Nitrogenase of *Klebsiella pneumoniae*

Hydrazine is a product of azide reduction

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Klebsiella pneumoniae nitrogenase reduced azide, at 30°C and pH 6.8–8.2, to yield ammonia (NH₃), dinitrogen (N₂) and hydrazine (N₂H₄). Reduction of (¹⁵N=¹⁴N=¹⁴N)[−] followed by mass-spectrometric analysis showed that no new nitrogen–nitrogen bonds were formed. During azide reduction, added ¹⁵N₂H₄ did not contribute ¹⁵N to NH₃, indicating lack of equilibration between enzyme-bound intermediates giving rise to N₂H₄ and N₂H₄ in solution. When azide reduction to N₂H₄ was partially inhibited by ¹⁵N₂, label appeared in NH₃ but not in N₂H₄. Product balances combined with the labelling data indicate that azide is reduced according to the following equations:

N₂ was a competitive inhibitor and CO a non-competitive inhibitor of azide reduction to N₂H₄. The percentage of total electron flux used for H₂ evolution concomitant with azide reduction fell from 26% at pH 6.8 to 0% at pH 8.2. Pre-steady-state kinetic data suggest that N₂H₄ is formed by the cleavage of the α–β nitrogen–nitrogen bond of bound azide to leave a nitride (≡N) intermediate that subsequently yields NH₃.

The six-electron reduction of dinitrogen (N₂) to NH₃ is catalysed by nitrogenase and is an important reaction in the biological nitrogen cycle. The enzyme when isolated from various bacteria comprises two oxygen-labile proteins; the tetrameric Mo–Fe protein (hereafter termed Kp1) has a mol.wt. of 218000 and the dimeric Fe protein (hereafter termed Kp2) a mol.wt. of 68000. Both proteins, a source of low-potential electrons (sodium dithionite *in vitro*) and MgATP are required for substrate reduction (Mortenson & Thorneley, 1979).

The mechanism of dinitrogen reduction by nitrogenase is not known, but the chemistry of low-oxidation-state molybdenum–dinitrogen complexes (Chatt *et al.*, 1978) suggests that N₂ is bound at the Mo and that reduction proceeds via a series of

co-ordinated dinitrogen hydride intermediates. The hydrazido(2[−]) species (=N–NH₂) is particularly stable in Mo complexes, and Thorneley *et al.* (1978) suggested that this intermediate was hydrolysed to give free hydrazine (N₂H₄) under the strong acid or alkali conditions used to quench functioning nitrogenase. Hydrazine is not normally a product of dinitrogen reduction by nitrogenase, but it is a substrate that is reduced to ammonia (Bulen, 1976).

Thorneley *et al.* (1980) favour a mechanism for dinitrogen reduction originally proposed by Chatt *et al.* (1977) in which the nitrogen–nitrogen bond is cleaved after partial reduction of co-ordinated N₂ to yield NH₃ and a nitride (≡N) or imido (=NH) group co-ordinated to Mo. An alternative mechanism (Newton *et al.*, 1976) requires the nitrogen–nitrogen bond to remain intact until the final two electrons and protons are added to co-ordinated hydrazine.

The mechanism of reduction of azide by nitrogenase is of interest, since in some chemical systems, molybdo-azides yield dinitrogen and the same co-ordinated nitride (Mo≡N) or imido (Mo=NH) (Chatt & Dilworth, 1977) groups, which are

Abbreviations used: in nitrogenase nomenclature, Kp refers to *Klebsiella pneumoniae*, Av to *Azotobacter vinelandii*, and Cp to *Clostridium pasteurianum*; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Ada, [(carbamoylmethyl)imino]diacetic acid.

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discussed above as potential intermediates in dinitrogen reduction. Henderson *et al.* (1981) have recently shown that ammonia can be obtained from a molybdo-nitride complex by base-, but not acid-, catalysed solvolysis.

Schöllhorn & Burris (1967) reported that Cp and Av nitrogenases reduce azide in a two-electron reaction to give stoichiometric amounts of dinitrogen (N_2) and ammonia, a product balance also found by Hardy & Knight (1967). Hwang *et al.* (1973) obtained a K_m of 1.43 mM for azide reduction to ammonia by Av nitrogenase and also showed that acetylene, hydrogen cyanide, methyl isocyanide and carbon monoxide were non-competitive inhibitors of this reaction. Parejko & Wilson (1971) obtained K_m values in the range 0.6–1.6 mM for azide reduction by Kp nitrogenase.

In the present paper we show that, in addition to ammonia and dinitrogen, hydrazine is also a major product when azide is reduced by Kp and Av nitrogenases. Kinetic data and product analyses showing the distribution of ^{15}N among the reduction products from terminally ^{15}N -labelled azide are presented. Mechanisms for the enzymic reduction of azide are discussed and some implications for the mechanism of reduction of dinitrogen are considered.

Methods and materials

Nitrogenase

Kp nitrogenase component proteins were purified as described by Eady *et al.* (1972), with a final additional chromatography on DEAE-cellulose (Smith *et al.*, 1976), and stored in liquid N_2 . Av nitrogenase proteins were a gift from Dr. R. R. Eady of this Unit.

All steady-state assays of azide reduction with Kp nitrogenase used Fe-Mo protein with specific activity $1700 \text{ nmol of } C_2H_4 \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ and Fe protein with specific activity $1700 \text{ nmol of } C_2H_4 \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ when assayed under the conditions used by Eady *et al.* (1972). Each assay (vol. 0.8 ml) contained (except where stated otherwise) $36 \mu\text{g}$ of Kp1 and $59 \mu\text{g}$ of Kp2 proteins.

Azide-reduction assays

The standard assay system for azide reduction contained, in a final volume of 0.8 ml, $7.2 \mu\text{mol}$ of ATP, $8 \mu\text{mol}$ of MgCl_2 , $20 \mu\text{mol}$ of Hepes buffer (pH 6.8 or 7.5), $8 \mu\text{mol}$ of sodium dithionite, $16 \mu\text{mol}$ of creatine phosphate and 0.2 mg of creatine phosphokinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2), with NaN_3 at appropriate concentration.

The assay vial was sealed with a Suba-Seal rubber closure and flushed with argon for 20 min before

injection of sodium dithionite. After a 5 min pre-incubation at 30°C and with shaking at $100 \text{ cycles} \cdot \text{min}^{-1}$ in a water bath, excess argon pressure was vented from the vials and reaction initiated by addition of pre-mixed nitrogenase components. Reaction was stopped by the addition of EDTA (0.2 ml, 0.4 M, at pH 7.5); nitrogenase activity (acetylene reduction) is immediately halted by this reagent, and this avoids the acid-catalysed conversion of azide to NH_3 found with acidic quenching reagents (Peters, 1979). Reaction time was normally 10 min. N_2H_4 production was linear with time up to at least 25 min, and completely dependent on both enzyme components, azide, ATP and dithionite ion.

Hydrogen assay

Water (0.3 ml) was injected into each vial containing a quenched assay and 0.5 ml samples assayed for H_2 on a Pye series-204 gas chromatograph fitted with a column (45 cm $\times$ 6 mm diam.) of molecular sieve 5A (80–100 mesh). The column was operated at 65°C with argon ($20 \text{ ml} \cdot \text{min}^{-1}$) as carrier gas, and the H_2 detected with a katharometer.

Ion-exchange-resin treatment

AG 1-X2 resin (Bio-Rad Laboratories, Richmond, CA, U.S.A.) was washed with 4 M-HCl and then exhaustively with water. Contents of assay vials were allowed to flow through individual columns (3.5 cm $\times$ 6 mm int. diam.) of AG 1-X2 resin, followed by two washes of vials and columns with 0.5 ml of water. Azide, dithionite ion, ATP, creatine phosphate, Hepes, EDTA and protein were adsorbed on to the resin, leaving creatine, N_2H_4 and NH_3 quantitatively recoverable in the effluent. Resulting solutions were collected in 30 ml Universal bottles and weighed. Samples were taken for N_2H_4 determination and 1 ml of saturated K_2CO_3 solution was added to the remaining solution to initiate microdiffusion of NH_3 on to glass rods carrying 1 M- H_2SO_4 , with overnight rotation of the bottles (Dilworth, 1966). The rods were eluted into water and ammonia determined by an indophenol method (Fawcett & Scott, 1960).

Determination of azide, N_2H_4 and hydroxylamine (NH_2OH)

Azide was measured spectrophotometrically as ferric azide in acid solution. The sample (1 ml) was treated with 2 ml of 0.1 M-glycine/HCl buffer, pH 2.0, and 1 ml of 0.2 M- FeCl_3 in 0.01 M-HCl (this reagent requires filtration) and the absorbance was read at 458 nm. Under these conditions, the ferric azide complex has a molar absorption coefficient of $1440 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

N_2H_4 was measured spectrophotometrically at

458 nm with a *p*-dimethylaminobenzaldehyde reagent (Watt & Crisp, 1952) by using an experimentally determined molar absorption coefficient of $70\,000\text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$.

NH_2OH was oxidized to nitrite with iodine in acetic acid as described by Yashpe *et al.* (1960) and nitrite measured by using the reagents developed by Trollope & Evans (1975).

Gases

Gases were added by flushing through Suba-Seal rubber closures (N_2 , N_2O , H_2) or by injection with a gas-tight micro-syringe (CO).

Isotopically labelled compounds

NaN_3 labelled in one terminal nitrogen atom (97.3 atom % excess ^{15}N and doubly labelled hydrazine sulphate (95 atom % excess ^{15}N) were from Prochem, London SW19 3UF, U.K.

Rapid-quench experiments

Rapid-quench studies were performed at 23°C in the rapid-mixing apparatus described by Gutteridge *et al.* (1978).

One syringe contained $31\mu\text{M}$ -Kp1 [specific activity $1800\text{ nmol of C}_2\text{H}_4\text{ produced}\cdot\text{min}^{-1}\cdot(\text{mg of protein})^{-1}$], $155\mu\text{M}$ -Kp2 [specific activity $1400\text{ nmol of C}_2\text{H}_4\text{ produced}\cdot\text{min}^{-1}\cdot(\text{mg of protein})^{-1}$], 10 mM - MgCl_2 , 1 mM - $\text{Na}_2\text{S}_2\text{O}_4$ and 25 mM -Hepes, pH 7.5. The other contained 18 mM -ATP, 10 mM - MgCl_2 , 20 mM - $\text{Na}_2\text{S}_2\text{O}_4$, 25 mM -Hepes, pH 7.5, and 40 mM - NaN_3 . Protein solutions were freed of N_2 by careful evacuation and flushing with purified argon on an all-glass vacuum line.

For measurement of H_2 production, the mixed solutions were fired from a needle jet through a Suba-Seal into HClO_4 (0.5 ml , 0.5 M) contained in glass vials (2.6 ml volume); the vials were pre-weighed and flushed with argon. To minimize azide breakdown, NaOH (0.1 ml , 5 M) and Na_2HPO_4 (0.2 ml , 0.5 M) were injected immediately after protein precipitation to return the pH to 7.4, and the samples kept on ice. Subsequently, all samples were equilibrated to 20°C for 10 min and 0.5 ml of the gas was analysed for H_2 by gas chromatography.

For measurement of N_2H_4 production, samples were fired into 1.6 ml of *p*-dimethylaminobenzaldehyde reagent; after centrifugation the absorbance of the supernatants was determined at 458 nm in 4 cm -light-path cuvettes in the spectrophotometer.

For NH_3 determination, samples were neutralized as described above and were frozen in solid- CO_2 /ethanol while the rapid-quench experiments were completed, then thawed, centrifuged to remove protein, and the supernatant run through the small columns of AG 1-X2 anion exchanger. Samples of the effluent were analysed for NH_3 by a modification of the method of Fawcett & Scott (1960). Reagents (sodium phenate, sodium nitroprusside

and sodium hypochlorite) were made up at double the usual concentrations, and 1.5 ml samples from experiments were treated sequentially with 0.3 ml of phenate, 0.45 ml of nitroprusside and 0.45 ml of hypochlorite. Absorbance at 630 nm was read in 4 cm cells after 20 min; the method is capable of measuring NH_3 to a precision of $\pm 2\text{ nmol}$ per sample.

Mass spectrometry

Enzyme incubations were performed with NaN_3 labelled with ^{15}N in one terminal nitrogen atom. Each incubation was four times the standard volume and run for 25 min at 30°C in 5 ml flasks fitted with a tap leading to a ground-glass socket allowing attachment to the mass spectrometer, but also allowing a Suba-Seal rubber closure to be fitted. After incubation, a sample was analysed for H_2 , and the tap closed. After attachment to the mass spectrometer, peaks corresponding to masses 28, 29 and 30 were recorded. The sample was then expanded about 10-fold, and the peaks at mass 28 (N_2) and 40 (argon) measured, thus allowing calculation of labelled N_2 in the reaction vessel by reference to argon. An ionization efficiency for argon of 1.6 times that of N_2 was used to correct the mass-40 peak.

After measurement of labelled N_2 , the contents of the incubation flasks were run through columns ($10\text{ cm}\times 6\text{ mm}$ int. diam.) of AG 1-X2 anion exchanger into bottles containing 0.02 ml of 1 M - H_2SO_4 (to prevent N_2H_4 decomposition) and the hydrazine concentration measured. A portion (160 nmol of N_2H_4 in 2 ml) was placed in one bulb of a 25 ml Rittenberg tube and a mixture of 0.7 ml of 0.4 M - $\text{K}_3\text{Fe}(\text{CN})_6$ and 0.3 ml of 1 M - NaOH in the other. After two cycles of freezing in liquid N_2 , evacuation and thawing, the sample and alkaline ferricyanide were mixed and allowed to react for 30 min. Under these conditions N_2H_4 is oxidized to N_2 without isotopic randomization between N_2H_4 molecules (Higginson & Sutton, 1953); the labelling pattern in N_2 is an accurate reflection of that in N_2H_4 .

NH_3 was recovered from column effluents by microdiffusion into 1 M - H_2SO_4 with overnight rotation, and oxidized to N_2 with alkaline hypobromite (Steinberg & Udenfriend, 1957) for determination of masses 28, 29 and 30.

All mass spectra were recorded on an MS10 mass spectrometer fitted with repeated-scan facilities.

Chromatography of N_2H_4 , NH_3 and creatine on Dowex 50W

A column ($10\text{ cm}\times 0.5\text{ cm}$) of Dowex 50W (X8; Na^+ form) was equilibrated with 20 mM -sodium phosphate buffer, pH 7.4, and loaded with 200 nmol of N_2H_4 (HCl)₂, 425 nmol of $(\text{NH}_4)_2\text{SO}_4$ and

10 μ mol of creatine in 1.8 ml of 10 mM-sodium phosphate buffer, pH 7.4. The column was eluted with 20 mM-sodium phosphate buffer, pH 7.4 (fractions 1–25, 0.6 ml/fraction), and then with 20 mM-sodium pyrophosphate buffer, pH 8.7 (fractions 26–55). Fractions 1–15 were analysed for creatine (Ennor, 1957) by using 0.1 ml samples, fractions 26–43 for N_2H_4 (Watt & Crisp, 1952) by using 0.1 ml samples, and fractions 35–55 for NH_3 (Fawcett & Scott, 1960) by using 0.2 ml samples.

A double-volume standard assay of azide reduction with Kp nitrogenase was run for 25 min at 30°C and stopped with EDTA. The incubation mixture was run through a column (5 cm $\times$ 0.5 cm) of Dowex 1X2 (Cl^- form), the first 0.5 ml of effluent being discarded, followed by two 0.5 ml water washes. This solution was then chromatographed on a column of Dowex 50W (X8; Na^+ form) as described above.

Paper chromatography

Product from enzymic reduction of azide was subjected to ascending paper chromatography on sheets (30 cm $\times$ 30 cm) of Whatman no. 1 paper, with diethyl ether / methanol / water / HCl (50:30:15:4, by vol.) as solvent (Bremner, 1954).

Glassware and reagents

All glassware was soaked in 2 M-HCl and rinsed three times with deionized water and once with

glass-distilled water before use. This was critically important in lowering the variability of the indophenol method for NH_3 determination.

Hepes was purified by treating the solution of the free acid with Dowex 50W (X8; H^+ form) at a ratio of 20 g/100 ml of 0.2 M-buffer to remove NH_4^+ .

Results

Identification of product

In experiments to determine if the same dinitrogen hydride intermediate is formed during azide reduction by nitrogenase as is during dinitrogen reduction to NH_3 , reaction mixtures reducing azide at 30°C were quenched after 10 s with the *p*-dimethylaminobenzaldehyde reagent under the same conditions as those of Thorneley *et al.* (1978). Quantities of *p*-dimethylaminobenzaldehyde-reactive material were detected that were in considerable molar excess over the enzyme present, indicating that the material was a product of nitrogenase action on azide and not an intermediate in the reaction.

The material has been identified as N_2H_4 on the basis of: (1) identity of the absorption spectrum of the enzyme product with *p*-dimethylaminobenzaldehyde with that of authentic N_2H_4 with the same reagent ($\lambda_{max.} = 458$ nm); (2) identity of behaviour of product with that of authentic N_2H_4 during chromatography on Dowex 50W (X8; Na^+ form) cation-exchange resin; (3) co-migration of the

Fig. 1. K_m determination for azide reduction at 30°C

The assay conditions and product-analysis procedures were as described in the Methods and materials section. The lines drawn are unweighted least-squares best fits of the data-points for NH_3 (Δ) and N_2H_4 ($\bullet$) production. The K_m values for azide at pH 6.8 were calculated from the abscissa intercepts of (a) to be 1.5 mM (N_2H_4 measured), and 0.83 mM (NH_3 measured). (b) Shows data at pH 7.4 from which K_m values for azide of 3.3 mM (N_2H_4 measured) and 1.2 mM (NH_3 measured) were calculated.

enzyme product with authentic N_2H_4 during paper chromatography.

Hydroxylamine was not found when azide reduction assays were analysed (limit of detection: 10 nmol/assay).

Determination of K_m for azide and pH-dependence of product ratio

The apparent K_m for azide was determined from double-reciprocal plots (Figs. 1a and 1b) obtained by measuring the rates of N_2H_4 and NH_3 production as a function of azide concentration (0.7–20 mM). The values obtained are, at pH 6.8, 1.5 mM for N_2H_4 production and 0.83 mM for NH_3 production, and at pH 7.5, 3.3 mM for N_2H_4 formation and 1.3 mM for NH_3 formation. These results show that the K_m depends on which product of reduction is monitored and also on the pH. If hydrazoic acid (HN_3), with a $\text{p}K_a$ of 4.6 at 30°C (Boughton & Keller, 1966) is the substrate, and not N_3^- , then lowering the pH from 7.5 to 6.8 should have decreased the apparent K_m by a factor of five. Since there is only a twofold change in the K_m for either product, additional proteolytic equilibria must be involved and, although N_3^- is the likely substrate, HN_3 cannot be excluded, with a K_m in the range of 1–15 μM .

The pH profiles for the three products NH_3 , N_2H_4 and H_2 (Fig. 2) show that lower pH favours the production of N_2H_4 relative to NH_3 and H_2 .

Fig. 2. Relative yields of H_2 , N_2H_4 and NH_3 from N_3^- reduction as a function of pH at 30°C

The relative yields of H_2 , N_2H_4 and NH_3 produced during reduction of N_3^- (20 mM) as a function of pH under the assay conditions given in the Methods and materials section are shown. Buffer systems contained 25 mM-Hepes and 25 mM-Ada at the appropriate pH. The maximum yield of each product has been normalized to 100%. ●, H_2 evolved in the absence of N_3^- ; ○, H_2 evolved concomitant with N_3^- reduction; △, NH_3 ; ▲, N_2H_4 .

Effect of changing the ratio of Kp2 to Kp1

The ratio of Kp2 to Kp1 was varied from 10 to 0.2 and the rates of N_2H_4 , NH_3 and H_2 production measured. Control experiments showed that, even at the lowest ratio of Kp2 to Kp1 (0.2), N_2H_4 , NH_3 and H_2 production were linear with time. The data-points for N_2H_4 production in Fig. 3 clearly lie on a different curve than that on which the points for NH_3 and H_2 formation fall. The insert to Fig. 3, in which the relative amounts of N_2H_4 and NH_3 are plotted as a function of component ratio, shows that a maximal yield of N_2H_4 relative to NH_3 was obtained with a Kp2 to Kp1 ratio of 1.0.

Inhibitors of N_2H_4 formation

CO , N_2 and N_2O were inhibitors of N_2H_4 formation from azide but H_2 was not (Table 1). N_2H_4 was not a product of enzymic reduction of N_2O . Inhibition patterns and constants were determined for CO and N_2 by using steady-state kinetics analysis.

Inhibition of azide reduction by CO

The data in Fig. 4 show that CO is a linear non-competitive inhibitor of N_2H_4 production from azide with $K_{is} = 121 \text{ Pa}$ (1.2 atm) and $K_{ii} = 2.42 \text{ kPa}$ (24 atm) (notation of Cleland, 1963). The same inhibition pattern and values of K_{is} and K_{ii} were obtained when the rates of NH_3 production were measured. Hwang *et al.* (1973) also reported linear non-competitive inhibition by CO of NH_3 formation from azide by Av nitrogenase, but their values of $K_{is} = 11.5 \text{ Pa}$ (114 μatm) and $K_{ii} = 446 \text{ Pa}$ (440 μatm) were an order of magnitude lower than those reported above for Kp nitrogenase.

Inhibition of azide reduction by N_2

The data presented in Fig. 5 show that N_2 is a competitive inhibitor of azide reduction with $K_i = 42.5 \text{ kPa}$ (0.42 atm). Since N_2 is an alternative substrate for nitrogenase which also yields NH_3 on reduction, it was not possible to use that product to determine the inhibition pattern. Hydrazine is not a product of N_2 reduction, and, since the concentration of Kp nitrogenase used in these experiments was so low, the amount of N_2H_4 produced from any enzyme-bound dinitrogen hydride intermediate in N_2 reduction (Thorneley *et al.*, 1978) would have been insignificant compared to that produced by reduction of azide.

The value of $K_i = 42.5 \text{ kPa}$ (0.42 atm) of N_2 is significantly larger than the apparent $K_m = 5.1 \text{ kPa}$ (0.05 atm) of N_2 for N_2 reduction to NH_3 under these conditions (R. R. Eady & B. E. Smith, unpublished work).

Inhibition of H_2 evolution by azide

The question of what proportion of H_2 evolution

Fig. 3. Effect of Kp2/Kp1 ratio on the yield of H_2 , N_2H_4 and NH_3 from N_3^- reduction at $30^\circ C$, pH 7.5

The assay conditions were as described in the Methods and materials section. When Kp2/Kp1 molar ratio was ≥ 1.0 , each assay contained $36 \mu g$ of Kp1, with the appropriate amount of Kp2. When the Kp2/Kp1 molar ratio was < 1.0 , each assay contained $11 \mu g$ of Kp2, with the appropriate amount of Kp1, and for these assays the yields of H_2 ($\square$), NH_3 (Δ) and N_2H_4 (O) have been multiplied by the Kp2/Kp1 molar ratio to correct for the increased concentration of Kp1. The insert shows the N_2H_4/NH_3 product ratio as a function of the Kp2/Kp1 molar ratio, with a maximum at Kp2/Kp1 = 1.0.

Table 1. Effect of inhibitors on N_2H_4 and NH_3 formation from azide

Inhibitor	Concentration	Percentage inhibition of formation of:	
		N_2H_4	NH_3
CO	202 Pa (0.002 atm)	16	18
	2.02 kPa (0.02 atm)	63	57
N_2	101 kPa (1 atm)	50	*
N_2O	101 kPa (1 atm)	48	*
H_2	101 kPa (1 atm)	0	0

* Not determined.

can be suppressed by the presence of substrate is of importance for the mechanism of N_2 reduction. Accordingly, we have plotted the difference between H_2 evolution under argon and H_2 evolution under argon in the presence of azide, as a function of azide concentration, by using a double-reciprocal plot

(Fig. 6). Extrapolation to infinite substrate concentration indicates that, even then, some 24–26% of the electron flux would be used to produce H_2 at pH 6.8, but that this value falls to 16% at pH 7.5, to 13% at pH 7.8 and to 0% at pH 8.2.

Mass spectrometry

Dinitrogen deriving from azide, terminally labelled with ^{15}N , would be composed of $^{28}N_2$ and $^{29}N_2$ unless new N–N bonds are formed by interaction of two different azide molecules or derivatives from them. N_2 produced from terminally labelled azide was found to be wholly $^{28}N_2$ and $^{29}N_2$, with no trace of $^{30}N_2$; total N_2 can therefore be calculated from the $^{29}N_2$ peak. Since no evidence can be found for N_2 being derived from two different azide molecules, we suggest that reduction of azide to give N_2 conforms to eqn. (1), originally formulated by Schöllhorn & Burris (1967) and Hardy & Knight (1967).

Degradation of authentic $^{15}N_2H_4$ by alkaline ferri-

Fig. 4. Non-competitive inhibition by CO of N_2H_4 formation from N_3^- , at $30^\circ C$, and pH 6.8

The standard assay conditions given in the Methods and materials section were used. $[N_3^-]$ was varied from 2 to 10 mM under various Ar/CO gas mixtures. The lines drawn are least-squares fits to the data-points with (O) 0 Pa (0 atm) of CO; (●) 505 Pa (5 matm) of CO; (Δ) 1.01 kPa (10 matm) of CO; (▲) 1.51 kPa (15 matm) of CO. Secondary plots of the slopes and intercepts gave $K_{is} = 121$ Pa (1.2 atm) and $K_{ii} = 2.42$ kPa (24 matm).

cyanide produced the $^{29}N_2$ and $^{30}N_2$ yields predicted from the ^{15}N concentration in the $^{15}N_2H_4$. This confirmed that no randomization of ^{15}N label occurs during oxidation to N_2 ; hence the labelling pattern in N_2 is an accurate reflection of that in N_2H_4 .

N_2H_4 produced enzymically from terminally labelled azide was degraded to give only $^{28}N_2$ and $^{29}N_2$, with no trace of $^{30}N_2$ detectable. Therefore N_2H_4 is also not produced by a process involving azide-azide interaction or reaction of azide with a single nitrogen species on the enzyme. We suggest that hydrazine is produced by a reduction according to eqn. (2):

Fig. 5. Competitive inhibition by N_2 of N_2H_4 formation from N_3^- at $30^\circ C$ and pH 6.8

The standard assay conditions given in the Methods and materials section were used. $[N_3^-]$ was varied from 2 to 10 mM under (●) 101 kPa (1.0 atm) of Ar and (O) 101 kPa (1.0 atm) of N_2 . The lines drawn are least squares-fits and the abscissa intercepts give $K_i = 42.5$ kPa (0.42 atm).

Product balance

N_2 production from azide can be measured in a variety of ways, many of which suffer from the problem of atmospheric- N_2 contamination. We have therefore measured N_2 production by the isotopic method described to avoid such contamination problems.

In Table 2, data are presented that summarize the product balance for azide reduction by Kp nitrogenase. The decrease in H_2 production due to azide reduction is also presented; even with 20 mM-azide (15 times the apparent K_m for N_2H_4 formation), a residual 20% of electrons is being used for H^+ reduction at pH 6.8.

The nitrogenous product balance is in the molar ratio $1N_2H_4:2N_2:5-6NH_3$ for both experiments; NH_3 formed as a consequence of N_2 and N_2H_4 formation (eqns. 1 and 2) is clearly insufficient to explain total NH_3 formation. We therefore consider an eqn. 3, representing total reduction of azide:

Calculation of electron pairs involved in the various reactions can be made from the N_2 , N_2H_4 and NH_3 formed, assuming that all NH_3 not stoichiometric with N_2 or N_2H_4 is formed by the reaction shown in eqn. (3). Table 2 gives these calculations and allows a comparison of the total calculated from the nitrogenous products with the electron pairs calculated from the decrease in H_2

Fig. 6. Dependence of H_2 evolution concomitant with N_3^- reduction on N_3^- concentration and pH 8.2 at $30^\circ C$. The data were obtained under the standard assay conditions described in the Methods and materials section. ΔH_2 was calculated from the difference between the H_2 produced at a particular concentration of N_3^- and the H_2 evolved in the absence of N_3^- . The ordinate intercept calculated from a least-squares fit of the data-points corresponds to a ΔH_2 of 370 nmol. Since 340 ± 20 nmol of H_2 were produced in the absence of N_3^- (-----), H_2 production is zero at infinite N_3^- concentration. The data-points represent average of four determinations at pH 8.2. At lower pH, the ΔH_2 at infinite N_3^- concentrations was less than the H_2 produced in the absence of N_3^- . These differences are plotted as a percentage of the total electron flux against pH in the insert to the Figure.

production in the presence of azide (ΔH_2). The correspondence is almost exact for expt. 1 and in reasonable agreement in expt. 2.

From eqns. 1, 2 and 3, and the relative concentrations of N_2 , N_2H_4 and NH_3 , the ^{15}N concentration in the NH_3 fraction can be calculated, assuming that NH_3 from eqn. 1 has half the atom % excess ^{15}N of the labelled azide nitrogen, that NH_3 from eqn. 2 will have a similar labelling, and that residual NH_3 will have one-third the atom % excess of the terminal nitrogen of azide. Since some NH_3 contamination occurs, this was measured and included in the calculation of the ^{15}N concentration in NH_3 .

Calculation gave a value for atom % excess ^{15}N of 38.3% for the data in expt. 2, Table 2; the experimental value from the $^{29}N_2/^{30}N_2$ ratio was 40.3%. The isotopic distribution is thus consistent with the product balance and the observed isotopic labelling pattern in N_2 and N_2H_4 .

$^{15}N_2H_4$ reduction in the presence of N_3^-

N_2H_4 is known to be reduced to NH_3 by nitrogenase, although the process requires a high pH and a high N_2H_4 concentration ($K_m = 15$ – 20 mM) (Bulen, 1976). We examined the question of whether N_2H_4 in solution was in equilibrium with intermediates on the enzyme leading to NH_3 formation from N_3^- .

Azide (20 mM) was reduced by Kp nitrogenase at pH 6.8 in the presence of 1 or 0.1 mM $^{15}N_2H_4$. After removal of azide etc. through columns of AG 1-X2 resin, N_2H_4 was oxidized to N_2 with alkaline ferricyanide [final concn. 0.1 M- $K_3Fe(CN)_6$, 0.1 M-

Table 2. Product and electron-balance studies on azide reduction by Kp nitrogenase at $30^\circ C$

Each assay contained, in 3.2 ml: 144 μg of Kp1, 236 μg of Kp2, 29 μmol of ATP, 32 μmol of $MgCl_2$, 80 μmol of Hepes buffer, pH 6.8, 32 μmol of sodium dithionite, 64 μmol of creatine phosphate, 0.8 mg of creatine phosphokinase, 64 μmol of NaN_3 . The assay time was 15 min in Expt. 1 and 25 min in Expt. 2.

Expt.	1		2	
Product	Amount found (nmol)	Electron pairs (nmol)	Amount found (nmol)	Electron pairs (nmol)
H_2 (no azide)	2573		4266	
H_2 (20 mM-azide)	513		832	
ΔH_2		2060		3434
N_2H_4	211	633*	359	1077
N_2	424	424†	685	685
Total	635		1044	
NH_3	1310		1094	
Minus $N_2 + N_2H_4$	675	900‡	900	1200
Total electron pairs		1957		2962

* Assumes that one N_2H_4 and one NH_3 molecule are produced by three electron pairs.

† Assumes that one N_2 and one NH_3 molecule are produced by each electron pair.

‡ Assumes that NH_3 not accounted for accompanying N_2 or N_2H_4 is produced at the rate of $3NH_3$ per four electron pairs.

NaOH], which also served to drive off NH_3 for collection by microdiffusion.

In control experiments, no enzymic NH_3 formation from N_2H_4 was detected under conditions optimal for N_2H_4 formation.

Ammonia was oxidized to N_2 with alkaline hypobromite; no ^{15}N was detected in the NH_3 produced. We conclude that exchange between free N_2H_4 and nitrogen hydrides on the enzyme does not occur.

Azide reduction in the presence of $^{15}\text{N}_2$

We have also investigated whether $^{15}\text{N}_2$ in solution is in equilibrium with an enzyme-bound N_2 intermediate that may be produced when azide is reduced to N_2H_4 and NH_3 .

Azide (5mM) was reduced by Kp nitrogenase under $^{15}\text{N}_2$ 100kPa (~1atm). With the methods described in the preceding section for determination of ^{15}N in N_2H_4 and NH_3 , ^{15}N was detected in the NH_3 produced by the enzyme, but not in the N_2H_4 . Thus $^{15}\text{N}_2$ can be reduced by the enzyme concomitantly with azide reduction, but does not contribute to N_2H_4 formation, implying that azide reduction to N_2H_4 may involve a different form of enzyme to that which reduces N_2 .

Reduction of azide by Av nitrogenase

Since previous studies (Schöllhorn & Burris, 1967; Hardy & Knight, 1967) in which N_2 and NH_3 were reported to be the only products of N_3^- reduction were with nitrogenase isolated from *A. vinelandii*, we were concerned whether N_2H_4 production was a unique property of Kp nitrogenase.

Standard assays containing 47 μg of Av1 and 90 μg of Av2 were run for 15 min at 30°C. In the absence of azide, 655 nmol of H_2 were produced, corresponding to a specific activity of 916 nmol H_2 /min per mg of Av1. When NaN_3 (20mM) was present, 158 nmol of H_2 , 260 nmol of NH_3 and 80 nmol of N_2H_4 were produced. Thus 76% of the electron flux went into N_3^- reduction (cf. 73% for Kp nitrogenase) and the ratio of NH_3 to N_2H_4 produced was 3.2 : 1 (cf. 6 : 1 for Kp nitrogenase).

Pre-steady-state kinetics of N_2H_4 , NH_3 and H_2 formation

A rapid-quench technique was used to determine the amount of N_2H_4 , NH_3 and H_2 produced at times in the range 0.02–5.00 s. A temperature of 23°C was used instead of 30°C used for the steady-state kinetic studies presented above in order that the data could be compared with those obtained for other substrates of nitrogenase (R. N. F. Thorneley & D. J. Lowe, unpublished work).

The data for H_2 evolution, presented in Fig. 7, show that a constant rate (200 nmol H_2 /min per mg of Kp1) was rapidly attained and that the pre-

Fig. 7. Pre-steady-state time course for H_2 evolution concomitant with N_3^- reduction at 23°C and pH 7.5. The data were obtained by using the rapid-quench technique as described in the Methods and materials section. The pre-steady-state lag phase was <100 ms and the steady-state rate of H_2 production calculated from the slope was 200 nmol of H_2 /min per mg of Kp1.

Fig. 8. Pre-steady-state time course for N_2H_4 production from N_3^- at 23°C and pH 7.5.

The data were obtained by using the rapid-quench technique as described in the Methods and materials section. The pre-steady-state lag phase was 180 ± 20 ms when calculated from a least-squares fit of the data at times longer than 200 ms. A steady-state rate of 57 nmol of N_2H_4 /min per mg of Kp1 was calculated from the slope.

steady-state lag, defined as the time-axis intercept, was less than 100 ms.

Fig. 8 shows that N_2H_4 production occurred with a pre-steady-state lag of 180 ± 20 ms. A steady-state rate of 57 nmol N_2H_4 /min per mg of Kp1 was calculated from the slope of the line in Fig. 8.

The data presented in Fig. 9 for NH_3 formation show an increased scatter relative to those for N_2H_4 and H_2 . This was due to the difficulty in measuring

NH_3 at the nanomolar concentrations and limited success in decreasing contamination by extraneous NH_3 during the rapid-quench and analytical procedures. An additional source of NH_3 was probably the decomposition of HN_3 produced on quenching the enzyme with HClO_4 , despite the neutralization procedure described. Although the EDTA quench at pH 7.4 was shown to be rapid on the seconds time scale, which was acceptable for the steady-state assays, it has not yet been shown to be capable of quenching functioning nitrogenase in the milli-second range required for rapid-quench experiments. The data of Fig. 9 show that NH_3 was produced with a calculated lag of 80ms from a least-squares fit of the data points from 0.02 to 1.15s. However, since NH_3 may be released at several points in the catalytic cycle, a straight line is clearly likely to be a simplification of a more complex time course, and 80ms must be regarded as a minimum and 200ms as a maximum value for the lag (Fig. 9).

The data-points from 1.15 to 5s lie on a different line of reduced slope corresponding to a steady-state rate of 350nmol NH_3 /min per mg of Kp1. Thus the lag in NH_3 production is followed by a 'burst' phase that is complete after about 1s. For the present we only wish to emphasize the NH_3 is formed with a lag of less than 200ms, which is significantly shorter than the lag of 400ms observed for NH_3 production from N_2 (R. N. F. Thorneley, M. J. Dilworth, D. J.

Lowe & J. Peters, unpublished work) and is equal to or less than the 180ms lag for N_2H_4 formation from N_3^- .

The steady-state rates of H_2 , N_2H_4 and NH_3 production calculated from the rapid-quench data give a total flux of 760nmol of electron pairs/min per mg of Kp1 by using the assumption stated in Table 1. This flux is lower than that predicted at 23°C from the specific activities of protein samples taken from the rapid-quench apparatus at the end of the experiment (1300–1600nmol of C_2H_2 reduced/min per mg of Kp1 at 30°C). Inhibition of nitrogenase at the high protein concentrations (15 μM -Kp1, 75 μM -Kp2) necessary for rapid-quench studies relative to those used for long-time steady-state assays (0.2 μM -Kp1, 1.0 μM -Kp2) has been previously observed for acetylene reduction and is attributed to inhibition of the dissociation reaction of Kp2 from Kp1 (R. N. F. Thorneley & D. J. Lowe, unpublished work). However, although the absolute rates of NH_3 and N_2H_4 production from N_3^- were lower in the rapid-quench experiments, the relative rates were the same as those reported above under the standard assay conditions with dilute proteins (Table 1).

Discussion

The reduction of azide by nitrogenase to give N_2 , NH_3 and N_2H_4 is represented by eqns. 1, 2 and 3. The product stoichiometry indicated by these equations has been measured and confirms a characteristic property of nitrogenase, namely that the total electron flux remains constant as the concentration of substrate increases to compete with protons for the available electrons (Table 2). Eqns. 1, 2 and 3 represent respectively two-, six- and eight-electron reductions of N_3^- .

Apparent inhibition of azide reduction measured by NH_3 formation has been reported (Schöllhorn & Burris, 1967; Peters, 1979) for higher azide concentrations. In other experiments (results not shown) we have found that the $\text{N}_2\text{H}_4/\text{NH}_3$ ratio increases by 50% over the range from 2 to 20mM-azide. Higher azide concentration thus favours N_2H_4 formation, and NH_3 formation associated with it is less efficient in utilization of electrons than NH_3 produced with N_2 . The apparent inhibition of NH_3 formation and total electron flux previously reported is therefore explicable in terms of N_2H_4 formation and shift in product balance with increasing azide concentration.

Two important questions concerned with the mechanism of these reactions arise: firstly, do they all occur at the same active site and what is the relationship between the site and that for N_2 reduction? secondly, what determines the relative rates of these reactions?

Fig. 9. Pre-steady-state time course for NH_3 formation from N_3^- at 23°C and pH 7.5

The data were obtained by using the rapid-quench technique as described in the Methods and materials section. The pre-steady-state lag of 80ms was obtained by a least-squares fit of the data-points from 20ms to 1150ms. The steady-state rate of NH_3 formation at times longer than 1.15s was 350nmol of NH_3 /min per mg of Kp1. Data from two separate experiments (●, ▲) are combined; significance of regression lines: 20–1150ms, $P < 0.001$; 0.95–5s, $P < 0.01$.

The different K_m values for N_3^- depending whether NH_3 or N_2H_4 production is measured, is not good evidence for the two products being produced at different sites. This is because the kinetic complexity of nitrogenase makes it unlikely that K_m will equal K_s . Since both the K_m values decrease by only a factor of two when the pH is raised from 6.8 to 7.5, it is unlikely that HN_3 is the exclusive substrate. The simplest interpretation of the pH-rate profiles for NH_3 and N_2H_4 production is that N_3^- is the substrate that binds at a single site and that the reaction of eqn. (2) is favoured at lower pH. The only other evidence for two substrate-reducing sites on nitrogenase is the inhibition by CO of all substrate reductions except that of protons to give H_2 . However since CO was a non-competitive inhibitor of both NH_3 and N_2H_4 production, these data again provide no evidence for NH_3 and N_2H_4 being produced at different sites.

Although molybdenum is the centre most likely to be involved in N_3^- reduction, Johnson & Holm (1978) have shown that Cl^- and $CH_3CO_2^-$ (acetate ion) can co-ordinate to Fe in a Fe_4S_4 cluster. Whether N_3^- will bind in a similar manner and be reduced is yet to be demonstrated. Chatt & Dilworth (1977) have shown that molybdenum in its higher oxidation states (III, IV or V) will bind N_3^- , although they did not show reduction to N_2H_4 or NH_3 .

The relative contributions of the reactions shown in eqns. (1), (2) and (3) to the overall product balance may be influenced by any of the following: (a) net oxidation level of the metal cluster involved; (b) rates of reduction of intermediates relative to their dissociation rates from the site; (c) pK_a values of intermediates and pH of the medium.

Both the lowering of pH and limiting the electron flux by decreasing the ratio of Kp2 to Kp1 favoured N_2H_4 production. This is consistent with reactions (1) and (2) occurring with a more oxidized form of the protein than that required for reaction (3). Since reaction (3) may involve a bound dinitrogen intermediate, the same low oxidation level may be required for this reaction as that required to reduce N_2 . Differential effects of decreased electron flux on two- and six-electron substrates have been reported previously (Silverstein & Bulen, 1970; Davis *et al.*, 1975; Hageman & Burris, 1978) and discussed in terms of the different reduction levels to which nitrogenase must be 'pumped' in order to be effective.

In the case of azide reduction, limiting electron flux by low Kp2/Kp1 ratios is likely to favour eqns. (1) and (2) rather than (3), whereas high Kp2/Kp1 ratios are likely to increase (2) and (3). A maximum in the N_2H_4/NH_3 ratio is therefore to be expected.

The competitive inhibition of azide reduction (N_2H_4 formation) by N_2 is consistent with N_2

binding to the same site as N_3^- . However, since the K_i for N_2 acting as an inhibitor is ten times larger than the K_m for N_2 reduction to NH_3 , N_3^- and N_2 cannot be binding to the same form of the enzyme. Nonetheless, $^{15}N_2$ can be reduced to $^{15}NH_3$ when it inhibits N_2H_4 production; since it does not contribute ^{15}N to N_2H_4 , N_2 reduction to NH_3 and azide reduction to N_2H_4 are likely to involve different oxidation levels of the enzyme.

The failure to detect doubly labelled N_2 either in the gas phase or after alkaline ferricyanide oxidation of the N_2H_4 produced in reaction (2) means that there is no evidence for the formation of new nitrogen-nitrogen bonds when ^{15}N -labelled azide is reduced. The mechanism of azide reduction cannot therefore involve any of the following steps: (1) the coupling of two molecules of azide; (2) the coupling of intermediates derived from one azide molecule (e.g. $=NH$) with a second molecule of azide; (3) the coupling of intermediates derived from two molecules of azide. We discuss these possibilities because Kane-Maguire *et al.* (1970) showed that the acid catalysed decomposition of $[Ru(NH_3)_5N_3]^{2+}$ to give $[(NH_3)_5Ru-N_2-Ru(NH_3)_5]^{4+}$ involves an association of two molecules of $[(NH_3)_5RuNH]^{3+}$ to form a new nitrogen-nitrogen bond. We conclude that this type of reaction is not involved in the enzymic reduction of azide to either N_2 or N_2H_4 .

Intermediates on the enzyme leading to NH_3 formation from azide are not in equilibrium with $^{15}N_2H_4$ in solution. Likewise intermediates leading to N_2H_4 formation from azide are not in equilibrium with $^{15}N_2$ in solution. These results are consistent with N_2H_4 being a poor substrate under the conditions of these experiments and also with N_2H_4 not being a product of N_2 reduction.

The complete suppression of H_2 evolution, calculated from the extrapolation of the dependence of H_2 -evolution rates on $[N_3^-]$ at pH 8.2, shows that H_2 production is not an integral part of the mechanism of N_3^- reduction. This is in contrast with H_2 evolution concomitant with N_2 reduction, which is not suppressed even under a 5.06 MPa (50 atm) pressure of N_2 (W. H. Orme-Johnson, personal communication) and accounts for at least 25% of the total electron flux. Chatt (1980) has proposed that N_2 displaces bound H_2 from molybdenum in a low oxidation state and that this reaction is obligatory for the reduction of N_2 to NH_3 . The failure to observe H_2 evolution under conditions when N_3^- is reduced is consistent with N_3^- binding to a higher oxidation state of molybdenum than that which will evolve H_2 and reduce N_2 .

The pre-steady-state kinetics of H^+ , C_2H_2 and N_2 reduction by nitrogenase have been analysed with a computer model (R. N. F. Thorneley & D. J. Lowe, unpublished work) in which a single electron is rapidly transferred from Kp2 to Kp1 protein

Scheme 1. Mechanism for reduction of azide to N_2H_4 and NH_3

($k = 2 \times 10^2 \text{ s}^{-1}$; Thorneley, 1975) with concomitant hydrolysis of MgATP (Eady *et al.*, 1978). Before the next electron can be transferred, a slow-rate limiting reaction with a computed first-order rate constant of $k = 3.5 \text{ s}^{-1}$ (at 23°C) has to occur. This reaction may be the dissociation of oxidized Kp2 from Kp1 protein. The model predicts lags of about 120, 280 and 680 ms for the appearance of two-, four- and six-electron reduction products. The lag of about 80 ms for NH_3 production from N_3^- is consistent with this model if at least some of the NH_3 is formed by eqn. 1.

The data do not allow a prediction of the cleavage pattern of azide in eqn. (1). However, the lag of 180 ms for N_2H_4 production suggests that the first three electrons transferred in eqn. (2) are used to produce N_2H_4 and that the subsequent three electrons produce the NH_3 . This implies that if the α -nitrogen atom of N_3^- is co-ordinated to a metal centre, then it is the β - and γ -nitrogen atoms that are reduced to give N_2H_4 . It also implies that a co-ordinated nitride ($\equiv\text{N}$) or imido ($=\text{NH}$) group is an intermediate in NH_3 production according to eqn. (2).

This mechanism is shown in Scheme 1 in which N_3^- is shown binding to a metal centre (a) to give the enzyme-substrate complex (b). The conversion of (b) into (c) is achieved by three single-electron transfers and coupled protonations. Since the position of the protons on the two partially reduced intermediates formed between (b) and (c) is not certain, these have not been included. The hydro-

lysis of (c) to (d), in which N_2H_4 is released is considered rapid compared with the rate of dissociation of Kp2_{ox} from Kp1, which is thought to limit the rate of electron transfers (R. N. F. Thorneley & D. J. Lowe, unpublished work). The nitride intermediate (d) may undergo stepwise reduction and protonation to give NH_3 [via (e), (f) and (g)] or may hydrolyse to give NH_3 and oxidized metal centre (h), which is reduced via (i) and (j) to complete the catalytic cycle. This latter mechanism is consistent with the observed pre-steady-state burst for NH_3 , since the NH_3 is released in the middle of the catalytic cycle. The stepwise reduction and protonation of the nitride intermediate with NH_3 released at the end of the catalytic cycle would not be consistent with a burst phase. However, interpretation of the pre-steady-state kinetics of NH_3 formation is complicated by NH_3 produced by eqns. 1 and 3.

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