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

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During the enzymic reduction of N_2 to NH_3 by Mo-nitrogenase, free hydrazine (N_2H_4) is not detectable, but an enzyme-bound intermediate can be made to yield N_2H_4 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 N_2H_4 (up to 0.5% of the electron flux resulting in N_2 reduction) as a product of the reduction of N_2. The amount of N_2H_4 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 N_2H_4 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 N_2 by V-nitrogenase.

INTRODUCTION

The reduction of N_2 by Mo-nitrogenase involves an enzyme-bound dinitrogen hydride intermediate, which can be made to yield hydrazine (N_2H_4) by quenching the enzyme with acid or alkali during turnover under N_2 (Thorneley *et al.*, 1978). Free N_2H_4 has never been detected as a product of N_2 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–N_2 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 N_2 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 H_2 during N_2 reduction than do the Mo-nitrogenases, which, when functioning under optimal conditions, have the limiting stoichiometry (Simpson & Burris, 1984) represented by the equation:

$$N_2 + 8H^+ + 8e^- = 2NH_3 + H_2$$

H_2 evolution accompanying N_2 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 N_2 occurring via a hydride displacement mechanism. The greater relative H_2 evolution from the V-nitrogenases during N_2 reduction (40–50% of the electron flux) may indicate differences in the detailed mechanism of N_2 reduction, as is also suggested by the ability of V-nitrogenase to reduce N_2 more effectively than Mo-nitrogenase at low temperatures (Miller & Eady, 1988). We demonstrate here that during the reduction of N_2 to NH_3 by V-nitrogenase free N_2H_4 is formed as a minor but significant product.

MATERIALS AND METHODS

$Ac1^V$ and $Ac2^V$, 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 H_2 produced/min per mg of protein for the Fe protein ($Ac2^V$) and 1516 nmol of H_2 produced/min per mg of protein for the VFe protein ($Ac1^V$).

$Ac1^{Mo}$ and $Ac2^{Mo}$, 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 H_2 produced/min per mg of protein for the Fe protein ($Ac2^{Mo}$) and 1541 nmol of H_2 produced/min per mg of protein for the MoFe protein ($Ac1^{Mo}$).

Enzyme assays at high enzyme concentrations as in Fig. 1 (12.8 μM-$Ac1^V$ and 33.4 μM-$Ac2^V$) utilized a 0.55 ml volume and $MgCl_2$ (12.5 mM), ATP (5 mM), phosphocreatine (20 mM) and $Na_2S_2O_4$ (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. H_2 in the gas headspace was determined by g.l.c., and, after centrifuging to remove precipitated protein, N_2H_4 was measured

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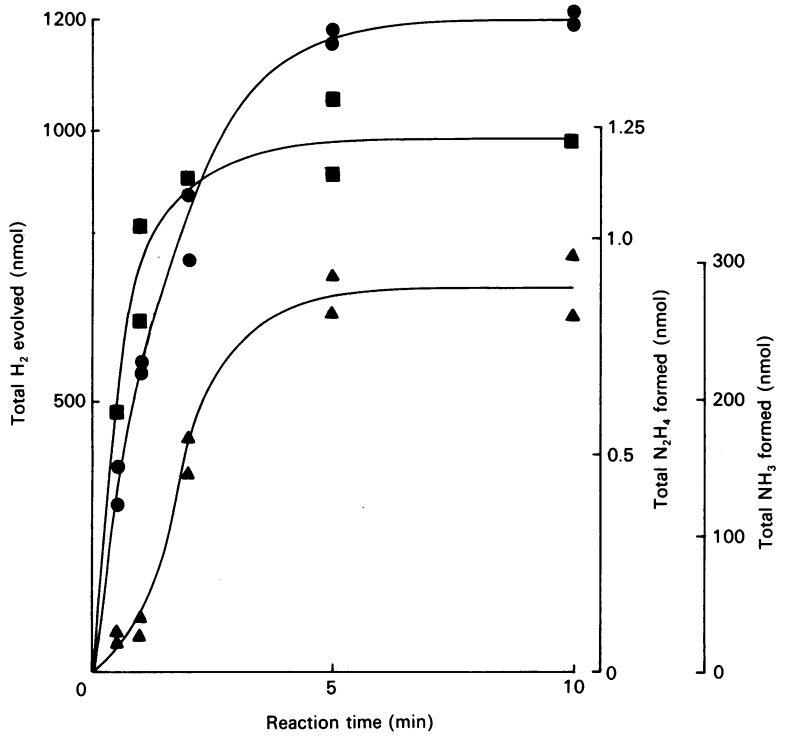


Fig. 1. Time course for the production of N_2H_4 (■), NH_3 (▲) and H_2 (●) during catalytic reduction of N_2 by the V-nitrogenase of *A. chroococcum* at pH 7.4 and 30 °C

Assay mixtures (0.55 ml) contained 2.7 mg of VFe protein (12.8 μM) and 2.15 mg of Fe protein (33.4 μM). Assays were quenched with acidic 4-dimethylaminobenzaldehyde at the times indicated; H_2, N_2H_4 and NH_3 were measured as described in the Materials and methods section.

as the 4-dimethylaminobenzaldehyde hydrazone (Thorneley *et al.*, 1978). NH_3 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 N_2 in a glove-box containing less than 1 p.p.m. of O_2. The enzyme-free filtrate was obtained in the lower compartment by centrifuging at 6000 *g* for 15 min. The N_2H_4 and NH_3 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 N_2, we observed a rapid increase in N_2H_4 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 N_2H_4 liberated from Mo-nitrogenase of *Klebsiella pneumoniae*, where there is good evidence that the concentration of the dinitrogen hydride–enzyme species from which N_2H_4 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 N_2H_4 detected. The failure to observe a decline in N_2H_4 concentration is presumably due to the amount of N_2H_4 formed by quenching the intermediate being small compared with the amount of N_2H_4 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 N_2H_4 on the basis of the absorption spectrum of its hydrazone with acidic 4-dimethylaminobenzaldehyde. The maximum rate of N_2H_4 production was about 1.25 nmol/min per mg of VFe protein of specific activity 177 nmol of NH_3 formed/min per mg of protein at 40 °C. N_2H_4 production was only observed under N_2, and was inhibited by H_2, a specific competitive inhibitor of N_2 reduction by Mo-nitrogenase (Table 1). H_2 also inhibits NH_3 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 H_2 inhibition have not yet been reported.

Steady-state assays at low levels of VFe protein (0.18 nmol) run for 5 h under N_2, during which time the reaction rate was linear, produced quantities of N_2H_4 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 N_2H_4 was free in solution was achieved by rapid ultrafiltration of a reacting enzyme solution through an Amicon Centricon-10 centrifugal micro-concentrator under N_2 (as described in the Materials and methods section). The N_2H_4 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 N_2H_4 must be a product. N_2H_4 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 N_2H_4 is a property of the VFe protein.

Table 1. Inhibition by H_2 of N_2H_4 formation from N_2 by V-nitrogenase

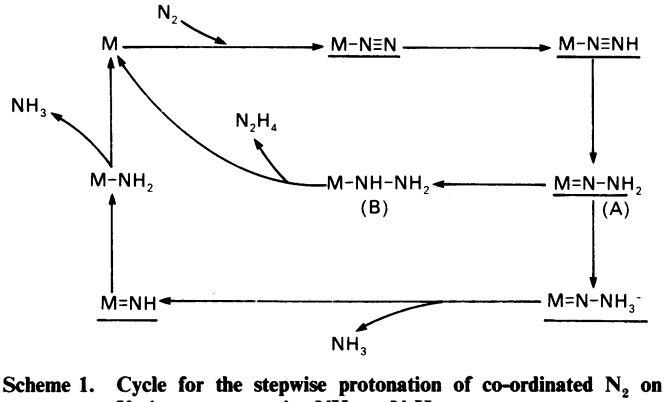
Nitrogenase activity was measured at 40 °C for 30 min under an atmosphere of N_2, 25% N_2 in Ar or 25% N_2 in H_2. Assay mixtures (1 ml) contained 120 μg of VFe protein (0.57 μM) and 103 μg of Fe protein (1.6 μM). The reaction was terminated by the addition of 0.05 ml of 30% (w/v) trichloroacetic acid, and products were determined as described in the legend of Fig. 1.

Gas phase	NH_3 formed (nmol)	N_2H_4 formed (nmol)
N_2	639	4.5
N_2/Ar	320	2
N_2/H_2	149	0.91

Table 2. Temperature-dependence of N_2H_4 formation as a product of N_2 reduction by V-nitrogenase

Assays were for 60 min at 20 °C and for 30 min at 30 °C and 40 °C. Other conditions are as in Table 1.

	Amount of product formed (nmol)		
Temperature ...	20.5 °C	30 °C	40 °C
H_2 evolved under Ar	562	1610	2553
H_2 evolved under N_2	221	667	1523
NH_3 formed	255	596	639
N_2H_4 formed	0.26	0.96	4.5



Scheme 1. Cycle for the stepwise protonation of co-ordinated N_2 on V-nitrogenase to give NH_3 or N_2H_4

For Mo-nitrogenase, species (A) has been proposed (see Thorneley & Lowe, 1985) to be the source of N_2H_4 detected on acid or alkali quenching of the enzyme during turnover under N_2. Chemical complexes containing the moieties underlined have been isolated from reactions of co-ordinated N_2 (see Leigh, 1988).

N_2H_4 could arise in the V-nitrogenase system if di-imide (HN=NH) were to be released from the enzyme and subsequently disproportionate to form N_2H_4 and N_2. However, when a large excess of reagents known to oxidize di-imide to N_2 (e.g. 100 mM-allyl alcohol, 25 mM-fumarate) was added to assays, total enzyme activity (H_2 evolution and NH_3 formation) was not affected and N_2H_4 accumulation did not decrease (results not shown). It therefore appears that N_2H_4 is released directly from the enzyme as a four-electron-reduced product. Although schemes for the enzymic reduction of N_2 to NH_3 propose formal intermediates reduced by two and four electrons before the stage where N_2H_4 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 N_2H_4.

N_2H_4 is reduced by Mo-nitrogenase to NH_3, but is a poor substrate, with an apparent K_m of 20–30 mM (Bulen, 1976; Davis, 1980). To test whether free N_2H_4 is involved in NH_3 formation by V-nitrogenase, the following experiments were performed. Exogenous N_2H_4, added to steady-state assays at concentrations detected during reduction of N_2, under either Ar or N_2 (103 kPa) was not reduced. Evidently at these concentrations (Tables 1 and 2) N_2H_4 cannot react with the enzyme to produce a species reducible to NH_3 and is not therefore a free intermediate on the route to NH_3 formation by V-nitrogenase.

Current chemical models for N_2 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 hydrazido^{2-} species (=N–NH_2^{2-}) (species A in Scheme 1) is the enzyme-bound intermediate proposed as the source of N_2H_4 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 NH_3, or be protonated at the α-N to give species B, capable of dissociating N_2H_4 as a product. However, other chemical species, e.g. M–NH–NH–M, potentially capable of releasing N_2H_4 are also possible intermediates in NH_3 and N_2H_4 formation.

The rate of NH_3 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 H_2 evolution under Ar are 2.86- and 1.58-fold (Table 2). The lower rate of NH_3 formation may well reflect an effect on the apparent K_m for N_2 reduction. At 30 °C the apparent K_m values for N_2 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 N_2 are saturated. However, at 40 °C the rates of N_2H_4 and NH_3 formation by V-nitrogenase were decreased markedly under an atmosphere of 25% N_2 in Ar compared with the rates under N_2 alone (Table 2). The effect of temperature on the apparent K_m for N_2 of the Mo-nitrogenases has not been reported; clearly this area merits further investigation. Our observations that the N_2H_4/NH_3 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 N_2H_4 formation differs from that leading to NH_3.

With Mo-nitrogenase, the proportion of electron flux used for N_2 reduction is strongly dependent on the Fe protein/MoFe protein ratio, low ratios favouring proton reduction to H_2. The detection of N_2H_4 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 N_2H_4 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 N_2 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

also results in an enzyme unable to reduce N_2 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 N_2H_4 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 N_2H_4 formation. The production of N_2H_4 as a minor product of N_2 reduction by V-nitrogenase is therefore an inherent part of the chemistry of N_2 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 (hydrazido^{2-})–enzyme intermediate to NH_3. Comparison with the reactivity of chemical complexes of N_2 suggests that subtle interactions between the metal atom and its ligands result in N_2H_4 or NH_3 being the products of N_2 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 N_2H_4 as a product is that it indicates the existence of an enzyme intermediate at the four-electron-reduced level during the reduction of N_2 by nitrogenase.

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