The molybdenum and vanadium nitrogenases of *Azotobacter chroococcum*: effect of elevated temperature on N_2 reduction

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During the reduction of N_2 by V-nitrogenase at 30 °C, some hydrazine (N_2H_4) is formed as a product in addition to NH_3 [Dilworth and Eady (1991) Biochem. J. **277**, 465–468]. We show here the following. (1) That over the temperature range 30–45 °C the apparent K_m for the reduction of N_2 to yield these products is the same, but increases from 30 to 58 kPa of N_2. On increasing the temperature from 45 °C to 50 °C, little change occurred in the rate of reduction of protons to H_2; the rate of N_2H_4 production increased, but the rate of NH_3 formation decreased 7-fold. (2) Temperature-shift experiments from 42 to 50 °C or from 50 to 42 °C showed that this selective loss of the ability to reduce N_2 to NH_3 was reversible. The effects we observe are consistent with the existence of different conformers of the VFe-protein at the two temperatures, that predominating at 50 °C being largely unable to reduce N_2 to ammonia. (3) Measurement of the ratio between H_2 evolution and N_2 reduced to NH_3 at N_2 pressures up to 339 kPa for both Mo- and V-nitrogenases gave limiting H_2/N_2 values of 1.13 ± 0.13 for Mo-nitrogenase and 3.50 ± 0.03 for V-nitrogenase. Since for Mo-nitrogenase our measured value for the ratio at 339 kPa is the same as that derived by Simpson and Burris [(1984) Science **224**, 1095–1097] at 5650 kPa, there appears to be little or no divergence from the predictions based on the apparent K_m for N_2. These data then suggest that there may be a fundamentally different mechanism for N_2 binding to V-nitrogenase compared with Mo-nitrogenase. (4) We did not detect any N_2H_4 as a product of N_2 reduction by Mo-nitrogenase over the temperature range investigated; however, at 50 °C this system reduced acetylene (C_2H_2) to yield some ethane (C_2H_6), in addition to ethylene (C_2H_4), a reaction normally associated with Mo-independent nitrogenases.

INTRODUCTION

Azotobacter chroococcum contains two types of nitrogenase: the extensively studied Mo-containing enzyme and the more recently discovered V-containing nitrogenase (Eady, 1990). Both enzymes have similar requirements for activity and similar physicochemical properties, both being two-component enzymes made up of a distinct Fe-protein and a MoFe- or VFe-protein in the Mo- and V-nitrogenases respectively. Despite this general overall similarity, the two enzymes display significant differences in substrate reduction in that the V-nitrogenase produces hydrazine (N_2H_4) as a minor product of N_2 reduction (Dilworth and Eady, 1991) and ethane as a minor product during acetylene reduction (Dilworth et al., 1988), reactions which are not normally shown by Mo-nitrogenase.

Studies on the effects of temperature on nitrogenase action are limited and have focused on the abrupt discontinuity at about 18–22 °C in the Arrhenius plot for the reduction of N_2, acetylene and H^+, and for ATP hydrolysis (see Hardy et al., 1968; Burns, 1969; Watt and Burns, 1977), which has been interpreted as arising from a sharp change in the temperature-dependence of complex-formation between the two protein components of Mo-nitrogenase (Thorneley et al., 1975). However, the underlying mechanism for this change is not understood. Comparative studies of the dependence of the rate of N_2 reduction on temperature with Mo- and V-nitrogenases have shown that N_2 remains a more effective substrate for the V-nitrogenase as the temperature is decreased from 30 to 5 °C than is the case for Mo-nitrogenase (Miller and Eady, 1988). N_2H_4 resulting from N_2 reduction by V-nitrogenase is also markedly influenced by temperature, increasing 17-fold between 20 and 40 °C (Dilworth and Eady, 1991), while Mo-nitrogenase does not produce free N_2H_4. Further, increasing temperature also increases the proportion of ethane relative to ethylene resulting from acetylene reduction with V-nitrogenase, while Mo-nitrogenase has been reported not to catalyse ethane formation from acetylene.

In the present work, we show that, at 50 °C, V-nitrogenase selectively and reversibly loses the ability to reduce N_2 to ammonia while continuing to form N_2H_4 and H_2. Over the temperature range 30–50 °C the apparent K_m for N_2 reduction to NH_3 or N_2H_4 increases for Mo- and V-nitrogenases, respectively. In contrast with its behaviour at 30 °C, Mo-nitrogenase is shown to produce ethane during acetylene reduction at 50 °C. The significance of these findings is discussed in the context of perturbations of cofactor–polypeptide interactions.

MATERIALS AND METHODS

The components of the V-nitrogenase of *A. chroococcum* MCD1155 were purified as described previously (Eady et al., 1987, 1988). When assayed under standard conditions at 30 °C, the specific activities were: VFe protein ($Ac1^V$), 1700 nmol of H_2 produced/min per mg of protein; Fe protein ($Ac2^V$), 1013 nmol of H_2 produced/min per mg of protein. The components of the Mo-nitrogenase were purified from strain MCD50 essentially as described by Yates and Planqué (1975) and had specific activities of 2350 nmol of H_2 produced/min per mg of MoFe protein ($Ac1^{Mo}$) and 1230 nmol H_2 produced/min per mg of Fe protein ($Ac2^{Mo}$). For reasons discussed below, all proteins were in 50 mM Hepes buffer, pH 7.4, containing 0.2 g/litre dithionite, but in the absence of $MgCl_2$.

Enzyme assays were conducted in glass serum vials (8 ml) capped with a rubber closure held in place by a screw cap perforated by a small hole to allow needle access for addition of gases and liquids, essentially as described by Eady et al. (1972). The assays contained, in a final volume of 1 ml, 25 μmol of Hepes buffer, pH 7.4, 5 μmol of ATP, 12.5 μmol of $MgCl_2$, 20 μmol of phosphocreatine, 100 μg of creatine phosphokinase

and 10 μmol of $Na_2S_2O_4$. Reactions were initiated by injection of nitrogenase components (0.015 ml of a mixture of nitrogenase components with a 4-fold molar excess of Fe protein). For assays conducted at 50 °C, and in assays subjected to temperature shifts, nitrogenase components (in buffer not containing $MgCl_2$), were pre-mixed with creatine phosphokinase and pre-equilibrated to the required temperature for 2 min before addition to a reaction mixture that did contain $MgCl_2$. This procedure was adopted because at 50 °C creatine kinase is unstable in the presence of Mg^{2+}, as indicated by precipitation occurring on prolonged incubation. We did not undertake a systematic study of this instability, but minimized the length of time that the kinase was exposed to Mg^{2+} at 50 °C. The linearity of product formation over the time period of the measurements (up to 6 min) indicates that inhibitory levels of MgADP did not accumulate and inhibit V-nitrogenase. In assays where the temperature was shifted up from 42 to 50 °C, or down over the same range, 95% temperature equilibration occurred within 30 s.

Assays were conducted in a Braun Certomat WR water bath shaking at 180 strokes/min over 2.5 cm. The change in pH of the assay mixtures at different temperatures as a consequence of the ΔpK_a/ °C of Hepes buffer is too small (0.28 pH) to affect the activity of V-nitrogenase significantly because of the broad pH optimum of the enzyme (see Dilworth et al., 1988).

In assays under an atmosphere of N_2 or Ar only, the gases were introduced by flushing for 15 min. Gas mixtures were prepared in the vial by injection followed by withdrawal of the same volume of mixed gases.

The reaction was terminated by injection of 0.3 ml of EDTA (pH 7.5; 0.1 M for V-nitrogenase and 0.4 M for Mo-nitrogenase); the higher EDTA concentration was necessary to completely inactivate Mo-nitrogenase. The products H_2, ethylene and ethane were measured by methods described in Ashby et al. (1987) and N_2H_4 as described by Dilworth and Eady (1991).

Where assays under hyperbaric pressures of N_2 (339 kPa) were required, a standard 0.1 ml of methane was injected, followed by the nitrogenase components, and then the appropriate volume of N_2. Excess pressure was relieved by venting immediately before injection of EDTA. After gas-chromatographic estimation of H_2 and methane on a molecular-sieve column (50 nm) fitted with a katharometer, the methane peak was used to calculate the full amount of H_2 produced during the assay. Although the katharometer was less sensitive for methane than flame-ionization detection, its use allowed both gases to be measured on the same sample.

To determine the amount of NH_3 produced, an aliquot of the EDTA-stopped reaction mixture was passed through a column (25 mm × 6 mm diam.) of Dowex-1X2 (Cl^-, 200–400 mesh) in a Pasteur pipette and the column washed with two 0.5 ml aliquots of water. The creatine concentration was measured in the combined eluate by the method of Ennor (1957), and the volume taken for NH_3 estimation adjusted to contain less than 1.5 μmol of creatine. NH_3 was then measured by the indophenol method as described by Dilworth and Thorneley (1981), and the values corrected for creatine interference from a standard calibration curve (Dilworth et al., 1992).

The ATP/2e ratio was determined from H_2 evolution under Ar and the amount of creatine in reaction mixtures which had been similarly passed through Dowex-1 columns.

RESULTS AND DISCUSSION

Effect of temperature on product formation by V-nitrogenase

Since with V-nitrogenase we have previously observed that the proportion of electron flux resulting in N_2H_4 formation increased over the temperature range 20–40 °C (Dilworth and Eady, 1991), the effect of higher temperatures was further studied. The data in Figure 1 show that N_2H_4 production increased 3-fold from 40 to 50 °C; surprisingly, while the rate of reduction of N_2 to NH_3 increased up to 45 °C, it decreased 7-fold between 45 and 50 °C. This abrupt decrease in the rate of reduction of N_2 to NH_3 cannot be due to simple inactivation of nitrogenase or creatine phosphokinase, since H_2 evolution under either N_2 or Ar does not change with this 5 °C temperature increase, and the rate of formation of H_2 was linear with time over the 5 min assay period. Further, NH_3 did not disappear when added to assays conducted at 50 °C, indicating that the low amounts of NH_3 found were not due to losses at high temperature.

While H_2 evolution increased steadily up to 45 °C, no further increase occurred between 45 and 50 °C. Under standard assay conditions at 30 °C and, indeed (as Figure 1 shows), up to 45 °C, the total of electron pairs utilized for the reduction of N_2 plus electron pairs resulting in H_2 evolution under N_2 is the same as for H_2 evolution under Ar. However, the decrease in NH_3 formation at 50 °C is not balanced by any increase in H_2 evolution.

The sudden decrease in NH_3 production at 50 °C is unlikely to be due to a large change in the efficiency of ATP utilization, a parameter known to affect product distribution, since the ATP/2e ratio measured for H_2 evolution under Ar varied only from 5.0 at 30 °C to 5.5 at 50 °C. The constancy of both the ATP/2e ratio and H_2 evolution under Ar indicates that the interaction of the Fe protein with MoFe protein and the coupling of ATP hydrolysis to electron transport is not seriously perturbed at the higher temperature.

Effect of temperature shifts on product formation by V-nitrogenase

We investigated how rapidly an increase in temperature was reflected in differences in the rates of product formation by shifting steady-state assays from 42 to 50 °C after 1.5 min reaction time. Figure 2(a) shows that the rate of H_2 evolution under N_2 showed only a marginal increase, as would be expected from the temperature profile shown in Figure 1. The adjustment in rate

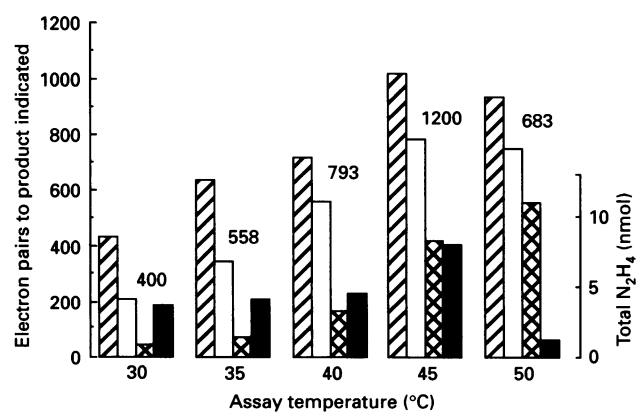


Figure 1 Effect of temperature on V-nitrogenase activity and product distribution

Assays were carried out for 5 min at the temperatures indicated, and assay mixtures contained 69 μg of $Ac1^V$ and 46 μg of $Ac2^V$. Products were determined as described in the Materials and methods section: ■, NH_3; ⊠, N_2H_4; □, H_2 under N_2; ▨, H_2 under Ar. Note that the scale for N_2H_4 as a product is expanded 50-fold. The numerical values for the total electron flux under N_2 at each temperature are shown on the Figure.

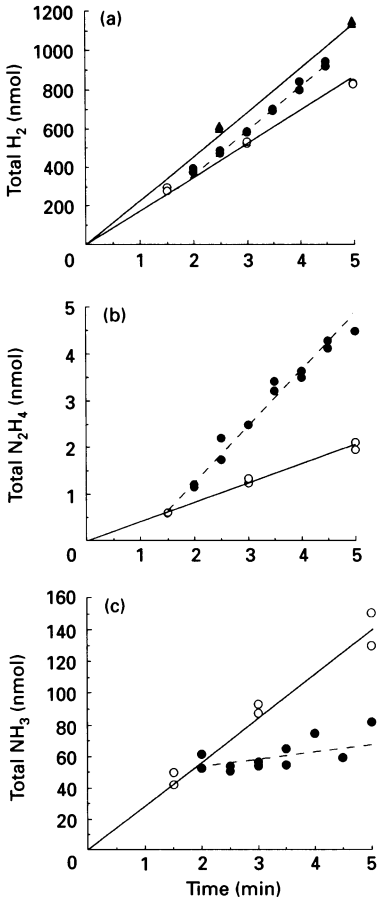


Figure 2 Effect of a temperature shift up from 42 to 50 °C on the rate of H_2 evolution under N_2 and product formation from N_2

Assay mixtures contained 130 μg of $Ac1^V$ and 154 μg of $Ac2^V$ under N_2 and the reactions were initiated at 42 °C; after 1.5 min the experimental assays were shifted to 50 °C, while the control assays under N_2 or Ar remained at 42 °C. The time course of the reaction was monitored over the subsequent 3.5 min, and NH_3, N_2H_4 and H_2 were measured as described in the Materials and methods section. (**a**): ▲, H_2 evolution under Ar at 50 °C; ○, H_2 evolution under N_2 at 42 °C; ●, H_2 evolution under N_2 following temperature shift to 50 °C. (**b**): ○, N_2H_4 production at 42 °C; ●, N_2H_4 production following temperature shift to 50 °C. (**c**): ○, NH_3 formation at 42 °C; ●, NH_3 formation following temperature shift to 50 °C.

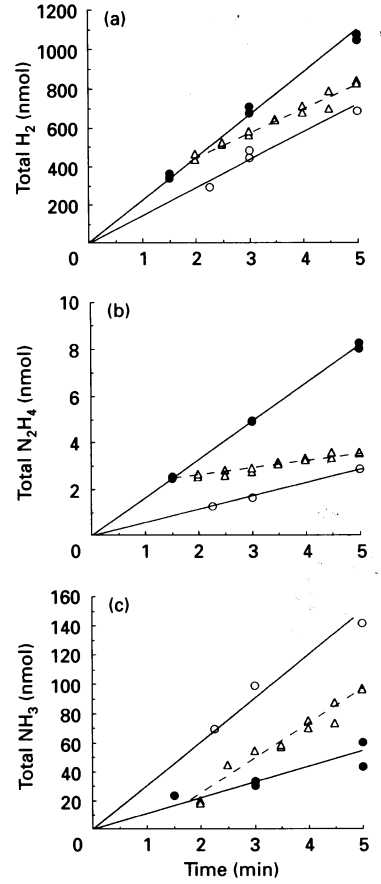


Figure 3 Effect of temperature shift-down from 50 to 42 °C on the rate of H_2 evolution under N_2 and product formation from N_2

Assay mixtures contained 130 μg of $Ac1^V$ and 154 μg of $Ac2^V$ and the reactions were initiated under N_2 at 50 °C; after 1.5 min the experimental assays were shifted to 42 °C, while control assays remained at 50 °C. The time course of the reaction was monitored over the subsequent 3.5 min, and NH_3, N_2H_4 and H_2 were measured as described in the Materials and method section. (**a**): ●, H_2 evolution under N_2 at 50 °C; ○, H_2 evolution under N_2 at 42 °C; △, H_2 evolution under N_2 following temperature shift to 42 °C. (**b**): ●, N_2H_4 production at 50 °C; △, N_2H_4 production following temperature shift to 42 °C; ○, N_2H_4 production in assays maintained at 42 °C. (**c**): ●, NH_3 formation at 50 °C; △, NH_3 formation following temperature shift to 42 °C; ○, NH_3 formation in assays maintained at 42 °C.

appeared to be complete within about 0.5 min. The rate of N_2H_4 formation increased nearly 3-fold, again within the same time frame (Figure 2b), while the rate of NH_3 production decreased to 27% of its rate at 42 °C, also within 0.5 min (Figure 2c).

Temperature shift-down experiments from 50 to 42 °C were used to determine whether the change(s) to the enzyme at 50 °C were reversible. The rate of H_2 evolution under N_2 fell back essentially to the control rate at 42 °C (Figure 3a), while N_2H_4 formation fell (Figure 3b). Although this step-down experiment has been performed several times, the rate of N_2H_4 formation after step-down was always less than the control rate of assays maintained at 42 °C throughout. The reasons for this difference remain unknown. However, NH_3 production by assays shifted from 50 to 42 °C returned to the control rate at 42 °C (Figure 3c). For all three products, the changes in rate appeared to be complete within 0.5 min.

Temperature clearly alters N_2 reduction by V-nitrogenase in a rapid and reversible manner. The effects we observe are consistent with the existence of different conformers of the VFe-protein at

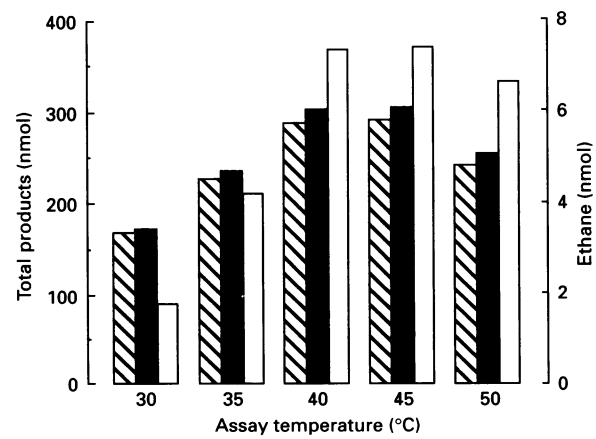


Figure 4 Effect of temperature on the rate of formation of ethylene and ethane as products of C_2H_2 reduction by V-nitrogenase

Assays were conducted for 5 min under Ar containing 10 kPa of C_2H_2 and 69 μg of $Ac1^V$, 46 μg of $Ac2^V$, and the products were separated as described in the Materials and methods section. ▨, ethylene; □, ethane; ■, total electron pairs used in acetylene reduction.

the two temperatures, that predominating at 50 °C being largely unable to reduce N_2 to NH_3.

Another characteristic of V-nitrogenase which has distinguished it from Mo-nitrogenase is its ability to catalyse the reduction of ethylene to ethane. The temperature-dependence of the reduction of acetylene to ethylene and ethane is shown in Figure 4. Over the temperature range 30–40 °C all products increased linearly, reaching a maximum at 45 °C, but showing only a marginal decrease between 45 and 50 °C. This is clearly different from the behaviour of the enzyme during N_2 reduction to NH_3.

Effect of temperature on kinetics of V-nitrogenase

Both Mo- and V-nitrogenases are routinely assayed at 30 °C, and there are consequently no data of which we are aware on the effect of temperature on apparent K_m for N_2. One possible explanation for the failure of N_2 to give rise to NH_3 at 50 °C is that N_2 binds with very different affinity to different sites on V-nitrogenase which lead to the formation of NH_3 or N_2H_4.

The apparent K_m of V-nitrogenase for N_2 was therefore measured at temperatures ranging from 30 to 50 °C. The data in Table 1 show that the apparent K_m for N_2 increases markedly over this temperature range, but is the same for both NH_3 and N_2H_4 (where both can be measured) at any particular temperature. These data suggest that N_2 binds at a single type of binding site and generates an intermediate with the potential to be reduced further either to NH_3 or N_2H_4 (as discussed by Dilworth and Eady, 1991). Consistent with this view is the finding that at 50 °C the inhibition by H_2 of N_2H_4 production is competitive with respect to N_2, as it is for NH_3 formation at 42 °C (results not shown). The differential effect of temperature on the rates of formation of NH_3 or N_2H_4 by V-nitrogenase over the range 30–45 °C would then imply that the activation energies for the conversion of an enzyme-bound dinitrogen hydride intermediate into these products must be significantly different (see Dilworth and Eady, 1991). At 50 °C, different activation energies cannot adequately explain why the rate of formation of NH_3 decreases to 27% of its rate at 45 °C. Similarly, an explanation involving different activation energies for reactions leading to H_2 evolution or to NH_3 formation is inconsistent with the fact that no compensating increase in H_2 evolution is observed when N_2 reduction to NH_3 is curtailed at 50 °C.

The best characterized aqueous system based on vanadium which reduces N_2 is the V^{II}–catechol complex formed at highly alkaline pH values (Nikonova and Shilov, 1977). This system provides a useful model for nitrogenase in that it reduces N_2 to NH_3, it also reduces H^+ to H_2, a reaction which is inhibited by N_2, and produces low levels of N_2H_4 when the reacting system is quenched with acid (as does Mo-nitrogenase; Thorneley et al., 1978). A second system based on vanadium is the heterogeneous V^{II} Mg alkaline gel, which catalyses the reduction of N_2 to both NH_3 and N_2H_4. The environment of the vanadium in this system is obviously very different from that in the VFe protein of V-nitrogenase; nevertheless it shows a temperature-dependence in product ratio. However, unlike our findings for V-nitrogenase, the product ratio shifts to favour NH_3 over N_2H_4 at higher temperatures (Shilov, 1977).

Effect of temperature on Mo-nitrogenase activity

For Mo-nitrogenase, for which the apparent K_m for N_2 reduction to NH_3 also increased 3-fold from 30 to 50 °C (Table 1), the rate of NH_3 formation under 56 kPa of N_2 at 50 °C is in fact higher than at 30 °C, showing that the dramatic decline in NH_3 formation shown by the V-nitrogenase does not occur.

Table 1 Apparent K_m (kPa) for N_2 as a function of temperature for Mo- and V-nitrogenases

The assay times varied between 5 and 30 min, depending upon the temperature; the times used were such that product formation was linear. The assay conditions and product analyses were as described in the Materials and methods section. Apparent K_m values were calculated using direct linear plots (Cornish-Bowden and Eisenthal, 1974).

		K_m (kPa)			
Enzyme	Temperature (°C)	30	35	42	50
V-nitrogenase					
N_2 to N_2H_4		30	40	58	93
N_2 to NH_3		29	44	57	N/A*
Mo-nitrogenase					
N_2 to NH_3		19	–	–	57

* N/A, not accessible due to low rates of N_2 conversion into NH_3.

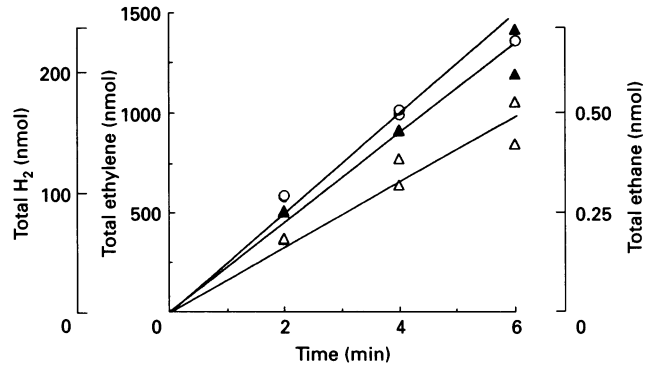


Figure 5 Time course of the reduction of acetylene to ethylene and ethane by Mo-nitrogenase at 50 °C

Assays were performed at 50 °C under an atmosphere of Ar containing 10 kPa of acetylene, $Ac1^{Mo}$ (109 μg) and $Ac2^{Mo}$ (126 μg); the reaction was terminated at the times indicated, and the reaction products were measured as described in the Materials and methods section. ○, H_2; ▲, ethylene; △, ethane. Note that the scale for the amount of ethane formed is expanded 2000-fold relative to that for ethylene as a product.

In addition, Mo-nitrogenase did not give rise to any detectable N_2H_4 as a product at temperatures up to 50 °C. However, temperature did affect product formation by Mo-nitrogenase; at 50 °C the enzyme catalysed the formation of a small amount of ethane from acetylene (Figure 5), a reaction not observed within the temperature range 20 °C to 40 °C (Dilworth et al., 1988).

H_2 evolution under N_2 by Mo- and V-nitrogenases

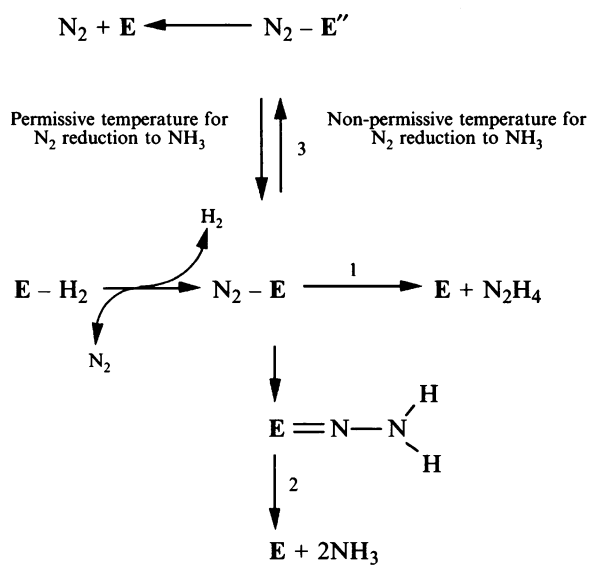
Extensive studies of the mechanism of Mo-nitrogenase have suggested that, under N_2, the evolution of a minimum of 1 mol of H_2/mol of N_2 reduced is obligatory (Thorneley and Lowe, 1985). A crucial experiment supporting such a conclusion was performed at 5650 kPa of N_2, giving a limiting result of 1.13 ± 0.13 (H_2/N_2 ratio) (Simpson and Burris, 1984). However, since the apparent K_m for N_2 reduction by Mo-nitrogenase at 30 °C is in the range 6–22 kPa, effective saturation should be achieved at much lower pressures than 5650 kPa. Using our measured value of 19 kPa of N_2 at 30 °C leads to the conclusion that, at 339 kPa (3 atm)-N_2, the enzyme should be functioning at 94% of its

maximum rate. Accordingly, we have measured the ratio between H_2 evolution and N_2 reduced to NH_3 at N_2 pressures up to 339 kPa for both Mo- and V-nitrogenases. The assays contained a 20-fold molar excess of Fe protein, conditions which maximize electron allocation to N_2. The limiting values we obtained were 1.13 ± 0.13 for Mo-nitrogenase and 3.50 ± 0.03 for V-nitrogenase. Since for Mo-nitrogenase our measured value for the ratio at 339 kPa is the same as that derived by Simpson and Burris (1984) at 5650 kPa, there appears to be little or no divergence from the predictions based on the apparent K_m for N_2. On the basis of similar calculations for V-nitrogenase and an apparent K_m of 29 kPa-N_2 at 30 °C (Table 1), the value of 3.5 for the H_2/N_2 ratio at 339 kPa-N_2 would suggest a limiting value of 3.0–3.2 mol of H_2 evolved/mol of N_2 reduced. These data then suggest that there may be a fundamentally different mechanism for N_2 binding or reduction for the V-nitrogenase compared with Mo-nitrogenase.

The most detailed model for the mechanism of reduction of N_2 by Mo-nitrogenase is consistent with H_2 being displaced from the MoFe protein by N_2 from a species which is three electrons reduced compared with the resting state (Thorneley and Lowe, 1985). Pre-steady-state analysis of the rates of product formation shows that the initial rate of H_2 evolution is inhibited as N_2 is reduced to NH_3. If the difference in stoichiometry between H_2 evolution and N_2 reduction between Mo- and V-nitrogenases discussed above is mechanistically significant, pre-steady-state studies of the V-nitrogenase should show different kinetics with respect to H_2 production.

Among the range of substrates reduced by nitrogenase, N_2 has the most stringent requirements for reduction, and this activity can be lost preferentially when Mo-nitrogenase is modified. When site-directed mutations are made at residues (Gln^{191} or His^{195}) located in the putative FeMoco (iron- and molybdenum-containing cofactor)-binding domain in the α-subunit of the MoFe protein, the ability to reduce N_2 is lost, but acetylene remains an effective substrate. Furthermore, acetylene reduction by the mutant MoFe protein results in the formation of significant amounts of ethane as well as ethylene (Scott et al., 1990). A similar pattern for substrate reduction is observed when homocitrate [(*R*)-2-hydroxybutane-1,2,4-tricarboxylic acid], a normal constituent of FeMoco, is replaced with other homocitrate analogues (Madden et al., 1990). These data indicate that perturbation of subtle interactions between FeMoco and the MoFe protein can result in the selective loss of the ability to reduce N_2 and in a change in the products of acetylene reduction. In the present case, it is apparent that, when Mo-nitrogenase is assayed at 50 °C, the MoFe protein assumes a conformation in which these interactions are only partially disrupted.

The VFe protein of the V-nitrogenase has a vanadium- and iron-containing cofactor (FeVaco) analogous to the FeMoco in MoFe proteins. Transfer of isolated FeVaco to the inactive MoFe protein synthesized by a *nifB* mutant of *Klebsiella pneumoniae* results in a hybrid protein which has the characteristic substrate-reducing properties of VFe protein with respect to acetylene, is capable of reducing H^+ to H_2, but is unable to reduce N_2 (Smith et al., 1988). These observations provided the first indication of the subtlety of the cofactor–polypeptide interactions which have subsequently been identified between FeMoco and the MoFe protein polypeptides. Comparison of the DNA sequences of the genes encoding the α-subunits of MoFe and VFe proteins (see Pau, 1991) show that the residues implicated as forming part of the FeMoco-binding site of MoFe proteins are conserved in the VFe proteins. These residues are therefore likely candidates for interacting with FeVaco and defining substrate specificity. In addition, there is strong circumstantial evidence that FeVaco contains homocitrate, based on the requirement for *nifV* (a gene showing DNA sequence similarity to that of homocitrate synthase; Dean and Jacobson, 1992) for the synthesis of functional N_2-reducing V-nitrogenase.



Scheme 1 How product formation from N_2 may occur with V-nitrogenase

This scheme assumes that the enzyme-bound dinitrogen hydride species proposed as intermediates in the reduction of N_2 to NH_3 by Mo-nitrogenase by the multi-step process 2 (Thorneley and Lowe, 1985) are also valid for V-nitrogenase. The bound dinitrogen species which gives rise to N_2H_4 as a product by reaction 1 is assumed to occur with V-nitrogenase. The reversible reaction 3 represents the temperature-dependent conformational change to E″ which results in the suppression the reduction of N_2 to NH_3 at temperatures above 45 °C, but does not perturb the rate of N_2H_4 formation.

Scheme 1 summarizes how product formation from N_2 may occur with V-nitrogenase. The binding of N_2 occurs by displacement of H_2 displacement, as suggested for Mo-nitrogenase; whatever the form of co-ordinated N_2, it can be reduced via a set of enzyme-bound intermediates to give NH_3, essentially as proposed (Thorneley and Lowe, 1985) for Mo-nitrogenase. Since bridging dinitrogen in metal complexes can under some circumstances give rise to N_2H_4, V-nitrogenase could give rise to N_2H_4 in such a way rather than from an intermediate directly involved in NH_3 formation, as previously suggested (Dilworth and Eady, 1991). In either case, the relative activation energies presumably control the effects of temperature on the balance between NH_3 and N_2H_4 as products. At 50 °C, the enzyme would be in a form essentially unable to produce NH_3; it remains to be seen whether this represents a form which can carry out none of the multi-step process 2 (as implied in Scheme 1) or a form where only the later ones, following a reduced intermediate common with the N_2H_4 pathway, are inhibited.

The effect of high temperature in apparently preventing N_2 reduction to NH_3 by V-nitrogenase could be explained as resulting from perturbation of interactions of FeVaco with important amino acid residues in the α-subunit of the VFe protein. If so, and bearing in mind that the liberation of N_2H_4 as a product implies that a four-electron reduced dinitrogen hydride species may exist on the enzyme, these residues are implicated in the final protonation step to yield NH_3. The reversible nature of the high-temperature switch-off in NH_3 formation is consistent with the predominant conformation of the VFe protein at 50 °C being one which does not allow these interactions to occur.

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