) (see Table 1). In the presence of C_2H_2, the concentration of the metal–alkyl intermediate ($M–CH_2CH_3$) that slowly yields C_2H_6 on reduction is decreased by suppression of its formation from the hydride–ethylene complex ($MH–C_2H_4$). This happens because C_2H_2 effectively displaces C_2H_4 to yield the hydride–acetylene complex ($MH–C_2H_2$). Since the rate of reduction of the hydride–acetylene complex ($MH–C_2H_2$) is slow [limited by the dissociation of $Kp2_{ox}(MgADP)_2$ from Kp1, $k = 6.4\ s^{-1}$, for each electron (Thorneley & Lowe, 1983)], the intermediate $MH–C_2H_2$, and others not shown in Scheme 1 (possibly metal hydride–carbynes and/or –carbenes), accumulate at the expense of $M–CH_2CH_3$ and $MH–C_2H_4$.

Thorneley & Lowe (1983) compared the steady-state rates of H^+, C_2H_2 and N_2 reduction with the independently measured rate of protein dissociation ($k = 6.4\ s^{-1}$) that occurs after each electron transfer from $Kp2(MgATP)_2$ to Kp1 and concluded that protein dissociation limits the rate of product formation. The low rate of C_2H_6 formation and inhibition of total electron flux shows that this is not the case when C_2H_4 is the substrate. Thus reduction of co-ordinated ethyl to yield C_2H_6 must be slow compared with the rate of protein dissociation ($k = 6.4\ s^{-1}$).

The one-electron-reduced form of Kp1 (E_1H), which is shown in Scheme 1 to bind C_2H_2 and C_2H_4, does not evolve H_2 in the steady state or on quenching with acid (Lowe & Thorneley, 1984*a*). Formation of $E_1H–C_2H_4$ prevents formation of E_2H_2 (the first form of Kp1 able to evolve H_2 in Scheme 2 of Lowe & Thorneley, 1984*a*), thereby inhibiting H_2 evolution and total electron flux. This is also consistent with the failure of H_2 (101 kPa) to inhibit C_2H_6 formation, since reversible binding of H_2 is only considered to be possible, when catalysed by N_2, on the more reduced forms E_3H_3 and E_4H_4 (Lowe & Thorneley, 1984*a*,*b*).

The ability of C_2H_4 to inhibit total electron flux, MgATP hydrolysis and H_2 evolution while being reduced to C_2H_6 at a low rate makes it potentially a valuable tool for investigating the active site of nitrogenase. H_2 evolution reactions normally prevent the generation of no more than low concentrations of reduced forms of Kp1. C_2H_4 at only moderate pressures should, by preventing H_2 evolution, allow such reduced forms with either C_2H_4 alone or C_2H_4 plus CO bound to reach concentrations adequate for spectroscopic analysis. Indeed, under conditions of low electron flux, C_2H_4 will completely suppress H_2 evolution while reduction to C_2H_6 occurs at an undetectable rate, with the result that all the Kp1 should be converted into a state with C_2H_4 bound.

We thank Dr. R. L. Richards, Dr. D. J. Lowe and Dr. R. R. Eady for helpful discussions and Professor J. R. Postgate and Professor G. J. Leigh for their comments on the manuscript. *Azotobacter chroococcum* nitrogenase component proteins were a gift from Dr. R. R. Eady and Mrs. M. Eldridge.

REFERENCES

Alt, H. G. & Eicher, M. E. (1982) Angew. Chem. Int. Ed. Engl. **21**, 205–206

Burgess, B. K. (1985) in Metal Ions in Biology Series vol. 7: Molybdenum Enzymes (Spiro, T. G., ed.), pp. 161–219, Wiley–Interscience, New York

Dilworth, M. J. (1966) Biochim. Biophys. Acta **127**, 283–294

Dilworth, M. J., Eady, R. R., Robson, R. L. & Miller, R. W. (1987) Nature (London) **327**, 167–168

Ennor, A. H. (1957) Methods Enzymol. **3**, 850–856

Hageman, R. V. & Burris, R. H. (1980) Biochim. Biophys. Acta **591**, 63–75

Hardy, R. W. F., Holsten, R. D., Jackson, E. K. & Burns, R. C. (1968) Plant Physiol. **43**, 1185–1207

Imam, S. & Eady, R. R. (1980) FEBS Lett. **110**, 35–38

Kelly, M. (1969) Biochim. Biophys. Acta **191**, 527–540

Koch, B. & Evans, H. J. (1966) Plant Physiol. **41**, 1748–1750

Li, J.-G., Burgess, B. K. & Corbin, J. L. (1982) Biochemistry **21**, 4343–4402

Lowe, D. J. & Thorneley, R. N. F. (1984*a*) Biochem. J. **224**, 877–886

Lowe, D. J. & Thorneley, R. N. F. (1984*b*) Biochem. J. **224**, 895–901

Lowe, D. J., Eady, R. R. & Thorneley, R. N. F. (1978) Biochem. J. **173**, 277–290

Lowe, D. J., Thorneley, R. N. F. & Smith, B. E. (1985) in Metallo Proteins (Harrison, P., ed.), vol. 1, pp. 207–249, Macmillan, London

McKenna, C. E., McKenna, M.-C. & Huang, C. W. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4773–4777

McLean, P. A., Smith, B. E. & Dixon, R. A. (1983) Biochem. J. **211**, 589–597

Nakamura, A. & Otsuka, S. (1972) J. Am. Chem. Soc. **94**, 1886–1894

Orme-Johnson, W. H. (1985) Annu. Rev. Biophys. Chem. **14**, 419–459

Robson, R. L., Eady, R. R., Richardson, T. H., Miller, R. W., Hawkins, M. & Postgate, J. R. (1986) Nature (London) **322**, 388–390

Rubinson, J. F., Corbin, J. L. & Burgess, B. K. (1983) Biochemistry **22**, 6260–6268

Schöllhorn, R. & Burris, R. H. (1967) Proc. Natl. Acad. Sci. U.S.A. **58**, 213–216

Schrauzer, G. N., Hughes, L. A. & Strampach, N. (1982) in Proceedings Int. Conf. Chemistry and Uses of Mo 4th (Berry, H. F. & Mitchell, P. C. H., eds.), pp. 145–149, Climax Molydenum Co., Ann Arbor

Smith, K. A. & Dowdell, R. J. (1973) J. Chromatogr. Sci. **11**, 655–658

Smith, K. A. & Restall, S. W. (1971) J. Soil Sci. **22**, 430–433
Stephens, P. J. (1985) in Metal Ions in Biology Series vol. 7: Molybdenum Enzymes (Spiro, T. G., ed.), pp. 117–159, Wiley–Interscience, New York
Thorneley, R. N. F. & Lowe, D. J. (1983) Biochem. J. **215**, 393–403
Thorneley, R. N. F. & Lowe, D. J. (1984*a*) Biochem. J. **224**, 886–894
Thorneley, R. N. F. & Lowe, D. J. (1984*b*) Biochem. J. **224**, 903–909
Thorneley, R. N. F. & Lowe, D. J. (1985) in Metal Ions in Biology Series vol. 7: Molybdenum Enzymes (Spiro, T. G., ed.) pp. 221–284, Wiley–Interscience, New York
Wherland, S., Burgess, B. K., Stiefel, E. I. & Newton, W. E. (1981) Biochemistry **20**, 5132–5140
Young, R. E., Pratt, H. K. & Biale, J. B. (1952) Anal. Chem. **24**, 551–555

Received 5 April 1987; accepted 15 July 1987