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The mechanism of *Klebsiella pneumoniae* nitrogenase action

Pre-steady-state kinetics of an enzyme-bound intermediate in N_2 reduction and of NH_3 formation

Roger N. F. THORNELEY and David J. LOWE
A.F.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, U.K.

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The reduction of N_2 to $2NH_3$ by *Klebsiella pneumoniae* nitrogenase was studied by a rapid-quench technique. The pre-steady-state time course for N_2H_4, formed on quenching by the acid-induced hydrolysis of an enzyme-bound intermediate in N_2 reduction, showed a 230ms lag followed by a damped oscillatory approach to a constant concentration in the steady state. The pre-steady-state time course for NH_3 formation exhibited a lag of 500ms and a burst phase that was essentially complete at 1.5s, before a steady-state rate was achieved. These time courses have been simulated by using a previously described kinetic model for the mechanism of nitrogenase action [Lowe & Thorneley (1984) *Biochem. J.* **224**, 877–886]. A hydrazido(2−) structure (=N–NH_2) is favoured for the intermediate that yields N_2H_4 on quenching. The NH_3-formation data indicate enzyme-bound metallo-nitrido (≡N) or -imido (=NH) intermediates formed after N–N bond cleavage to produce the first molecule of NH_3 and which subsequently give the second molecule of NH_3 by hydrolysis on quenching. The simulations require stoichiometric reduction of one N_2 molecule at each Mo and the displacement of one H_2 when N_2 binds to the MoFe protein. Inhibition by H_2 of N_2-reduction activity occurs before the formation of the proposed hydrazido(2−) species, and is explained by H_2 displacement of N_2 at the active site.

Nitrogenase catalyses the reduction of N_2 to NH_3 with the concomitant evolution of H_2 and hydrolysis of MgATP. The rate-limiting step in the catalytic cycle is the dissociation of oxidized Fe protein [$Kp2_{ox.}$ $(MgADP)_2$] from reduced MoFe protein (Kp1) (Thorneley & Lowe, 1983). This protein-dissociation reaction occurs after each electron transfer from the Fe protein to the MoFe protein. The electron-transfer reaction is coupled to the hydrolysis of MgATP (Eady *et al.*, 1978; Hageman *et al.*, 1980). Lowe & Thorneley (1984*a*) showed how, by coupling together eight consecutive electron-transfer cycles, each involving a slow protein-dissociation reaction, a scheme, whose kinetics were defined by independently determined rate constants, could be constructed that is capable of simulating both steady-state and pre-steady-state kinetics of H_2 evolution.

Although Lowe & Thorneley (1984*a*) presented a complete scheme that included N_2 reduction to NH_3, they only discussed in detail those aspects relevant to H_2 evolution. In the present paper we concentrate on N_2 reduction and justify the conclusions implicit in Scheme 2 of Lowe & Thorneley (1984*a*) by comparison of experimentally determined pre-steady-state time courses for N_2H_4 and NH_3 production with computer simulations. A number of the rate constants in Table 1 of Lowe & Thorneley (1984*a*) that have been used in these simulations were determined by analysis of steady-state kinetic data for N_2 reduction and H_2 inhibition of N_2 reduction under high and low electron-flux conditions. The analysis of these data is contained in an accompanying paper (Lowe & Thorneley, 1984*b*).

N_2H_4 is obtained from nitrogenase that is reducing N_2 when the enzyme is quenched with acid or alkali (Thorneley *et al.*, 1978, 1980). It does not accumulate in solution during N_2 reduction under physiological conditions. N_2H_4 arises from the reaction of protons or solvent, in an acid/base-catalysed reaction, with a dinitrogen hydride, which is an intermediate in N_2 reduction. Scheme 1 shows how this occurs and the use of *p*-dimethylaminobenzaldehyde to detect the very low concentrations of N_2H_4 produced.

Abbreviations used: Kp1 and Kp2, MoFe-containing protein and Fe-containing protein components respectively of *Klebsiella pneumoniae* nitrogenase.

The species E_n in Scheme 1 represents one of two independently functioning halves ($Kp1^{\dagger}$) of the tetrameric Kp1 protein. E_n contains one N_2-binding site. The subscript n indicates the number of electron equivalents by which $Kp1^{\dagger}$ has been reduced relative to the resting state E_0, which is that of Kp1 as isolated in the presence of $S_2O_4^{2-}$ ion (see Schemes 1 and 2 of Lowe & Thorneley, 1984*a*). We have previously suggested that the intermediate that gives N_2H_4 on quenching contains a bound hydrazido(2−) group (Thorneley *et al.*, 1978, 1980), i.e. $n+x=4$ in Scheme 1. This suggestion was based entirely on the observation that similar yields of N_2H_4 were obtained from the enzyme under acid and alkali quench conditions. The hydrazido(2−) complexes of W with phosphine ligands are the only complexes (in a series involving bound dinitrogen and dinitrogen hydrides) that also give N_2H_4 in equal yield after treatment with either acid or alkali (Anderson *et al.*, 1981). In addition, the hydrazido(2−) level of reduction of dinitrogen appears to have greater stability than other metal-bound partially reduced dinitrogen derivatives (Richards, 1983; Henderson *et al.*, 1983). The pre-steady-state kinetics for N_2H_4 formation presented below, together with the mechanism outlined in Scheme 2 of Lowe & Thorneley (1984*a*), provide further evidence for the intermediate being a hydrazido(2−) species. A comparison of the experimentally determined time course for N_2H_4 formation with computer simulations have allowed the value of $n+x$ in Scheme 1 to be determined.

NH_3 is the only product of N_2 reduction that is released by nitrogenase during turnover at neutral pH. Pre-steady-state kinetic data for NH_3 formation, together with computer simulations based on Table 1 and Scheme 2 of Lowe & Thorneley (1984*a*), were used to determine the position in the catalytic cycle at which NH_3 is released from the enzyme on quenching with acid. These data were obtained by using the rapid-quench technique described by Lowe & Thorneley (1984*a*). It is therefore necessary to discuss, not only the chemistry occurring at pH7.4 with functioning enzyme, but also those reactions that are induced by the acid quench. This is particularly important when using the data to decide at which point in the catalytic cycle the N–N bond is cleaved and the two molecules of NH_3 are released.

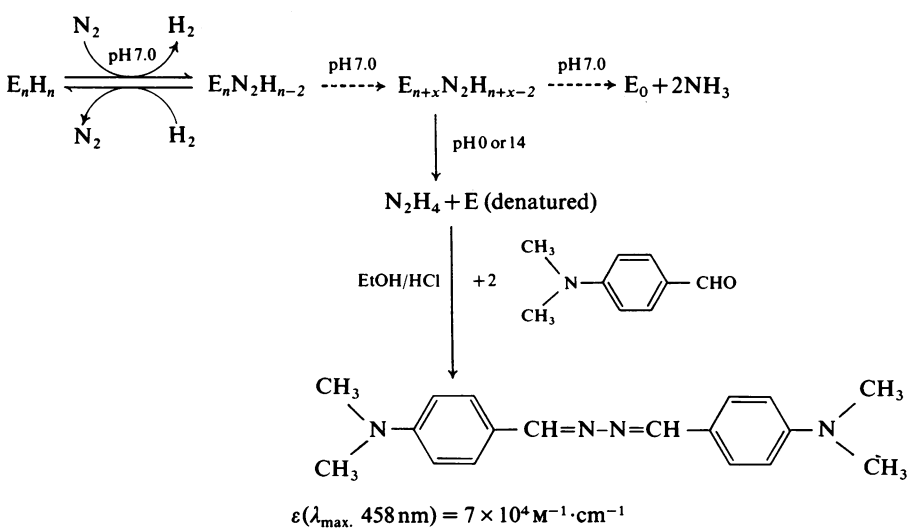
Materials and methods

Protein preparation

The nitrogenase component proteins were purified, assayed and their metal content determined as previously described (Lowe & Thorneley, 1984*a*).

N_2H_4 determination

Samples were prepared by using the rapid-quench technique as previously described (Lowe & Thorneley, 1984*a*). The quenching agent [1.6ml of aq. 50% (v/v) ethanol] contained 60mM-*p*-dimethylaminobenzaldehyde and 1M-HCl. After the quenching, the precipitated protein was removed by centrifugation, and the absorbance of the supernatant was determined at 458nm in a 4cm-light-path cuvettes in the spectrophotometer. An experimentally determined molar absorption coefficient



$\varepsilon(\lambda_{max.}\ 458\,nm) = 7 \times 10^4\,M^{-1}\cdot cm^{-1}$

Scheme 1. *Detection of an intermediate in N_2 reduction that yields N_2H_4 on quenching with acid or alkali*

of $7 \times 10^4 M^{-1} \cdot cm^{-1}$ for the *p*-dimethylaminobenzaldehyde derivative of N_2H_4 was used to determine the N_2H_4.

NH_3 determination

The sample of quenched protein (0.4ml of reacting enzyme shot into 0.5ml of 1.0M-$HClO_4$) was centrifuged to remove precipitated protein, and the pH of the supernatant was adjusted to 7.0 with NaOH. Supernatant (0.7ml) was applied to a small column (Pasteur pipette, 40mm × 5mm) of AG-1-X2 anion-exchange resin. NH_4^+ ion was eluted with two washes of water (0.5ml each). 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 1.5ml samples from experiments were treated sequentially with 0.3ml of phenate, 0.45ml of nitroprusside and 0.45ml of hypochlorite. Absorbance at 630nm was determined in a 4cm-pathlength cuvette after 20min; the method is capable of measuring NH_3 to a precision of ± 2nmol per sample.

Computing

This was done as described in the preceding paper (Lowe & Thorneley, 1984*a*).

Results and discussion

N_2H_4 derived from an enzyme bound intermediate in N_2 reduction

Figs. 1 and 2 show data obtained with the rapid-quench technique for N_2H_4 formation over the time range 0.01–10s. After a lag phase of approx. 200ms, the concentration of N_2H_4 increases to a maximum of 6.3 ± 0.2 μM at 1s and then falls with a damped oscillation to a steady-state concentration of 4.8 ± 0.2 μM, which is maintained from 3 to 10s. This oscillatory behaviour is explained by reference to Scheme 2, in which N_2H_4 is released from $E_4N_2H_2$ and related protein complexes present in this part of the cycle.

The lag phase of 230ms, the time at which the maximum amplitude occurs (1s) and the extent of the overshoot before a steady-state concentration of N_2H_4 is attained all determine that only species associated with E_4 yield N_2H_4 on quenching. Species associated with E_3 would exhibit a shorter lag phase, a maximum amplitude occurring too early and too large an overshoot. Similarly, species associated with E_5 would have too long a lag phase, a maximum amplitude occurring too late and too small an overshoot. This is shown by the simulations in Fig. 2. The shape of these curves is similar to the response of an electrical circuit containing a low-pass filter to a step function. In our circuit (Scheme 2 of Lowe & Thorneley, 1984*a*), the step pulse is applied at $t = 0$, since at this time all the $Kp1^{\dagger}$ is present in the form E_0. A damped 'wave' of

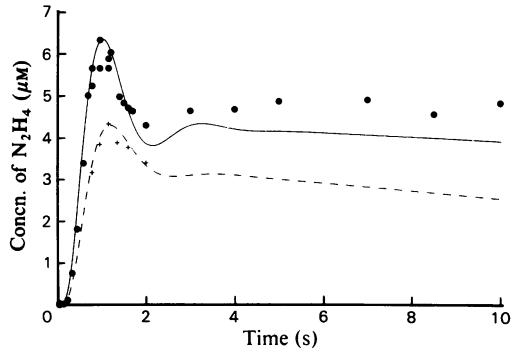


Fig. 1. *Pre-steady-state time course for N_2H_4 formation from N_2 at 23°C at pH7.4*

The continuous line (——) through the ● data points is under 100% N_2 and the broken line (----) through the + data points is under 50% N_2/50% H_2. Both lines are simulations made by using Scheme 2 and the rate constants in Table 1 of Lowe & Thorneley (1984*a*) with N_2H_4 released from $E_4N_2H_2$ and associated complexes with Kp2 (see Scheme 3). [Kp1] = 37 μM, [Kp2] = 125 μM, [MgATP] = 9mM, and [$Na_2S_2O_4$] = 10mM.

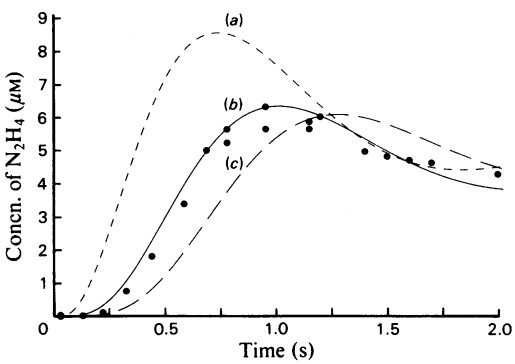
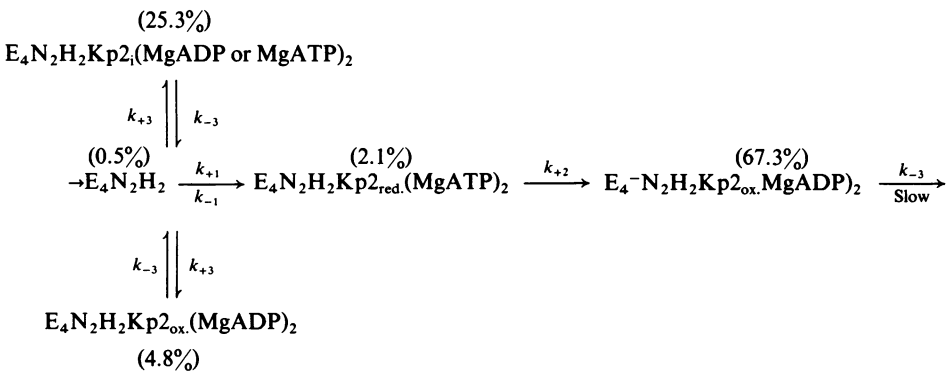


Fig. 2. *Determination of which species in Scheme 2 give rise to N_2H_4 on quenching*

The data points are taken from Fig. 1. Curve (*b*) is a simulation in which species $E_4N_2H_2$ and associated complexes with Kp2 (see Scheme 3) give N_2H_2 on quenching. Curves (*a*) and (*c*) are simulations in which species E_3N_2H and $E_5N_2H_3$ and associated complexes with Kp2 respectively are assumed to give N_2H_4 on quenching. Statistical analysis shows that curve (*b*) is more than 1000 times more likely to be a fit to the data than curve (*a*) (variance ratio $a/b = 45$) or curve (*c*) (variance ratio $c/b = 8.6$) for 18 degrees of freedom.



Scheme 2. *Intermediates in N_2 reduction that yield N_2H_4 on quenching with acid*

$Kp1^{\dagger}$ concentration passes round the circuit with an amplitude that decreases until a steady state is attained for all the states of $Kp1^{\dagger}$.

The application of an *F*-test to the curves and data in Fig. 2 shows that it is at least 1000 times more likely that N_2H_4 arises from species E_4 than from species E_3 or E_5. We note the systematic (approx. 10%) deviation of the continuous simulated curve in Fig. 1 at times greater than 3s; this may indicate that our assumption that the rate constants do not vary with oxidation level of Kp1 (E_n, $n = 0$ to 7 in Scheme 2 of Lowe & Thorneley, 1984*a*) is only accurate to approx. 5%. Since this is within the experimental error in the determination of these rate constants, we have chosen to disregard it in the interests of simplicity.

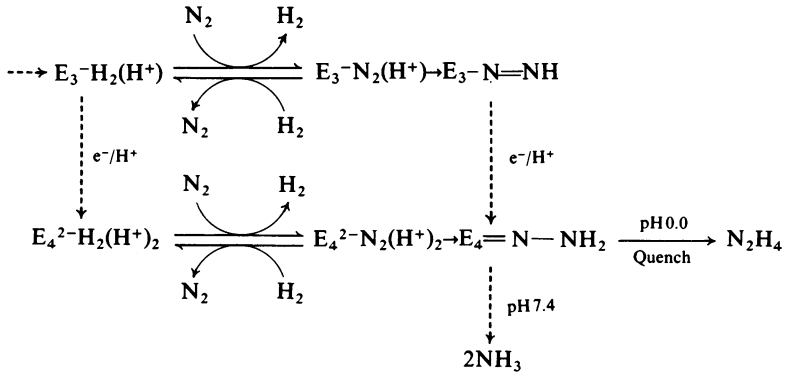
Species $E_4N_2H_2$ in Scheme 2 is free $Kp1^{\dagger}$ that has been reduced by 4 electron equivalents relative to its oxidation level when isolated in the presence of $S_2O_4{}^{2-}$. Two of these electrons are lost when N_2 binds by the displacement of H_2 (see the section on N_2 binding and H_2 inhibition below and Lowe & Thorneley, 1984*a*,*b*).

Lowe & Thorneley (1984*a*) showed that H_2 is not evolved until at least two protein dissociations have occurred. They explained this by assuming that bound $Kp2_{ox.}(MgADP)_2$ restricts access of protons to the reduction site on $Kp1^{\dagger}$ and considered that the protein-dissociation reaction induces proton transfer to the reduction site after each electron transfer from Kp2 to $Kp1^{\dagger}$. This is important in understanding the number of protons shown to be associated with the intermediates in N_2 reduction in Scheme 2. These species are all formed after four slow dissociation steps (k_{-3}). Species $E_4{}^{-}N_2H_2Kp2_{ox.}(MgADP)_2$, although it is reduced by 3 electron equivalents, is shown with only two protons associated with the N_2. It is only after the next slow protein dissociation that in our model $E_4{}^{-}N_2H_2$ protonates to give $E_5N_2H_3$. E_5 and its complexes cannot yield N_2H_4 on quenching, since this is incompatible with the experimentally determined time course (Figs. 1 and 2). We stress we have no spectroscopic evidence for the assignment of structures to these dinitrogen hydride intermediates. However, since a two-electron reduction of N_2 bound to a metal centre and the addition of two protons can yield the bound hydrazido(2−) species, which is capable of yielding N_2H_4 on quenching, we favour this structure for the intermediate bound to E_4. The two additional electrons required to convert a bound hydrazido(2−) intermediate into free N_2H_4 come from the metal centres of $Kp1^{\dagger}$, i.e. on quenching the metal centres of E_4 become oxidized by 2 equivalents relative to E_0.

In order to simulate the dependence of the apparent $K_m^{N_2}$ on H_2 partial pressure, it was necessary to assume that $E_4N_2H_2$ derived from E_3N_2H does not react with H_2 to yield E_4H_4 and N_2 (Lowe & Thorneley, 1984*b*). If hydrazido(2−) is an intermediate in N_2 reduction, then irreversibility may arise from protonation of the N_2 bound to E_4. The tungsten complexes $[WX(N\text{–}NH_2)(dppe)_2]^+$ (X = F, Cl or Br; dppe = $Ph_2PCH_2CH_2PPh_2$) only deprotonate as far as the diazenido stage $[WX(N\text{–}NH)(dppe)_2]$ on addition of triethylamine or K_2CO_3 (Chatt *et al.*, 1974, 1976). These complexes may be good models for the proposed enzyme-bound hydrazido(2−) intermediate at E_4, since it is the W complexes, not the Mo complexes $[M(N_2)_2(PR_3)_4]$ (M = W or Mo; $PR_3 = PMe_2Ph$ or $PMePh_2$), that when treated with H_2SO_4 in methanol give the higher yield of NH_3 (Chatt *et al.*, 1977; Chatt, 1980).

Scheme 3 is an expansion of the equilibria defined by k_{+10}, k_{+10}, k_{-11} and k_{-11} in Scheme 2 of Lowe & Thorneley (1984*a*).

The species E_3H_3 and E_4H_4 have been written as $E_3H_2(H^+)$ and $E_4H_2(H^+)_2$ respectively to indicate a metal dihydride with one or two protons on adjacent groups. In our model these protons can



Scheme 3. *Proposed irreversible protonation of bound N_2 to give a hydrazido(2−) intermediate that yields N_2H_4 on quenching*

react with the dihydride to yield E_1H (k_{+8}) or E_2H_2 (k_{+9}) and H_2, or they may be transferred to bound N_2. They cannot react directly with the metal centre to yield tri- or tetra-hydrides. The level of reduction of bound N_2 and the location of protons at the active site of the MoFe protein (free and complexed with reduced, oxidized and inactive Fe protein) at all stages in Scheme 2 of Thorneley & Lowe (1984) must be speculative. However, the above suggestion that a metal dihydride is the intermediate from which H_2 is displaced when N_2 binds is attractive because it also provides a mechanism by which all the experimentally derived criteria for the formation of $^1H^2H$ from $^2H_2$ can be satisfied (Lowe & Thorneley, 1984*b*).

The steady-state concentration of N_2H_4 simulated by Scheme 2 and Table 1 of Lowe & Thorneley (1984*a*) and shown in Fig. 1 requires that each Mo can bind and reduce only one N_2 molecule. The coincidence between the simulated and experimental steady-state concentrations of N_2H_4 confirms the 1:1 stoichiometry for N_2 binding to Mo. Therefore the two Mo centres in Kp1 must function independently, and mechanisms involving N_2 bridging between the Mo centres cannot be valid (Luneva *et al.*, 1982). However, we cannot exclude N_2 bridging between Mo and Fe centres.

Scheme 2 shows that free $E_4N_2H_2$ is present at a relatively low concentration. This is a consequence of the same kinetic constraints that minimize H_2 evolution from species E_2 during the reduction of Kp1 to the redox state that binds N_2 (see the discussion and Scheme 2 in Lowe & Thorneley, 1984*a*). The complexes present in highest concentration are those in which $Kp1^{\dagger}$ is complexed with $Kp2_{ox.}(MgADP)_2$ or with inactive $Kp2_i$. The affinity of $Kp2_i$ for $Kp1^{\dagger}$ (defined by k_{+3} and k_{-3}; Table 1 of Lowe & Thorneley, 1984*a*) has been assumed to be the same as that of active $Kp2_{ox.}(MgADP)_2$. The ratio of measured specific activity at 30°C of Kp2 (1500nmol of C_2H_4 produced/min per mg of protein) to an assumed maximum specific activity of 3500nmol of C_2H_2 reduced/min per mg of protein was used to determine the ratio of active Kp2 to inactive $Kp2_i$. If $Kp2_i$ is assumed not to bind to $Kp1^{\dagger}$, then it is not possible to simulate the extensive inhibition of H_2 formation observed under conditions of high protein concentration (Lowe & Thorneley, 1984*b*).

H_2 inhibition of N_2H_4 formation

Fig. 1 also shows data for N_2H_4 formation from enzyme equilibrated with 50% N_2/50% H_2. The line drawn through the data points is a simultation made by using Scheme 2 and Table 1 of Lowe & Thorneley (1984*a*).

The decrease in N_2H_4 concentration at all the times measured shows that H_2 inhibition must occur before the formation of the intermediate that yields N_2H_4 on quenching (E_4). H_2 is a competitive inhibitor of N_2 reduction to NH_3 (Hwang *et al.*, 1973; Lowe & Thorneley, 1984*b*). This supports the suggestion by Chatt (1980) that N_2 binds to $Kp1^{\dagger}$ by the displacement of H_2 and that the inhibition by H_2 of NH_3 formation is a mass-action effect on this equilibrium. In Scheme 3, this occurs at species E_3 and E_4 in order to explain the results reported by Silverstein & Bulen (1970) and Hageman & Burris (1980), which showed that the relative steady-state rates of H_2 and NH_3 formation depended on the electron flux through the MoFe protein.

NH_3 formation

The pre-steady-state time course for NH_3 formation is shown in Fig. 3. Five simulations that assume NH_3 release on quenching from various species in Scheme 2 of Lowe & Thorneley (1984*a*) are shown in Fig. 4. Table 1 shows the results of a

Table 1. *Statistical comparison of simulations made by using Scheme 2 of Lowe & Thorneley (1984a) to determine which intermediates yield NH_3 on quenching functioning nitrogenase with acid*

Variances were calculated by using the data points in Fig. 4 weighted equally.

Species giving first NH_3	Species giving second NH_3	Variance ratio	% point of *F*-distribution with 16 × 16 degrees of freedom	Comment	Key to Fig. 4
E_5	E_5	1.00	–	Best simulation used as basis for *F*-test comparison	(*a*)
E_6	E_6	1.25	35	Not significant, therefore possible	(*b*)
E_5	E_7	1.67	20	Not significant, therefore possible	(*c*)
E_5	$*E_0$	2.4	3.0	Significant, therefore unlikely	(*d*)
E_6	$*E_0$	2.8	1.5	Significant, therefore unlikely	(*e*)
E_7	E_7	3.1	1.0	Highly significant, therefore most unlikely	(*b*)
$*E_0$	$*E_0$	5.7	<0.1	Very highly significant, therefore extremely unlikely	(*g*)

* NH_3 released at eighth slow step.

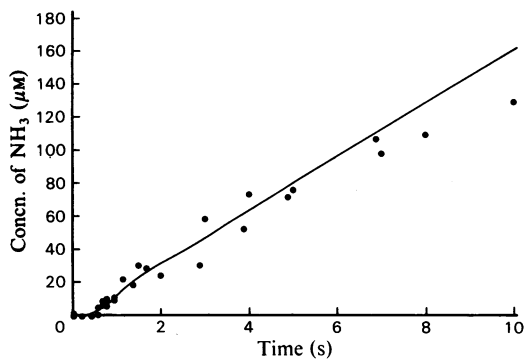


Fig. 3. *Pre-steady-state time course for NH_3 formation at 23°C at pH7.4*

The data show a pre-steady-state lag phase of 500ms, then a burst phase that is essentially complete after 2s, followed by a steady-state rate of 213nmol of NH_3/min per mg of Kp1. The data were obtained with the rapid-quench technique as described in the Materials and methods section. Before mixing syringe *A* contained: 66μM-Kp1, 200μM-Kp2, 1mM-$Na_2S_2O_4$, 10mM-$MgCl_2$ and 25mM-Hepes buffer, pH7.4. Syringe *B* contained: 18mM-ATP, 19mM-$Na_2S_2O_4$, 10mM-$MgCl_2$ and 25mM-Hepes buffer, pH7.4. Both syringes were saturated with N_2 (101kPa, 1.0atm). The line is a simulation made by using Scheme 2 and the rate constants from Table 1 of Lowe & Thorneley (1984*a*) assuming that both molecules of NH_3 are released from species E_5 [simulation (*a*) in Table 1].

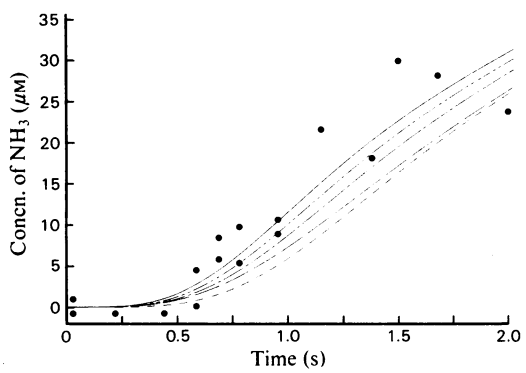


Fig. 4. *NH_3-formation data and simulations for NH_3 release on quenching*

The various intermediates that give NH_3 are shown in Scheme 2 of Lowe & Thorneley (1984*a*) and are defined by the key in Table 1: —— (*a*); —·-·— (*b*); —·— (*c*); —·— (*d*); ---- (*g*). The data points are taken from Fig. 3.

statistical comparison of seven simulations, some of which have been omitted from Fig. 4 in the interest of clarity.

We conclude that it is not necessary to reduce the MoFe protein, after N_2 has bound by H_2 displacement, by 6 electron equivalents relative to the oxidation level at E_0 in order to obtain 2 molecules of NH_3 on quenching functioning enzyme [simulation (*g*) in Table 1 and Fig. 4]. The best simulation (*a*) assumes that both molecules of NH_3 are released from species E_4 on quenching. Simulations (*b*) and (*c*), although less likely than (*a*), cannot be excluded, since by the *F*-test criterion they are not significantly different from (*a*). We exclude the other possibilities given in Table 1, namely simulations (*d*), (*e*) and (*f*), with increasing certainty, as indicated in the comment column.

We have presented data for H_2 evolution (Lowe & Thorneley, 1984*a*) and N_2H_4 formation in the present paper that support the assumptions in Scheme 2 of Lowe & Thorneley (1984*a*) that N_2

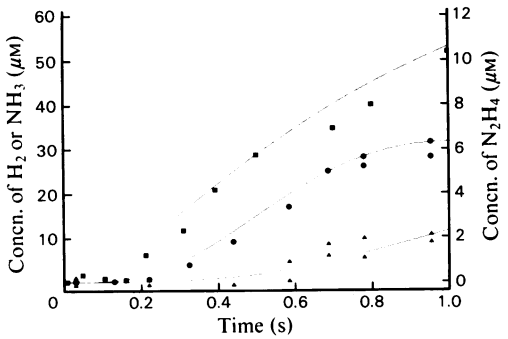


Fig. 5. *Comparison of pre-steady-state phases of H_2, N_2H_4 and NH_3 formation*

The H_2-evolution data (■) and simulated curve (——) are taken from Fig. 3 of Lowe & Thorneley (1984*a*). The N_2H_4 data (●) and simulated curve (—·—) are taken from Fig. 1 of the present paper. The NH_3 data (▲) and simulated curves (——) are taken from Fig. 3 of the present paper.

binds to Kp1† by H_2 displacement on species E_3H_3 or E_4H_4. Consequently Kp1† that has been reduced by only 2 (E_5) or 3 (E_6) electron equivalents relative to E_3 must become oxidized on quenching. This is necessary for the provision of the additional 4 or 3 electrons required to produce $2NH_3$ from N_2 on quenching the intermediates bound to E_5 or E_6 with acid. This strongly supports the suggestion by Chatt (1980) that the triple bond of N_2 is weakened by progressive protonation of the β-nitrogen atom. This is induced by an increase in bond order between the Mo and the α-nitrogen atom. Thus the intermediate bound to E_5 is likely to be a hydrazido(2−) derivative (Mo=N–NH_3^+) and that bound to E_6 a nitrido derivative (Mo≡N). Both these types of intermediate would be expected to hydrolyse rapidly under acid conditions to yield NH_3. It is difficult to see, if E_5 and E_6 have N_2H_4 or $N_2H_5^+$ bound to Mo, why these intermediates would not yield N_2H_4 on quenching. The time course for N_2H_4 formation (Fig. 1) excludes this possibility.

We are unable to say from our acid-quench data whether a similar mechanism for NH_3 release also occurs at physiological pH. However, if both molecules of NH_3 are released from species E_5 and E_6 at pH7.4, then species E_6 and E_7 should be oxidized forms of Kp1† (relative to E_0, which is that of Kp1 as isolated in the presence of $S_2O_4^{2-}$). It may be possible to detect these oxidized states by using the change in c.d. of the 'p' centres that occurs on oxidation (Orme-Johnson *et al.*, 1981).

Fig. 5 is a composite of data from Fig. 3 of Lowe & Thorneley (1984*a*) and Figs. 1 and 3 of the present paper. It shows that, when nitrogenase that is reducing N_2 to NH_3 with concomitant H_2 evolution is quenched with acid, the release sequence is H_2, N_2H_4 and then $2NH_3$; we have related this to the number of electrons transferred from Fe protein to MoFe protein. This conclusion is fundamental to the understanding of the mechanism of nitrogenase action, and can easily be lost in the kinetic complexity of this series of papers.

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