Hydrazine is a product of dinitrogen reduction by the vanadium-nitrogenase from *Azotobacter chroococcum*

Michael J. DILWORTH* and Robert R. EADY†

*School of Biological and Environmental Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia, and †A.F.R.C. Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, U.K.

During the enzymic reduction of N₂ to NH₃ by Mo-nitrogenase, free hydrazine (N₂H₄) is not detectable, but an enzyme-bound intermediate can be made to yield N₂H₄ by quenching the enzyme during turnover [Thorneley, Eady & Lowe (1978) Nature (London) 272, 557–558]. In contrast, we show here that the V-nitrogenase of *Azotobacter chroococcum* produces a small but significant amount of free N₂H₄ (up to 0.5% of the electron flux resulting in N₂ reduction) as a product of the reduction of N₂. The amount of N₂H₄ formed increased 15-fold on increasing the assay temperature from 20 °C to 40 °C. Activity cross-reactions between nitrogenase components of Mo- and V-nitrogenases showed that the formation of free N₂H₄ was associated with the VFe protein. These data provide the first direct evidence for an enzyme intermediate at the four-electron-reduced level during the reduction of N₂ by V-nitrogenase.

**INTRODUCTION**

The reduction of N₂ by Mo-nitrogenase involves an enzyme-bound dinitrogen hydride intermediate, which can be made to yield hydrazine (N₂H₄) by quenching the enzyme with acid or alkali during turnover under N₂ (Thorneley *et al.*, 1978). Free N₂H₄ has never been detected as a product of N₂ reduction by nitrogenase, despite repeated attempts to do so (Bach, 1957; Garcia-Rivera & Burris, 1967). The generally accepted mechanism for nitrogenase action assumes a series of enzyme-bound dinitrogen hydride species, though the existence and identity of these species can only be inferred by analogy to the chemistry of defined transition-metal–N₂ complexes (Thorneley & Lowe, 1985). The recently characterized V-nitrogenase of *Azotobacter* has very similar physicochemical properties to those of Mo-nitrogenase (Robson *et al.*, 1986; Hales *et al.*, 1986), but differs in the specificity of product formation from a number of substrates (Dilworth *et al.*, 1987; Miller & Eady, 1988).

The Mo- and V-nitrogenases of *Azotobacter chroococcum* and *Azotobacter vinelandii* are similar in that both are two-protein systems. The Mo-nitrogenase consists of a specific Fe protein and a MoFe protein, whereas the V-nitrogenase has a genetically distinct Fe protein associated with a VFe protein. The site of N₂ reduction on the MoFe protein is thought to be an FeMo cofactor (FeMoco) (Hawkes *et al.*, 1984); an analogous cofactor containing iron and vanadium (FeV cofactor, FeVaco) is present in the VFe protein (Smith *et al.*, 1988), and X-ray absorption spectroscopy has shown that the chemical environments of Mo and V in these enzymes are very similar (Arber *et al.*, 1987; George *et al.*, 1988). The MoFe protein is active when assayed with the Fe protein from the V-nitrogenase, and the VFe protein forms an active nitrogenase with the Fe protein of Mo-nitrogenase.

Despite these similarities, the V-nitrogenases evolve much more H₂ during N₂ reduction than do the Mo-nitrogenases, which, when functioning under optimal conditions, have the limiting stoichiometry (Simpson & Burris, 1984) represented by the equation:

\[
N_2 + 8H^+ + 8e^- = 2NH_3 + H_2
\]

H₂ evolution accompanying N₂ reduction by Mo-nitrogenase, which accounts for at least 25% of the electron flux, is likely to be unavoidable, since it is thought to be a consequence of the binding of N₂ occurring via a hydride displacement mechanism. The greater relative H₂ evolution from the V-nitrogenases during N₂ reduction (40–50% of the electron flux) may indicate differences in the detailed mechanism of N₂ reduction, as is also suggested by the ability of V-nitrogenase to reduce N₂ more effectively than Mo-nitrogenase at low temperatures (Miller & Eady, 1988). We demonstrate here that during the reduction of N₂ to NH₃ by V-nitrogenase free N₂H₄ is formed as a minor but significant product.

**MATERIALS AND METHODS**

AcI⁺ and Ac₂⁺, the components of the V-nitrogenase of *A. chroococcum*, were purified from strain MCD 1155 as described previously (Eady *et al.*, 1987, 1988). When assayed under standard conditions at 30 °C the specific activities were 1309 nmol of H₂ produced/min per mg of protein for the Fe protein (Ac₂⁺) and 1516 nmol of H₂ produced/min per mg of protein for the VFe protein (AcI⁺).

Ac₁⁺ and Ac₂⁺, the components of the Mo-nitrogenase of *A. chroococcum*, used in control experiments, were purified essentially as described by Yates & Planqué (1975) from strain MCD 50 and had specific activities of 2424 nmol of H₂ produced/min per mg of protein for the Fe protein (Ac₂⁺) and 1541 nmol of H₂ produced/min per mg of protein for the MoFe protein (Ac₁⁺).

Enzyme assays at high enzyme concentrations as in Fig. 1 (12.8 μm-AcI⁺ and 33.4 μm-Ac₂⁺) utilized a 0.55 ml volume and MgCl₂ (12.5 mM), ATP (5 mM), phosphocreatine (20 mM) and Na₂S₂O₄ (10 mM) in 50 mM-Hepes buffer, pH 7.4. The reaction at 30 °C was terminated by injection of 1.45 ml of ethanol containing 1 M-HCl and 0.07 M-4-dimethylaminobenzaldehyde. H₂ in the gas headspace was determined by g.l.c., and, after centrifuging to remove precipitated protein, N₂H₄ was measured.

† To whom correspondence should be addressed.
as the 4-dimethylaminobenzaldehyde hydrazone (Thorneley et al., 1978). NH₃ was measured after micro-distillation (Miller & Eady, 1988).

Micro-concentration of reacting enzyme solutions was carried out under the conditions described above. After 5 min reaction time the liquid contents of an assay vial were transferred by syringe to the upper chamber of an Amicon Centricon 10 capsule previously assembled under N₂ in a glove-box containing less than 1 p.p.m. of O₂. The enzyme-free filtrate was obtained in the lower compartment by centrifuging at 6000 g for 15 min. The N₂H₄ and NH₃ contents of the filtrate were measured as described above.

All other assays utilized a 1 ml reaction volume and were terminated by injection of 0.05 ml of 30% (w/v) trichloroacetic acid. Product analysis was as described above.

RESULTS AND DISCUSSION

During experiments designed to detect potential enzyme-bound intermediates on quenching of V-nitrogenase during reduction of N₂, we observed a rapid increase in N₂H₄ concentration that did not decline when enzyme turnover ceased owing to the accumulation of the product inhibitor MgADP (Fig. 1). This pattern was in marked contrast with that for the N₂H₄ liberated from Mo-nitrogenase of Klebsiella pneumoniae, where there is good evidence that the concentration of the dinitrogen hydrde–enzyme species from which N₂H₄ is generated declines to zero as enzyme turnover ceases (Thorneley et al., 1978). With V-nitrogenase, labile intermediates do not appear to contribute significantly to the N₂H₄ detected. The failure to observe a decline in N₂H₄ concentration is presumably due to the amount of N₂H₄ formed by quenching the intermediate being small compared with the amount of N₂H₄ formed as a product, and is therefore not detectable on the time scale of these experiments. The material from assays of V-nitrogenase was identified as N₂H₄ on the basis of the absorption spectrum of its hydrazone with acidic 4-dimethylaminobenzaldehyde. The maximum rate of N₂H₄ production was about 1.25 nmol/min per mg of VFe protein of specific activity 177 nmol of NH₃ formed/min per mg of protein at 40 °C. N₂H₄ production was only observed under N₂, and was inhibited by H₂, a specific competitive inhibitor of N₂ reduction by Mo-nitrogenase (Table 1). H₂ also inhibits NH₃ formation by V-nitrogenase [see Dilworth et al. (1988) and Table 1], but does not inhibit acetylene reduction to either ethylene or ethane (Dilworth et al., 1988); detailed studies of the kinetics of H₂ inhibition have not yet been reported.

Steady-state assays at low levels of VFe protein (0.18 nmol) run for 5 h under N₂, during which time the reaction rate was linear, produced quantities of N₂H₄ 10-fold greater than the molar amount of enzyme added, consistent with it being a product, rather than being derived from quenching of a bound intermediate. A direct demonstration that the N₂H₄ was free in solution was achieved by rapid ultrafiltration of a reacting enzyme solution through an Amicon Centricon-10 centrifugal micro-concentrator under N₂ (as described in the Materials and methods section). The N₂H₄ was quantitatively recoverable in the enzyme-free filtrate, indicating that chemical quenching was not necessary to liberate it. Both lines of evidence therefore indicate that N₂H₄ must be a product. N₂H₄ was detected when VFe protein was complemented with purified Fe protein of either Mo-nitrogenase or V-nitrogenase, but not when purified MoFe protein was complemented with either Fe protein (results not shown). This indicates that dissociation of N₂H₄ is a property of the VFe protein.

![Fig. 1. Time course for the production of N₂H₄ (■), NH₃ (▲) and H₂ (●) during catalytic reduction of N₂ by the V-nitrogenase of A. chroococcum at pH 7.4 and 30 °C](image-url)
Hydrazine formation from N₂ by V-nitrogenase

Table 1. Inhibition by H₂ of N₂H₄ formation from N₂ by V-nitrogenase

<table>
<thead>
<tr>
<th>Gas phase</th>
<th>NH₃ formed (nmol)</th>
<th>N₂H₄ formed (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂</td>
<td>639</td>
<td>4.5</td>
</tr>
<tr>
<td>N₂/Ar</td>
<td>320</td>
<td>2</td>
</tr>
<tr>
<td>N₂/H₂</td>
<td>149</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Table 2. Temperature-dependence of N₂H₄ formation as a product of N₂ reduction by V-nitrogenase

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>H₂ evolved under Ar</th>
<th>H₂ evolved under N₂</th>
<th>NH₃ formed</th>
<th>N₂H₄ formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.5</td>
<td>562</td>
<td>221</td>
<td>255</td>
<td>0.26</td>
</tr>
<tr>
<td>30</td>
<td>1610</td>
<td>667</td>
<td>596</td>
<td>0.96</td>
</tr>
<tr>
<td>40</td>
<td>2553</td>
<td>1523</td>
<td>639</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Scheme 1. Cycle for the stepwise protonation of co-ordinated N₂ on V-nitrogenase to give NH₃ or N₂H₄

For Mo-nitrogenase, species (A) has been proposed (see Thorneley & Lowe, 1985) to be the source of N₂H₄ detected on acid or alkali quenching of the enzyme during turnover under N₂. Chemical complexes containing the moieties underlined have been isolated from reactions of co-ordinated N₂ (see Leigh, 1988).

N₂H₄ could arise in the V-nitrogenase system if di-imide (HN = NH) were to be released from the enzyme and subsequently disproportionate to form N₂H₃ and N₂. However, when a large excess of reagents known to oxidize di-imide to N₂ (e.g. 100 mM allyl alcohol, 25 mM fumarate) was added to assays, total enzyme activity (H₂ evolution and NH₃ formation) was not affected and N₂H₃ accumulation did not decrease (results not shown). It therefore appears that N₂H₄ is released directly from the enzyme as a four-electron-reduced product. Although schemes for the enzymic reduction of N₂ to NH₃ propose formal intermediates reduced by two and four electrons before the stage where N₂H₄ can be released by quenching with consequent oxidation of the metal centres of the MoFe protein (see Thorneley & Lowe, 1985), our observation provides direct evidence for a four-electron-reduced dinitrogen hydride species on the VFe protein capable of dissociating N₂H₄.

N₂H₄ is reduced by Mo-nitrogenase to NH₃, but is a poor substrate, with an apparent Kₘ of 20–30 mM (Bulen; Davis, 1980). To test whether free N₂H₄ is involved in NH₃ formation by V-nitrogenase, the following experiments were performed. Exogenous N₂H₄, added to steady-state assays at concentrations detected during reduction of N₂, under either Ar or N₂ (103 kPa) was not reduced. Evidently at these concentrations (Tables 1 and 2) N₂H₄ cannot react with the enzyme to produce a species reducible to NH₃ and is not therefore a free intermediate on the route to NH₃ formation by V-nitrogenase.

Current chemical models for N₂ reduction on transition-metal sites involve sequential protonation at the terminal β-N atom and concerted strengthening of the bond between the metal and the α-N atom resulting in a series of intermediates as shown in Scheme 1 (see Leigh, 1988; Richards, 1991). The hydrazido2⁻ species (=N—NH₂⁻) (species A in Scheme 1) is the enzyme-bound intermediate proposed as the source of N₂H₄ derived from the Mo-nitrogenase during turnover by acid quench. On the basis of the structural similarities of Mo- and V-nitrogenases suggestive of a common mechanism for dinitrogen reduction, we propose that in the V-nitrogenase this species can either undergo further reduction to yield NH₃, or be protonated at the α-N to give species B, capable of dissociating N₂H₄ as a product. However, other chemical species, e.g. M—N—NH—M, potentially capable of releasing N₂H₄ are also possible intermediates in NH₃ and N₂H₄ formation.

The rate of NH₃ formation increased 2–3-fold between 20 °C and 30 °C, but only 1.07-fold between 30 °C and 40 °C. These differences are not as marked as those in total electron flux, since corresponding ratios for H₂ evolution under Ar are 2.86- and 1.58-fold (Table 2). The lower rate of NH₃ formation may well reflect an effect on the apparent Kₘ for N₂ reduction. At 30 °C the apparent Kₘ values for N₂ of Mo- and V-nitrogenases are similar (0.3–2 kPa and 6 kPa respectively; see Dilworth et al., 1988) and under an atmosphere of N₂, are saturated. However, at 40 °C the rates of N₂H₄ and NH₃ formation by V-nitrogenase were decreased markedly under an atmosphere of 25 % N₂ in Ar compared with the rates under N₂ alone (Table 2). The effect of temperature on the apparent Kₘ for N₂ of the Mo-nitrogenases has not been reported; clearly this area merits further investigation. Our observations that the N₂H₄/NH₃ ratio increases as the temperature is raised from 20 °C to 40 °C (Table 2) imply that the activation energy for the reactions after the formation of the hydrazido precursor (species A, Scheme 1) leading to N₂H₄ formation differs from that leading to NH₃.

With Mo-nitrogenase, the proportion of electron flux used for N₂ reduction is strongly dependent on the Fe protein/MoFe protein ratio, low ratios favouring proton reduction to H₂. The detection of N₂H₄ as a product of V-nitrogenase is not due to limiting electron or proton flux, since increasing the Fe protein/VFe protein ratio from 1:1 to 3:1 did not alter the amount of N₂H₄ produced.

Significant changes in the substrate-specificity of Mo-nitrogenase have been shown to result from minor perturbations of the structure of the FeMo cofactor or of its protein environment. Thus mutation of a single glutamine residue to lysine results in the loss of the ability to reduce N₂ while acetylene reductase activity is retained, but with an altered balance between ethylene and ethane as reduction products (Scott et al., 1990). The insertion of FeVaco into Mo-nitrogenase polypeptides

Vol. 277
also results in an enzyme unable to reduce N₂ but that retains the characteristic product distribution of the VFe protein for acetylene reduction (Smith et al., 1988). We have shown that the formation of N₂H₄ as a product is not a consequence of contamination of VFe protein with species containing FeMoco, because assays with preparations of VFe protein containing 0.5 g-atom of Mo/mol did not result in additional N₂H₄ formation. The production of N₂H₄ as a minor product of N₂ reduction by V-nitrogenase is therefore an inherent part of the chemistry of N₂ reduction with this system. However, in a protein as complex as the VFe protein, it cannot be excluded that it arises from a small fraction of the enzyme defective in some specific interaction required for the further reduction of the putative (hydrazido²⁻)-enzyme intermediate to NH₃. Comparison with the reactivity of chemical complexes of N₂ suggests that subtle interactions between the metal atom and its ligands result in N₂H₄ or NH₃ being the products of N₂ reduction (Anderson et al., 1981). The differences that we report here for V-nitrogenase could well result from minor changes of the environment of FeMoco and FeVacO at the active site. The importance of the detection of N₂H₄ as a product is that it indicates the existence of an enzyme intermediate at the four-electron-reduced level during the reduction of N₂ by nitrogenase.

We gratefully acknowledge financial assistance to M. J. D. from the British Council, technical assistance from Marie Eldridge, helpful discussions with Professor G. J. Leigh and Dr. R. L. Richards, and Professor B. E. Smith for critical reading of the manuscript.

REFERENCES


Received 19 November 1990/21 January 1991; accepted 25 January 1991


