Biochem. J. (1988) 249, 745–751 (Printed in Great Britain)

The vanadium nitrogenase of *Azotobacter chroococcum*

Reduction of acetylene and ethylene to ethane

Michael J. DILWORTH,* ROBERT R. EADY† and Marie E. ELDRIDGE
AFRC Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, U.K.

1. The vanadium (V-) nitrogenase of *Azobacter chroococcum* transfers up to 7.4% of the electrons used in acetylene (C_2H_2) reduction for the formation of ethane (C_2H_6). The apparent K_m for C_2H_2 (6 kPa) is the same for either ethylene (C_2H_4) or ethane (C_2H_6) formation and much higher than the reported K_m values for C_2H_2 reduction to C_2H_4 by molybdenum (Mo-) nitrogenases. Reduction of C_2H_2 in ^2H_2O yields predominantly [*cis*-$^2H_2$]ethylene. 2. The ratio of electron flux yielding C_2H_6 to that yielding C_2H_4 (the C_2H_6/C_2H_4 ratio) is increased by raising the ratio of Fe protein to VFe protein and by increasing the assay temperature up to at least 40 °C. pH values above 7.5 decrease the C_2H_6/C_2H_4 ratio. 3. C_2H_4 and C_2H_6 formation from C_2H_2 by V-nitrogenase are not inhibited by H_2. CO inhibits both processes much less strongly than it inhibits C_2H_4 formation from C_2H_2 with Mo-nitrogenase. 4. Although V-nitrogenase also catalyses the slow CO-sensitive reduction of C_2H_4 to C_2H_6, free C_2H_4 is not an intermediate in C_2H_6 formation from C_2H_2. 5. Propyne ($CH_3C{\equiv}CH$) is not reduced by the V-nitrogenase. 6. Some implications of these results for the mechanism of C_2H_6 formation by the V-nitrogenase are discussed.

INTRODUCTION

Azotobacter chroococcum (Robson, 1986) and *Azotobacter vinelandii* (Bishop *et al.*, 1986) have two genetically distinct systems for nitrogen fixation. One is the well-characterized molybdenum-containing nitrogenase (Mo-nitrogenase); the other is a recently isolated vanadium-containing nitrogenase (V-nitrogenase) that is synthesized in cells given vanadium in place of molybdenum (Robson *et al.*, 1986; Hales *et al.*, 1986).

The V-nitrogenase of *A. chroococcum* is a two-component system consisting of a VFe protein (Ac1*) and an Fe protein (Ac2*). Ac1* is an $\alpha_2\beta_2$ tetramer of native M_r 210000 containing 2 V, 23 Fe and 20 acid-labile sulphide atoms per tetramer (Eady *et al.*, 1987). Ac2* is a dimer of native M_r 60000 containing 4 Fe and 4 acid-labile sulphide atoms per molecule (Robson *et al.*, 1986). For activity, both proteins, MgATP, a low-potential electron donor and the absence of O_2 are required.

The substrate specificity of V-nitrogenase differs from that of Mo-nitrogenase in that neither acetylene (C_2H_2) nor dinitrogen (N_2) competes as effectively with H^+ in V-nitrogenase as in the Mo-nitrogenase system (Robson *et al.*, 1986; Eady *et al.*, 1987). A V-nitrogenase with similar properties has been isolated from *Azotobacter vinelandii* (Hales *et al.*, 1986).

With Mo-nitrogenase, C_2H_2 is reduced only to ethylene (C_2H_4) (Dilworth, 1966; Schollhorn & Burris, 1967; Hardy *et al.*, 1968). We recently reported that isolated V-nitrogenase forms not only C_2H_4, but also ethane (C_2H_6), from C_2H_2, a reaction that also occurs *in vivo* in *A. chroococcum*, *A. vinelandii* and *Clostridium pasteurianum* grown under Mo-deficient conditions in the presence of vanadium (Dilworth *et al.*, 1987).

C_2H_2 can also be reduced to both C_2H_4 and C_2H_6 in chemical systems involving vanadium, such as $V(OH)_2$ in $Mg(OH)_2$ (Denisov *et al.*, 1981; Schrauzer *et al.*, 1981) or V^{II}–catechol complexes (Schrauzer & Palmer, 1981). The yield of C_2H_6 relative to C_2H_4 was improved at low C_2H_2 pressure or at high V^{II} concentration.

A detailed biochemical study of the factors affecting the yield of C_2H_6 may provide insight into (1) the conditions under which C_2H_6 production may be used as an assay for V-nitrogenase *in vivo*, and (2) the difference between the chemical mechanisms of V- and Mo-nitrogenases.

In the present paper we define the parameters that affect the relative yields of C_2H_4 and C_2H_6 from C_2H_2 and present data indicating that free C_2H_4 is not an intermediate in the production of C_2H_6 from C_2H_2. We also show that V-nitrogenase can nevertheless slowly reduce C_2H_4 to C_2H_6.

MATERIALS AND METHODS

Nitrogenase

The nitrogenase components Ac1* and Ac2* were purified from *A. chroococcum* strain MCD1155, which carries a deletion of *nifHDK*, the structural genes for Mo-nitrogenase, as described by Robson *et al.* (1986) and Eady *et al.* (1987). The nitrogenase components Ac1 and Ac2 were purified from *A. chroococcum* MCD50 (Robson, 1986), grown under Mo-sufficient conditions, essentially as described by Yates & Planque (1975).

Abbreviations used: nitrogenase components of *Azotobacter chroococcum* are abbreviated using the notation of Eady *et al.* (1972), in which Ac1* denotes the VFe protein and Ac2* the Fe protein of the V-nitrogenase, and Ac1 the MoFe protein and Ac2 the Fe protein of Mo-nitrogenase.

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

Assays

Nitrogenase was assayed as described by Eady *et al.* (1972), except that the liquid volume was 1.0 ml and the dithionite concentration 10 mM. Time courses for substrate reduction were determined on assays stopped at appropriate times.

Gases

C_2H_2 was generated from CaC_2 (BDH, Poole, Dorset, U.K.). Ar, H_2, N_2 and CO were purchased from Air Products Ltd. (Walton-on-Thames, Surrey, U.K.). Cylinder C_2H_4 from Air Products Ltd. was purified as described by Ashby *et al.* (1987). Ethane, propene (CH_2=CH—CH_3) and propane ($CH_3CH_2CH_3$) were commercial products from Messer Griesheim, Düsseldorf, Germany. Propyne ($CH_3C{\equiv}CH$) was synthesized by Dr. R. L. Richards by the method described by Brandsma (1971).

Product analysis

Ethane, ethylene, propane, propene, acetylene and propyne were separated by chromatography on a column (1 m × 6 mm diam.) of chromatographic alumina (type H_{CL}; Chromatographic Specialties). The alumina was deactivated by treatment with 10% (w/v) NaI solution, washed with deionized water and dried (Smith & Dowdell, 1973). The column was activated by heating overnight at 200 °C while dry N_2 was passed through it. For chromatography, the column was operated at 110 °C with a flow rate of 45 ml of $N_2 \cdot min^{-1}$ (Smith & Restall, 1971). H_2 was measured by gas chromatography (Eady *et al.*, 1972).

NH_3 was measured after microdistillation using the indophenol method as described by Dilworth & Thorneley (1981).

Mass spectrometry

For m.s. analysis, V-nitrogenase was incubated in a 5 ml round-bottomed flask containing three times the normal volumes of reagents. The flask was equipped with a stopcock leading to a B10 socket, which could be closed with a SubaSeal or connected to the mass-spectrometer inlet. After injection of protein and incubation, the headspace gas was assayed by gas chromatography, the stopcock closed, the SubaSeal removed, and the flask connected to the mass spectrometer (AEI model MS 10) inlet.

Stereochemistry of C_2H_4 formation

A 100 ml round-bottomed flask, with a SubaSeal in one side neck which carried a stopcock between the flask and the rubber seal, contained reagents corresponding to 20 times the standard assay, 1.4 mg of Ac1* and 9.6 mg of Ac2*, under an atmosphere of C_2H_2 (10 kPa) and Ar (91 kPa), with 86% ^2H_2O in the liquid phase. The reaction was stopped after 60 min with trichloroacetic acid, and the flask contents frozen in a solid CO_2/methylated spirit bath before transfer of the headspace gas to a 100 ml i.r. gas cell with a 10 cm path length and NaCl windows. I.r. spectra were recorded on a Perkin–Elmer model 1710 Fourier-transform i.r. spectrometer before and after reaction.

RESULTS AND DISCUSSION

C_2H_2 reduction by V-nitrogenase

The major product of C_2H_2 reduction by V-nitrogenase was C_2H_4. The dependence of the rate of C_2H_4 production on C_2H_2 concentration gave an apparent K_m of 6 kPa (Fig. 1), a value which is 3-fold greater than the highest

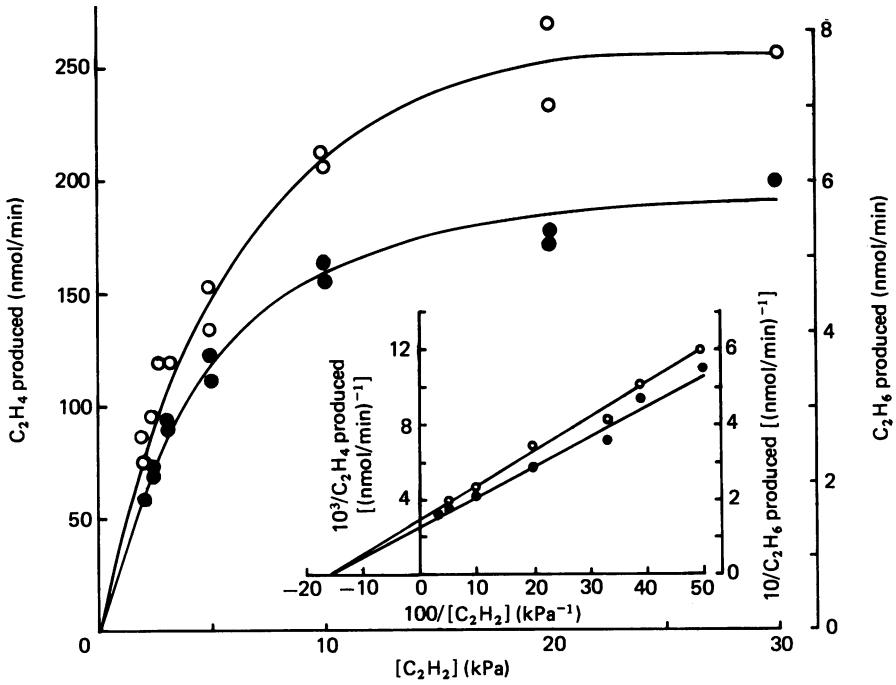

Fig. 1. Dependence of C_2H_4 (○) and C_2H_6 (●) formation on C_2H_2 partial pressure

Ac2* and Ac1* (molar ratio 9.3:1) were assayed under standard conditions, except that the partial pressure of C_2H_2 was as indicated. The inset shows a double-reciprocal plot of the mean values of the same data.

value of the range quoted for Mo-nitrogenase (0.3–2.0 kPa) (Hardy, 1979). The high apparent K_m for C_2H_2 does not, however, explain the failure of C_2H_2 to inhibit H_2 evolution completely (at 30 kPa of C_2H_2, 5 times the apparent K_m, evolution of H_2 was still 33% of the rate under Ar).

Mo-nitrogenase in ^2H_2O reduces C_2H_2 predominantly to [*cis*-$^2H_2$]ethylene (Dilworth, 1966; Hardy *et al.*, 1968; Kelly, 1969), an observation widely interpreted as indicating side-on bonding of C_2H_2 to a metal in the active site (Hardy, 1979). With the V-nitrogenase system, analysis of the products of C_2H_2 reduction in ^2H_2O by i.r. spectroscopy showed a strong band at 843 cm^{-1} characteristic of [*cis*-$^2H_2$]ethylene (Crawford *et al.*, 1953), whereas the band at 988 cm^{-1} for [*trans*-$^2H_2$]ethylene (Crawford *et al.*, 1953) was only just detectable. The reduction of C_2H_2 therefore appears to be highly stereospecific in the V-nitrogenase, just as it is for Mo-nitrogenase.

In addition to the production of C_2H_4, the V-nitrogenase forms C_2H_6 as a minor product (Dilworth *et al.*, 1987). The identification of the additional gaseous product as C_2H_6 is based on data presented here. Its retention time on an alumina column was identical with that of authentic C_2H_6 (45 s), whereas the retention time for C_2H_4 was 62 s. Analysis of the head-space gases by m.s. before and after incubation showed that a species with a molecular mass of 30 Da was formed from C_2H_2 by V-nitrogenase.

Gas chromatography also showed that V-nitrogenase did not form detectable quantities of methane, propene, propane, 2-methylpropane, butane, but-1-ene or but-2-ene from C_2H_2. Unlike the Mo-nitrogenase from *A. vinelandii*, which reduces propyne to propene (Hardy *et al.*, 1971; McKenna *et al.*, 1979), V-nitrogenase from *A. chroococcum* produced neither propene nor propane from propyne (16 kPa) under standard assay conditions.

We have previously reported (Dilworth *et al.*, 1987) that formation of C_2H_6 from C_2H_2 occurs only with combinations of Ac1* with either Ac2 or Ac2*, but not with Ac1. Since Mo-nitrogenase components from *Klebsiella pneumoniae* (*oxytoca*) also do not produce C_2H_6 from C_2H_2 (Ashby *et al.*, 1987), this reaction appears to be a characteristic of the V Fe protein. The parameters of this reaction have therefore been investigated further with a view to its use as an assay for V-nitrogenase both *in vivo* and *in vitro*.

Factors affecting ethane formation from acetylene

Under standard assay conditions of pH 7.4 and 30 °C with C_2H_2 at 15 kPa, C_2H_6 formation accounted for 2.35% of the total electron flux through V-nitrogenase, measured as $H_2+C_2H_4+2\ C_2H_6$. In determining the total electron flux the yield of C_2H_6 was multiplied by 2, since C_2H_6 is a 4e reduction product of C_2H_2, whereas C_2H_4 and H_2 are both 2e reduction products of C_2H_2 and H^+ respectively. As a percentage of the electron flux resulting in C_2H_2 reduction, C_2H_6 accounted for 5.5%. The rate of C_2H_6 formation was proportional to the V-nitrogenase added to the assay under conditions where the time course remained linear.

Since Mo-nitrogenase does not reduce C_2H_2 to C_2H_6 (Hardy, 1979), the possibility arises that the V-nitrogenase has distinct binding sites for C_2H_2 for these reactions. Determination of the apparent K_m for C_2H_2 reduction to C_2H_6 lends no support to this hypothesis,

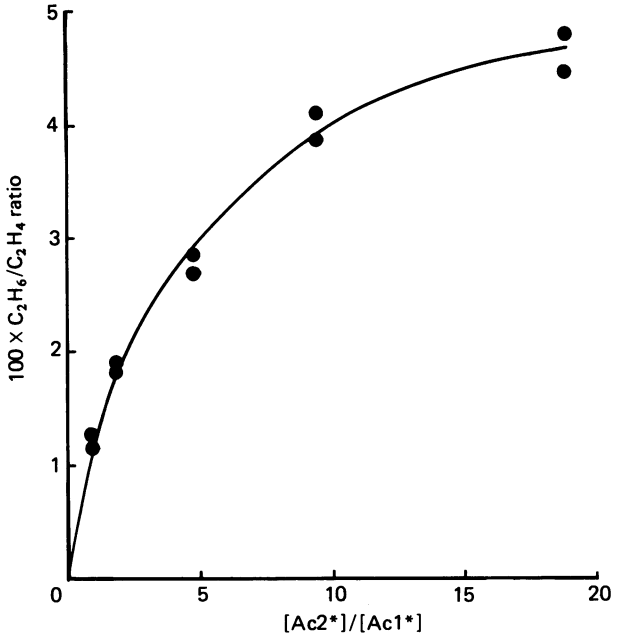

Fig. 2. Effect of the Ac2*/Ac1* ratio on the electron-flux-to-C_2H_6/electron-flux-to-C_2H_4 ratio

Each assay mixture contained Ac1* (65 μg) and appropriate amounts of Ac2* to give the component ratios shown.

since it was experimentally indistinguishable from that for the C_2H_2-to-C_2H_4 reaction at 5.9 kPa (Fig. 1). The electron-flux-to-C_2H_6/electron-flux-to-C_2H_4 ratio (C_2H_6/C_2H_4 ratio) was essentially constant over the range of C_2H_2 partial pressure from 2 to 30 kPa. This implies that C_2H_2 is itself not an inhibitor of the further reduction of free C_2H_4 or an enzyme-bound intermediate.

Since C_2H_6 is a four-electron reduced product, its formation might be expected to be favoured under conditions where electron flux through the enzyme is high (a high molar ratio of Ac2* to Ac1*). It has previously been shown that activity for both H_2 evolution and C_2H_4 formation increases rapidly up to an approx. 20-fold molar excess of Ac2* over Ac1* (Eady *et al.*, 1987). In a similar titration where C_2H_4 and C_2H_6 formation were monitored, the C_2H_6/C_2H_4 ratio increased from 1.2 to 4.65% (Fig. 2). By analogy with Mo-nitrogenase, high component ratios and consequent high electron flux would favour the formation of more highly reduced states of the substrate-reactive sites (Thorneley & Lowe, 1985). For V-nitrogenase, our data suggest that a more reduced form of the enzyme is required for C_2H_6 formation than for C_2H_4 production. Although the C_2H_6/C_2H_4 ratios observed *in vivo* in intact cells of *A. chroococcum* fall within the range in Fig. 2, the difference in electron donor(s) makes it impractical to draw conclusions about the component ratio of Ac2* to Ac1* *in vivo*.

The effect of pH on V-nitrogenase was studied over the pH range 6.5–8.5 in 50 mM-Hepes/NaOH buffer. The profiles for H_2 evolution under Ar, residual H_2 evolution in the presence of 15 kPa C_2H_2, and C_2H_2 reduction to C_2H_4 and C_2H_6, are shown in Fig. 3. All activities show a broad pH optimum in the region 7.0–7.5, whereas the C_2H_6/C_2H_4 ratio decreases at pH values above 7.5 (Fig.

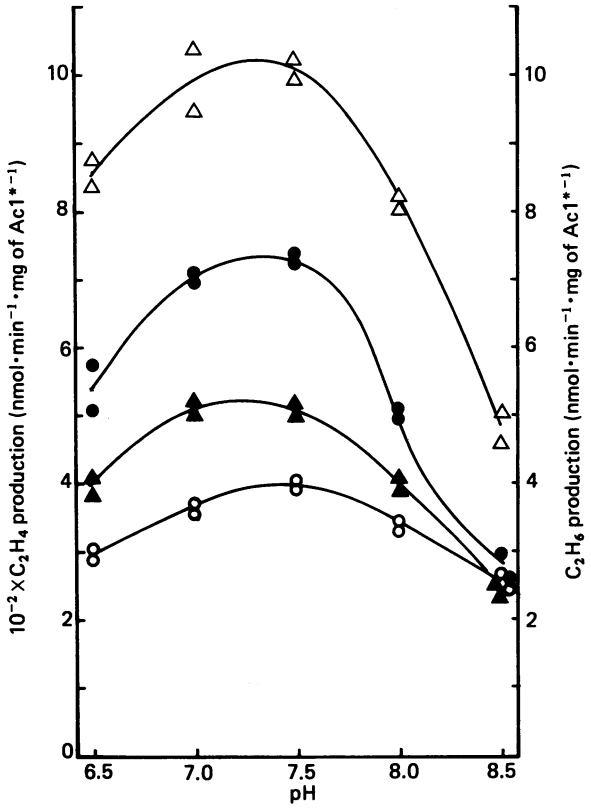

Fig. 3. Effect of pH on product formation by V-nitrogenase

Ac2* and Ac1* (molar ratio 9.1:1) were assayed under standard conditions, except that the initial pH was varied as indicated. △, H_2 evolution under Ar; ▲, H_2 evolution under C_2H_2 (16 kPa); ●, C_2H_6 formation and ○, C_2H_4 production, from C_2H_2 (16 kPa).

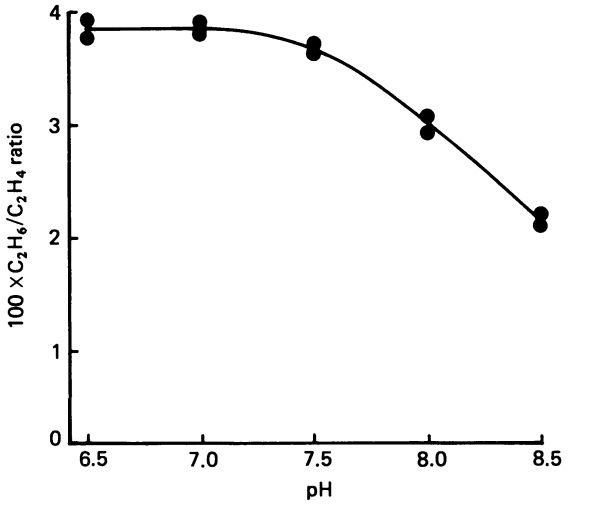

Fig. 4. Effect of pH on the ratio of C_2H_6 to C_2H_4 produced from C_2H_2

Values are derived from the data in Fig. 3.

4). The similarities of these pH profiles to those observed for Mo-nitrogenase (e.g. Dilworth & Thorneley, 1981) suggest that the variation in activity with pH is due more to its effect on the protein than on the intrinsic chemistry of substrate reduction.

Table 1. Effect of temperature on product distribution during C_2H_2 reduction to C_2H_4 and C_2H_6 by V-nitrogenase

The reduction of C_2H_2 to C_2H_4 and C_2H_6 by Ac2* and Ac1* (molar ratio 9.2:1) was measured under the standard assay conditions, except that the temperature was varied as indicated. Activity was measured over 3–15 min at the two highest temperatures, 5–30 min at 19.8 °C and 10–45 min at 10.8 °C to establish that reaction rates were linear. Total flux is expressed in total electron pairs (nmol)·min^{-1}·mg of protein^{-1}.

	Percentage flux			
Temp. (°C) ...	10.8	19.8	30.0	39.7
Total flux ...	2.96	20.8	51.9	75.9
Flux to H_2	37.9	45.7	54.6	61.3
Flux to C_2H_4	61.7	52.9	43.0	35.9
Flux to C_2H_6	0.4	1.4	2.4	2.9

The production of C_2H_6 and C_2H_4 from C_2H_2, and of H_2 evolution under Ar, were investigated over the temperature range 10.8–39.7 °C (Table 1). A marked change in electron allocation occurred over this range; C_2H_2 was a better substrate than H^+ at the lower temperatures, since as the temperature was lowered the rate of C_2H_2 reduction increased relative to the rate of H^+ reduction. The activity of the V-nitrogenase, as measured by H_2 evolution under Ar, extended to lower temperatures than the corresponding Mo-nitrogenase from *A. chroococcum* (R. W. Miller & R. R. Eady, unpublished work).

The C_2H_6/C_2H_4 ratio increased 11-fold over the same temperature range, implying that the activation energy for the formation of C_2H_6 is significantly higher than that for C_2H_4. If C_2H_6 production is to be used for an assay for V-nitrogenase *in vivo*, such assays should obviously be attempted at as high a temperature as practicable.

With the exception of the inhibitory effect of C_2H_2 on H_2 evolution, there are no published data concerning the inhibition of V-nitrogenase. In the Mo-nitrogenase system, H_2 is a specific competitive inhibitor of N_2 reduction, but affects the reduction of no other substrate (see Burgess, 1985), whereas CO inhibits the reduction of all substrates except H^+ (with the exception of the nitrogenase from the *nifV* mutant of *K. pneumoniae*; McLean *et al.*, 1983).

In the V-nitrogenase system, H_2 at 50 kPa inhibited N_2 reduction, but at 85 kPa had no effect on C_2H_2 reduction to either C_2H_4 or C_2H_6 (Table 2). C_2H_4 has recently been shown to inhibit H_2 evolution by the Mo-nitrogenase from *K. pneumoniae* (Ashby *et al.*, 1987). It is also an inhibitor of H_2 evolution by the V-nitrogenase of *A. chroococcum* (Table 2). It may also inhibit the reduction of C_2H_2 to C_2H_6, but this situation is far from clear. When C_2H_4 (85 kPa) was added to C_2H_2 (15 kPa), the resultant rate of C_2H_6 formation reached 10.2 nmol·min^{-1}·mg of Ac1*$^{-1}$ compared with the rate of 8.6 nmol·min^{-1}·mg of Ac1*$^{-1}$ under Ar (86 kPa) and C_2H_2 (15 kPa). Since C_2H_4 (86 kPa) itself led to a rate of C_2H_6 formation of 3.7 nmol·min^{-1}·mg of Ac1*$^{-1}$, the rates of C_2H_6 formation from C_2H_2 *plus* C_2H_4 were not additive, but it is not possible to decide which compound

Table 2. Effects of H_2 and C_2H_4 on reduction of C_2H_2 and N_2

The effect of H_2 and C_2H_4 as potential inhibitors of the reduction of Ac2* and Ac1* (molar ratio 10.4:1) of C_2H_2 to C_2H_6, and of N_2 to NH_3, was measured under standard assay conditions with the gas head-space composition as indicated.

Gas head-space composition	Specific activity (nmol·min^{-1}·mg of protein^{-1})			
Substrate ...	H^+	C_2H_2 (15 kPa)		N_2 (50 kPa)
Product ...	H_2	C_2H_4	C_2H_6	NH_3
Ar (101 kPa)	1094	426	8.5	265
H_2 (50 kPa)	–	–	–	137
H_2 (85 kPa)	–	430	8.6	–
C_2H_4 (85 kPa)	860	–	6.5	–

is an inhibitor of which reaction. It is clear, however, that a high partial pressure of C_2H_4 does not result in any large increase in C_2H_6 formation from C_2H_2.

CO did not inhibit H_2 evolution by V-nitrogenase at pressures up to 5 kPa (Fig. 5); it did, however, inhibit the reduction of C_2H_2 to either C_2H_4 or C_2H_6. Unlike the Mo-nitrogenase system, where 0.02 kPa of CO produced 50% inhibition of C_2H_2 reduction (Hardy, 1979), 1 kPa of CO resulted in only 45% inhibition (Fig. 5) of either C_2H_4 or C_2H_6 formation.

In metal carbonyls, the bond energies of the metal–carbon bonds increase with the atomic number of the metal (Connor, 1975). Weaker binding of CO to vanadium than to molybdenum would therefore be expected.

The time courses for H_2 evolution and C_2H_4 formation from C_2H_2 showed no detectable lag period before product formation became linear with time (Fig. 6). By contrast, C_2H_6 formation from C_2H_2 showed a consistent lag of several minutes before linear rates were attained. This lag suggests that an intermediate on the route to C_2H_6 accumulates slowly.

We can exclude the most obvious candidate, free C_2H_4, on the following grounds. Ethylene is itself slowly reduced to C_2H_6 by V-nitrogenase (Fig. 7), as it is by the Mo-nitrogenases of both *K. pneumoniae* and *A.*

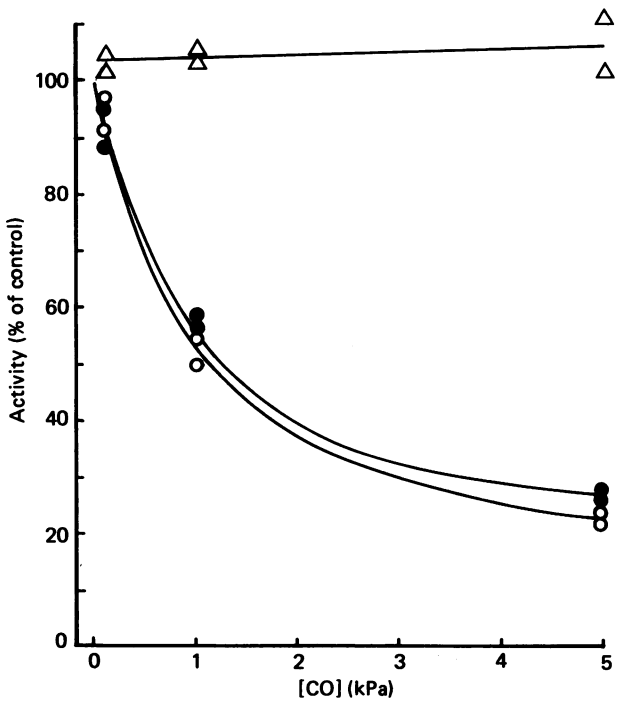

Fig. 5. Effect of CO on H_2 evolution and on the reduction of C_2H_2 to C_2H_4 and C_2H_6 by V-nitrogenase

Ac2* and Ac1* (molar ratio 9.4:1) were assayed under an atmosphere of Ar or Ar plus C_2H_2 (16 kPa) with the partial pressure of CO as indicated. △, H_2 under Ar; ●, C_2H_6 formation and ○, C_2H_4 production, from C_2H_2.

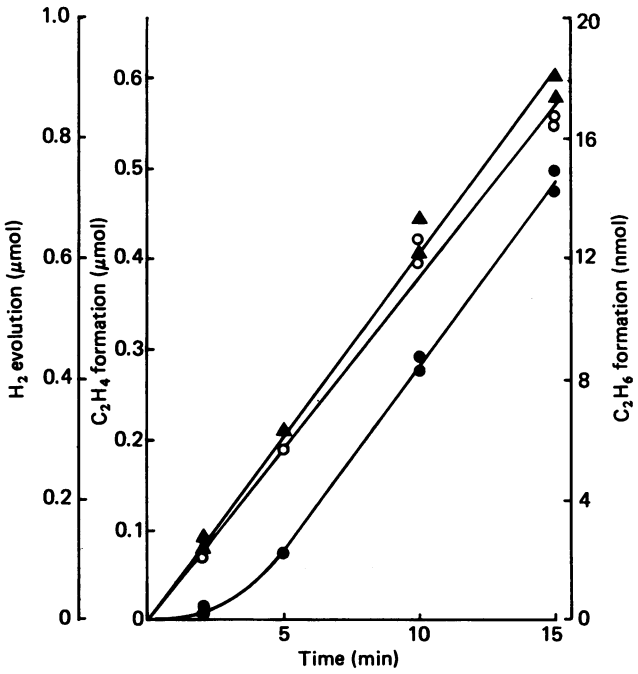

Fig. 6. Time courses of H_2 evolution, C_2H_4 and C_2H_6 formation from C_2H_2

Ac2* and Ac1* (molar ratio 6.6:1) were assayed for the times indicated under standard conditions before product analysis: ▲, H_2 evolution under C_2H_2; ○, C_2H_4 formation and ●, C_2H_6 formation, from C_2H_2.

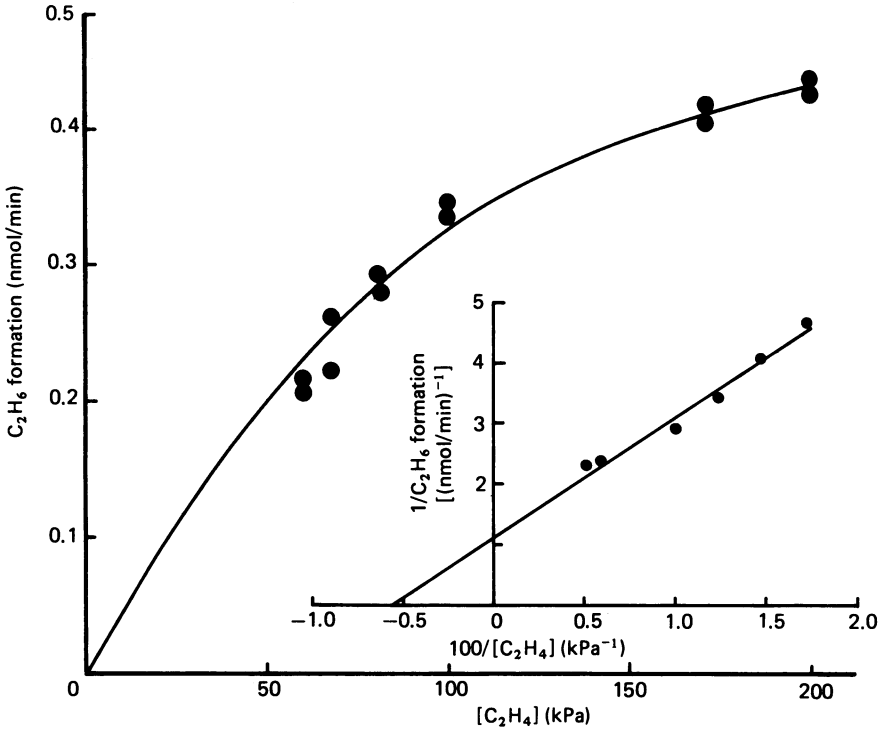

Fig. 7. C_2H_6 formation from C_2H_4 as a function of partial pressure of C_2H_4

Ac2* and Ac1* (molar ratio 9.4:1) were assayed under standard conditions, except that the gas phase contained C_2H_4 at partial pressures ranging from 60 to 200 kPa. Values in excess of 100 kPa were obtained as described by Ashby *et al.* (1987). The inset shows a double-reciprocal plot of the mean values of the same data.

chroococcum (Ashby *et al.*, 1987). With V-nitrogenase, the apparent K_m for C_2H_4 in this process is 172 kPa (cf. 130 kPa for Mo-nitrogenase from *K. pneumoniae*). After the lag time in assays of C_2H_2 reduction to C_2H_6, the total C_2H_4 formed from C_2H_2, namely 97 nmol in the experiment shown in Fig. 6, would, even if it were all to remain in solution, give rise to a solution concentration of only 0.097 mM. As this concentration is only about 1% of the apparent K_m (7.8 mM) for the reduction of C_2H_4 to C_2H_6, free C_2H_4 cannot be the intermediate whose synthesis produces the lag shown in C_2H_6 formation from C_2H_2. Further, the rate of C_2H_6 formation from C_2H_2 (15 kPa) is greater than that from C_2H_4 (101 kPa) (see Table 2).

The lag cannot at present be explained in terms of the Thorneley–Lowe (1985) model for nitrogenase function. C_2H_6 formation from C_2H_4, though slow, shows no lag (results not shown), so the route from C_2H_4 to C_2H_6 does not appear to require the slow accumulation of any intermediate. C_2H_4 formation from C_2H_2 is also linear with time (Fig. 6), so the formation of any intermediate common to C_2H_4 and C_2H_6 production seems unlikely to cause a lag in C_2H_6 formation. If C_2H_6 were formed from the same intermediate generated from C_2H_2 or C_2H_4, the same lag in C_2H_6 formation would be expected. To explain the lag in C_2H_6 formation from C_2H_2, it may be necessary to postulate different routes to C_2H_6 from C_2H_2 and C_2H_4.

Support for the existence of different routes to C_2H_6 from C_2H_2 and C_2H_4 comes from two further observations. Firstly, CO very effectively inhibits C_2H_6 formation from C_2H_4 (90% inhibition of 2 kPa; results not shown), whereas 40% of the activity for C_2H_6 formation from C_2H_2 remains at 2 kPa of CO (Fig. 5).

The second line of support comes from an experiment where V-nitrogenase was allowed to reduce C_2H_4 (101 kPa) for 6 min before injection of C_2H_2 (16 kPa). If C_2H_4 is able to generate intermediates in C_2H_6 formation which are only slowly formed from C_2H_2, preincubation of V-nitrogenase under C_2H_4 should eliminate the lag in C_2H_6 production from C_2H_2. However, the new rate of C_2H_6 formation after the injection of C_2H_2 still showed a lag of 2 min.

Taken together, these experiments support the idea of different routes to C_2H_6 depending on whether the substrate is C_2H_2 or C_2H_4. They also raise the question of whether entirely different metal centres are involved.

The failure of the V-nitrogenase to reduce propyne is consistent with the kinetics of the enzyme. For Mo-nitrogenase, the apparent K_m for C_2H_2 reduction to C_2H_4 of 0.6–2 kPa (Hardy, 1979) increases to about 50 kPa for propyne (McKenna, 1980). If there is a similar relative loss of substrate affinity for the V-nitrogenase, the apparent K_m for propyne reduction would be as high as 300 kPa, and reduction consequenty barely detectable.

The reason why V-nitrogenase produces C_2H_6 from C_2H_2, whereas Mo-nitrogenase does not, may well lie in the kinetics of C_2H_2 binding. In V-nitrogenase, C_2H_2 appears unable to affect the further reduction of whatever bound form of hydrocarbon exists on the enzyme, since increasing the C_2H_2 pressure from 2 to 30 kPa did not affect the C_2H_6/C_2H_4 ratio. By contrast, C_2H_2 (1 kPa) markedly inhibited the reduction of C_2H_4 to C_2H_6 with the Mo-nitrogenase from *K. pneumoniae* (Ashby *et al.*, 1987). Since both C_2H_2 and C_2H_4 can give rise to C_2H_6 with V-nitrogenase, the same experiment could only be attempted with labelled C_2H_2 or C_2H_4. Nevertheless it

seems probable that C_2H_2 effectively displaces C_2H_4 from the enzyme in Mo-nitrogenase as soon as it is formed, but does not affect V-nitrogenase in the same way, possibly because of weaker binding of C_2H_2 to the enzyme, indicated by the higher apparent K_m for C_2H_2.

A further indication that C_2H_2 can displace C_2H_4 from Mo-nitrogenase of *K. pneumoniae* comes from the e.p.r. studies of Lowe *et al.* (1978). The e.p.r. signal seen on exposure of the Mo-nitrogenase to C_2H_4 at 10 °C under turnover conditions was abolished by addition of C_2H_2.

What then are the likely chemical mechanisms of C_2H_2 reduction to C_2H_4 and to C_2H_6? Recent studies of the chemistry of alkene release from metal centres after alkyne reduction suggest that release occurs by a reversible β-elimination from a metal alkyl (Alt & Eicher, 1982). The metal alkyl itself can be further reduced to yield free alkane and metal centre (Nakamura & Otsuka, 1972).

With the Mo-nitrogenase from *K. pneumoniae*, where C_2H_6 can be formed from C_2H_4 but not from C_2H_2, Ashby *et al.* (1987) suggested that rapid binding of C_2H_2 to the metal hydride effectively displaces C_2H_4 from the metal centre in the enzyme, resulting in low concentrations of the metal alkyl intermediate for C_2H_6 formation. The higher apparent K_m for C_2H_2 for V-nitrogenase could therefore explain why C_2H_2 does not prevent C_2H_6 formation in this system.

We thank Dr. D. J. Lowe, Dr. R. L. Richards, and Dr. R. N. F. Thorneley for helpful and prolonged discussions, Professor J. R. Postgate for his comments on the manuscript, and Ms. Beryl Scutt for typing it.

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Received 6 May 1987/28 August 1987; accepted 1 October 1987