During the reduction of \( N_2 \) by V-nitrogenase at 30 °C, some hydrazine (NH\(_2\)H\(_2\)) 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 \( NH_4 \) 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\(_2\)H\(_2\)) to yield some ethane (C\(_2\)H\(_6\)), in addition to ethylene (C\(_2\)H\(_4\)), a reaction normally associated with Mo-independent nitrogenases.

**INTRODUCTION**

*Azo*batrer* 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\(_2\)H\(_4\)) 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\(_2\), 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\(_2\)H\(_4\) 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 \( N_2\)H\(_4\). 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\(_2\)H\(_4\) and H\(_2\). Over the temperature range 30–50 °C the apparent \( K_m \) for \( N_2 \) reduction to NH\(_3\) or N\(_2\)H\(_4\) 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 *Azotobacter 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 (Ac\(_{1+}^V\)), 1700 nmol of H\(_2\) produced/min per mg of protein; Fe protein (Ac\(_{2+}^V\)), 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 Planché (1975) and had specific activities of 2350 nmol of H\(_2\) produced/min per mg of MoFe protein (Ac\(_{1+}^M\)) and 1230 nmol of H\(_2\) produced/min per mg of Fe protein (Ac\(_{2+}^M\)). 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 Na₂S₂O₄. 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 MgCl₂), 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 MgCl₂. This procedure was adopted because at 50 °C creatine kinase is unstable in the presence of Mg²⁺, 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 Mg²⁺ 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 ΔpKₐ °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 N₂ 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 H₂, ethylene and ethane were measured by methods described in Ashby et al. (1987) and N₂H₄ as described by Dilworth and Eady (1991).

Where assays under hyperbaric pressures of N₂ (339 kPa) were required, a standard 0.1 ml of methanol was injected, followed by the nitrogenase components, and then the appropriate volume of N₂. Excess pressure was relieved by venting immediately before injection of EDTA. After gas-chromatographic estimation of H₂ and methane on a molecular-sieve column (50 nm) fitted with a katharometer, the methane peak was used to calculate the full amount of H₂ 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 NH₃ produced, an aliquot of the EDTA-stopped reaction mixture was passed through a column (25 mm x 6 mm diam.) of Dowex-1×2 (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 NH₃ estimation adjusted to contain less than 1.5 μmol of creatine. NH₃ 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 H₂ 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 N₂H₄ 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 N₂H₄ production increased 3-fold from 40 to 50 °C; surprisingly, while the rate of reduction of N₂ to NH₃ increased up to 45 °C, it decreased 7-fold between 45 and 50 °C. This abrupt decrease in the rate of reduction of N₂ to NH₃ cannot be due to simple inactivation of nitrogenase or creatine phosphokinase, since H₂ evolution under either N₂ or Ar does not change with this 5 °C temperature increase, and the rate of formation of H₂ was linear with time over the 5 min assay period. Further, NH₃ did not disappear when added to assays conducted at 50 °C, indicating that the low amounts of NH₃ found were not due to losses at high temperature.

While H₂ 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 N₂ plus electron pairs resulting in H₂ evolution under N₂ is the same as for H₂ evolution under Ar. However, the decrease in NH₃ formation at 50 °C is not balanced by any increase in H₂ evolution.

The sudden decrease in NH₃ 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 H₂ 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 H₂ 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 H₂ evolution under N₂ 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](image-url)
Assay mixtures contained 130 μg of Ac1 and 154 μg of Ac2 under N2 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 N2 or Ar remained at 42 °C. The time course of the reaction was monitored over the subsequent 3.5 min, and NH3, N2H4 and H2 were measured as described in the Materials and methods section. 

(a): Δ, H2 evolution under Ar at 50 °C; ○, H2 evolution under N2 at 42 °C; ●, H2 evolution under N2 following temperature shift to 50 °C. 

(b): ○, N2H4 production at 42 °C; ●, N2H4 production following temperature shift to 50 °C. 

(c): ○, NH3 formation at 42 °C; ●, NH3 formation following temperature shift to 50 °C.

Figure 2 Effect of a temperature shift up from 42 to 50 °C on the rate of H2 evolution under N2, and product formation from N2.

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 H2 evolution under N2 fell back essentially to the control rate at 42 °C (Figure 3a), while N2H4 formation fell (Figure 3b). Although this step-down experiment has been performed several times, the rate of N2H4 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, NH3 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 N2 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 42 °C. Although the rate at 42 °C decreased about 27% of its rate at 42 °C, also within 0.5 min (Figure 2c).

Figure 3 Effect of temperature shift-down from 50 to 42 °C on the rate of H2 evolution under N2, and product formation from N2.

Effect of elevated temperature on N2, reductase activity by Mo- and V-nitrogenases

Assay mixtures contained 130 μg of Ac1 and 154 μg of Ac2 and the reactions were initiated under N2 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 NH3, N2H4 and H2 were measured as described in the Materials and methods section. 

(a): ●, H2 evolution under N2 at 50 °C; ○, H2 evolution under N2 at 42 °C; Δ, H2 evolution under N2 following temperature shift to 42 °C. 

(b): ●, N2H4 production at 50 °C; Δ, N2H4 production following temperature shift to 42 °C; ○, N2H4 production in assays maintained at 42 °C. 

(c): ○, NH3 formation at 50 °C; Δ, NH3 formation following temperature shift to 42 °C; ○, NH3 formation in assays maintained at 42 °C.

Figure 4 Effect of temperature on the rate of formation of ethylene and ethane as products of C2H2 reduction by V-nitrogenase

Assays were conducted for 5 min under Ar containing 10 kPa of C2H2 and 69 μg of Ac1, 46 μg of Ac2, 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 \( \text{N}_2 \) to \( \text{N}_3 \).

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 \( \text{N}_2 \) reduction to \( \text{NH}_3 \).

**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 \( \text{N}_2 \). One possible explanation for the failure of \( \text{N}_2 \) to give rise to \( \text{NH}_3 \) at 50 °C is that \( \text{N}_2 \) binds with very different affinity to different sites on V-nitrogenase which lead to the formation of \( \text{NH}_3 \) or \( \text{N}_2\text{H}_4 \).

The apparent \( K_m \) of V-nitrogenase for \( \text{N}_2 \) was therefore measured at temperatures ranging from 30 to 50 °C. The data in Table 1 show that the apparent \( K_m \) for \( \text{N}_2 \) increases markedly over this temperature range, but is the same for both \( \text{NH}_3 \) and \( \text{N}_2\text{H}_4 \) (where both can be measured) at any particular temperature. These data suggest that \( \text{N}_2 \) binds at a single type of binding site and generates an intermediate with the potential to be reduced further either to \( \text{NH}_3 \) or \( \text{N}_2\text{H}_4 \) (as discussed by Dilworth and Eady, 1991). Consistent with this view is the finding that at 50 °C the inhibition by \( \text{H}_2 \) of \( \text{N}_2\text{H}_4 \) production is competitive with respect to \( \text{N}_2 \), as it is for \( \text{NH}_3 \) formation at 42 °C (results not shown). The differential effect of temperature on the rates of formation of \( \text{NH}_3 \) or \( \text{N}_2\text{H}_4 \) by V-nitrogenase over the range 30–45 °C would then imply that the activation energies for the conversion of an enzyme-bound dihydrogen 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 \( \text{NH}_3 \) decreases to 27% of its rate at 45 °C. Similarly, an explanation involving different activation energies for reactions leading to \( \text{H}_2 \) evolution or to \( \text{NH}_3 \) formation is inconsistent with the fact that no compensating increase in \( \text{H}_2 \) evolution is observed when \( \text{N}_2 \) reduction to \( \text{NH}_3 \) is curtailed at 50 °C.

The best characterized aqueous system based on vanadium which reduces \( \text{N}_2 \) is the \( \text{V}^{11-} \)-catechol complex formed at highly alkaline pH values (Nikonova and Shilov, 1977). This system provides a useful model for nitrogenase in that it reduces \( \text{N}_2 \) to \( \text{NH}_3 \), it also reduces \( \text{H}^+ \) to \( \text{H}_2 \), a reaction which is inhibited by \( \text{N}_2 \), and produces low levels of \( \text{N}_2\text{H}_4 \) 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 \( \text{V}^{11-} \) \( \text{Mg} \) alkaline gel, which catalyses the reduction of \( \text{N}_2 \) to both \( \text{NH}_3 \) and \( \text{N}_2\text{H}_4 \). 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 \( \text{NH}_3 \) over \( \text{N}_2\text{H}_4 \) at higher temperatures (Shilov, 1977).

**Effect of temperature on Mo-nitrogenase activity**

For Mo-nitrogenase, for which the apparent \( K_m \) for \( \text{N}_2 \) reduction to \( \text{NH}_3 \) also increased 3-fold from 30 to 50 °C (Table 1), the rate of \( \text{NH}_3 \) formation under 56 kPa of \( \text{N}_2 \) at 50 °C is in fact higher than at 30 °C, showing that the dramatic decline in \( \text{NH}_3 \) formation shown by the V-nitrogenase does not occur.

In addition, Mo-nitrogenase did not give rise to any detectable \( \text{N}_2\text{H}_4 \) 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 \( \text{N}_2 \), the evolution of a minimum of 1 mol of \( \text{H}_2 \)/mol of \( \text{N}_2 \) reduced is obligatory (Thorneley and Lowe, 1985). A crucial experiment supporting such a conclusion was performed at 5650 kPa of \( \text{N}_2 \), giving a limiting result of 1.13 ± 0.13 (\( \text{H}_2 \)/\( \text{N}_2 \) ratio) (Simpson and Burris, 1984). However, since the apparent \( K_m \) for \( \text{N}_2 \) 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 \( \text{N}_2 \) at 30 °C leads to the conclusion that, at 339 kPa (3 atm)-\( \text{N}_2 \), the enzyme should be functioning at 94% of its

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**Table 1. Apparent \( K_m \) (kPa) for \( \text{N}_2 \) 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).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Temperature (°C)</th>
<th>30</th>
<th>35</th>
<th>42</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-nitrogenase</td>
<td>( \text{N}_2 ) to ( \text{N}_2\text{H}_4 )</td>
<td>30</td>
<td>40</td>
<td>58</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>( \text{N}_2 ) to ( \text{NH}_3 )</td>
<td>29</td>
<td>44</td>
<td>57</td>
<td>N/A*</td>
</tr>
<tr>
<td>Mo-nitrogenase</td>
<td>( \text{N}_2 ) to ( \text{NH}_3 )</td>
<td>19</td>
<td>–</td>
<td>–</td>
<td>57</td>
</tr>
</tbody>
</table>

* N/A, not accessible due to low rates of \( \text{N}_2 \) conversion into \( \text{NH}_3 \).

**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 \( \text{Ar} \) containing 10 kPa of acetylene, \( \text{Ac}^{18} \) (109 μg) and \( \text{Ac}^{16} \) (126 μg); the reaction was terminated at the times indicated, and the reaction products were measured as described in the Materials and methods section. ◇, \( \text{H}_2 \); ▲, ethylene; △, ethane. Note that the scale for the amount of ethane formed is expanded 2000-fold relative to that for ethylene as a product.

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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 $1.13 \pm 0.13$ for Mo-nitrogenase and $3.50 \pm 0.03$ 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 $K_a$ for $N_2$. On the basis of similar calculations for V-nitrogenase and an apparent $K_a$ 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 (Thornerley 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$^{190}$ or His$^{360}$) located in the putative FeMoco (iron- and molybdenum-containing cofactor)-binding domain in the $\alpha$-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-1,4,2-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 nifV 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 $H_2$ to $H_2$, but is unable to reduce $N_2$ (Smith et al., 1988). These observations provide the first indication of the subtlety of the cofactor–peptide interactions which have subsequently been identified between FeMoco and the MoFe protein. The amino acid sequences of the DNA sequences of the genes encoding the $\alpha$-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 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 $\alpha$-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.

![Scheme 1](image-url)
We thank Professor B. E. Smith, Dr. R. L. Richards and Dr. D. J. Lowe for helpful discussions, and the Underwood Fund of the Agriculture and Food Research Council for financial assistance to M. J. D.

REFERENCES


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