The mechanism of *Klebsiella pneumoniae* nitrogenase action

The determination of rate constants required for the simulation of the kinetics of N₂ reduction and H₂ evolution

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Kinetic data for *Klebsiella pneumoniae* nitrogenase were used to determine the values of nine of the 17 rate constants that define the scheme for nitrogenase action described by Lowe & Thorneley [(1984) *Biochem. J.* **224**, 877–886]. Stopped-flow spectrophotometric monitoring of the MgATP-induced oxidation of the Fe protein (Kp2) by the MoFe protein (Kp1) was used to determine the rates of association (k_{+1}) and dissociation (k_{-1}) of reduced Kp2(MgATP)₂ with Kp1. The dependences of the apparent K_m^N on Fe protein/MoFe protein ratio and H₂ partial pressure were used to determine the mutual displacement rates of N₂ and H₂ (k_{+10} , k_{-10} , k_{+11} and k_{-11}). These data also allowed the rate constants for H₂ evolution from progressively more reduced forms of Kp1 to be determined (k_{+7} , k_{+8} and k_{+9}). A mechanism for N₂-dependent catalysis of ¹H²H formation from ²H₂ that requires H₂ to be a competitive inhibitor of N₂ reduction is also presented.

In the three previous papers in this series (Thorneley & Lowe, 1983; Lowe & Thorneley, 1984; Thorneley & Lowe, 1984a) we have developed a kinetic model for the mechanism of nitrogenase action. An encouraging feature of this model was that it simulated the kinetically complex pre-steady-state phases of H₂, N₂H₄ (derived from an enzyme-bound intermediate) and NH₃ formation. The rate constants used in these simulations were determined independently, such that the curves presented by Lowe & Thorneley (1984) and Thorneley & Lowe (1984a) were simulations and not 'fits' to the data points. A number of these rate constants (Table 1 of Lowe & Thorneley, 1984) were determined from the kinetic data presented in the current paper.

Materials and methods

Protein preparation

Nitrogenase component proteins (Kp1 and Kp2) were purified and characterized as previously described (Lowe & Thorneley, 1984).

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

Assay procedure

The steady-state rates of substrate reduction at 23°C at pH 7.4 were determined by the method of Eady *et al.* (1972) as described by Dilworth & Thorneley (1981) with medium containing 9 mM-ATP, 10 mM-MgCl₂, 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer and 10 mM-Na₂S₂O₄.

Assays were initiated by the syringe addition of pre-mixed nitrogenase component proteins. The duration of the assays was varied from 5 to 40 min to ensure that the time course for product formation was linear and to optimize the yield of product for subsequent analysis.

Assays (1 ml total volume) were terminated by addition of either 0.2 ml of 0.4 M-EDTA or 0.1 ml of 30% (w/v) trichloroacetic acid. Gas mixtures of H₂, N₂ and Ar were prepared in a calibrated syringe (50 ml), which was then used to flush the Ar-filled gas space (6.7 ml) above the prepared assay mixture (0.8 ml).

H₂ and NH₃ determination

A gas sample (0.5 ml) was removed from a quenched assay for H₂ analysis as described previously (Lowe & Thorneley, 1984). A sample of the quenched assay (typically 0.85 ml) was transferred to a Universal bottle (30 ml), and 1 ml of

saturated K_2CO_3 solution was added to initiate microdiffusion of NH_3 on to glass rods carrying 1 M- H_2SO_4 (Dilworth, 1966). After overnight rotation of the stoppered bottles, the rods were eluted into water, and NH_3 was determined by an indophenol method (Fawcett & Scott, 1960).

Analysis of kinetic data

Apparent K_m and K_i values were determined by using the program of Cleland (1979) run on a PDP 1134A computer. Standard errors are shown on the Figures. Rate constants were determined from the dependence of the apparent K_m and K_i on the $[Kp2]/[Kp1]$ ratio as described in the Results and discussion section.

Stopped-flow spectrophotometry was performed as described by Thorneley & Lowe (1983), except that the storage oscilloscope was replaced by an analogue-to-digital converter operated at 3 kHz with a BBC micro-computer (Acorn Computers, Cambridge, U.K.). Data were subsequently transferred to a PDP 1134A computer for analysis.

Results and discussion

Rate of association of reduced $Kp2(MgATP)_2$ with $Kp1^\dagger$ (k_{+1})

The value of $k_{+1} \geq 5 \times 10^7 M^{-1} \cdot s^{-1}$ was determined by stopped-flow spectrophotometric monitoring of the electron transfer from $Kp2(MgATP)_2$ to $Kp1^\dagger$ as a function of $Kp2(MgATP)_2$ and $Kp1^\dagger$ concentration. Scheme 2 of Lowe & Thorneley (1984) shows that, if the concentrations of $Kp2(MgATP)_2$ and $Kp1^\dagger$ are low enough, then the second-order protein-association reaction (k_{+1}) will limit the rate of electron transfer. This will be reflected in a decrease in the observed rate of oxidation of $Kp2(MgATP)_2$ by $Kp1^\dagger$ (k_{obs}). Thorneley (1975) was able to estimate $k_{+1} \geq 10^7 M^{-1} \cdot s^{-1}$, since k_{obs} was independent of component protein concentration ($[Kp2(MgATP)_2] = [Kp1] \geq 5 \mu M$). Two technical developments have enabled us to determine the value of k_{+1} more accurately. Firstly, the improved signal-to-noise ratio of the computer-interfaced stopped-flow apparatus allowed lower protein concentrations to be used. Secondly, Schemes 1 and 2 of Lowe & Thorneley (1984) enabled us to simulate the time course for the oxidation of $Kp2(MgATP)_2$, which is essentially complete after 50 ms. Over this time range, the important rate constants are k_{+1} , k_{-1} , k_{+2} , k_{+3} , k_{-3} and k_{+4} . The values of k_{+2} , k_{+3} , k_{-3} and k_{+4} have been determined previously (see Table 1 of Lowe & Thorneley, 1984). The simulation is most sensitive to the chosen value of k_{+1} . Fig. 1 shows that $k_{+1} \geq 5 \times 10^7 M^{-1} \cdot s^{-1}$. The simulations used

Fig. 1. Time course for the oxidation of $Kp2(MgATP)_2$ by $Kp1$ at $23^\circ C$ at pH 7.4 used to determine the rate of association of $Kp2(MgATP)_2$ with $Kp1^\dagger$ (k_{+1})

The data trace was obtained by the stopped-flow technique as described in the Materials and methods section with a mixture containing $2.5 \mu M$ - $Kp1$, $2.5 \mu M$ - $Kp2$, $9 mM$ - $MgATP$, $10 mM$ - $Na_2S_2O_4$ and $25 mM$ -Hepes buffer, pH 7.4. The simulations were obtained by using Schemes 1 and 2 and the rate constants in Table 1 of Lowe & Thorneley (1984), with the exception of k_{+1} and k_{-1} , which were chosen for each simulation. (a) $k_{+1} = 1 \times 10^7 M^{-1} \cdot s^{-1}$, $k_{-1} = 15 s^{-1}$ (— · —); (b) $k_{+1} = 3 \times 10^7 M^{-1} \cdot s^{-1}$, $k_{-1} = 15 s^{-1}$ (— · — · —); (c) $k_{+1} = 5 \times 10^7 M^{-1} \cdot s^{-1}$, $k_{-1} = 100 s^{-1}$ (— · — · — · —); (d) $k_{+1} = 5 \times 10^7 M^{-1} \cdot s^{-1}$, $k_{-1} = 15 s^{-1}$ (— · —); (e) $k_{+1} = 1 \times 10^8 M^{-1} \cdot s^{-1}$, $k_{-1} = 15 s^{-1}$ (— · — · —). The values of k_{+1} and k_{-1} used in simulation (d) are those used in this series of papers (Table 1 of Lowe & Thorneley, 1984).

the experimentally determined value of $\Delta\epsilon_{430} = 8.4 mM^{-1} \cdot cm^{-1}$ for active $Kp2$, which constitutes 45% of the total $Kp2$ present (see below and Thorneley & Lowe, 1984b). Inactive $Kp2$ and $Kp1$ were assumed not to contribute significantly to the absorbance change at 430 nm. Inactive $Kp2$ ($Kp2_i$) was assumed to bind to, and dissociate from, $Kp1^\dagger$ with the same rate constants (k_{+3} and k_{-3}) as active oxidized $Kp2$. The amplitude of the absorbance change in Fig. 1 depends critically on the ratio of active to inactive $Kp2$ protein, the $[Kp2]/[Kp1]$ ratio and the absolute concentrations of $Kp2$ and $Kp1$. Because active $Kp2$ binds tighter to $Kp1^\dagger$ than does $Kp2_i$, increasing the $[Kp2]/[Kp1]$ ratio causes a greater proportion of the $Kp1^\dagger$ to be bound to active $Kp2$. This explains why Thorneley (1975) and Eady *et al.* (1978) observed an increase in amplitude when $[Kp2]$ was increased while $[Kp1]$ was kept constant until $[Kp2]/[Kp1]$ was approx. 4:1. The value of this ratio does not therefore measure the stoichiometry

of binding of Kp2 to Kp1, nor the number of mol of Kp2 than can be oxidized by Kp1 within 50 ms.

The simulations in Fig. 1 show that, if $k_{+1} = 5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, then $k_{-1} < 100 \text{ s}^{-1}$; values of $k_{-1} > 100 \text{ s}^{-1}$ significantly slow down the rate of oxidation of $\text{Kp2}(\text{MgATP})_2$. It is possible to simulate the data in Fig. 1 by using higher values of k_{+1} provided that a proportionate increase in the value of k_{-1} is used. However, we have used the lower limit for the value of k_{+1} ($5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) in this series of papers because of the constraints of diffusion limitation and steric factors, which make the higher values less likely. The value of $k_{-1} = 15 \text{ s}^{-1}$ was chosen for historical reasons, and is entirely arbitrary, since any value for $k_{-1} < 100 \text{ s}^{-1}$ would suffice for the simulations. Indeed, it is possible that all Fe protein-MoFe protein dissociation rates are the same, i.e. $k_{-1} = k_{-3} = 6.4 \text{ s}^{-1}$.

Dependence of the apparent $K_m^{\text{N}_2}$ on $[\text{Kp2}]/[\text{Kp1}]$ ratio

The rate of NH_3 formation was determined as a function of N_2 partial pressure over the range 101 kPa (1.0 atm) to 3.03 kPa (0.03 atm) at six $[\text{Kp2}]/[\text{Kp1}]$ ratios. At each component-protein ratio, a hyperbolic dependence was obtained, and the apparent $K_m^{\text{N}_2}$ for NH_3 formation was determined as described in the Materials and methods section. The difference between the H_2 -evolution rate at a particular N_2 concentration and that observed in the absence of N_2 under Ar can be used as an indirect measure of the electron flux used to reduce N_2 (Hageman & Burris, 1980; Wherland *et al.*, 1981). These H_2 -evolution data were used to calculate an apparent $K_m^{\text{N}_2}$ at each $[\text{Kp2}]/[\text{Kp1}]$ ratio. Both sets of apparent $K_m^{\text{N}_2}$ data are shown in Fig. 2.

A surprising feature of Fig. 2 is that, as the total electron flux through the Kp1 increases, owing to the large excess of Kp2 at high $[\text{Kp2}]/[\text{Kp1}]$ ratios, the apparent $K_m^{\text{N}_2}$ increases, as also reported by Guth & Burris (1983). A simplistic conclusion would be that, the more reduced the MoFe protein becomes, the weaker the binding of N_2 at the active site. This might seem to contradict the data of Silverstein & Bulen (1970), Hageman & Burris (1980) and Wherland *et al.* (1981), which show that high electron flux favours N_2 reduction relative to H_2 evolution. Two points need to be made at this stage before a detailed explanation is given. Firstly, the data of Silverstein & Bulen (1970), Hageman & Burris (1980) and Wherland *et al.* (1981) were obtained with N_2 partial pressure at least 5 times higher than any apparent $K_m^{\text{N}_2}$ in Fig. 2; thus under these conditions the 2-fold variation of apparent $K_m^{\text{N}_2}$ with $[\text{Kp2}]/[\text{Kp1}]$ ratio shown in Fig. 2 has little effect on the observed rates of H_2 evolution and NH_3 formation. Secondly, the

Fig. 2. Dependence of the apparent $K_m^{\text{N}_2}$ on the $[\text{Kp2}]$ ratio at pH 7.4 at 23°C

Data points were obtained by using the assay procedure described in Materials and methods section. Each assay mixture contained 9 mM-ATP, 16 mM-phosphocreatine, 0.2 mg of creatine kinase (EC 2.7.3.2), 10 mM- MgCl_2 , 25 mM-Hepes buffer, pH 7.4, 10 mM- $\text{Na}_2\text{S}_2\text{O}_4$, 0.57 μM -Kp1 and 0.57–11.4 μM -Kp2. $K_m^{\text{N}_2}$ was determined from NH_3 -formation rates (●) and from ΔH_2 rates (▲) as described in the text. The line through the data points is a simulation made by using Scheme 1 and the rate constants in Table 1 of Lowe & Thorneley (1984).

apparent $K_m^{\text{N}_2}$ should not be equated with the affinity of the reduction site of N_2 .

Scheme 1 is an expansion of the N_2 -binding section of Scheme 2 of Lowe & Thorneley (1984). It shows that the three main features of Fig. 2, namely the value of $K_m^{\text{N}_2}$ at low $[\text{Kp2}]$, the value of $K_m^{\text{N}_2}$ at high $[\text{Kp2}]$, and the Kp2 concentration at which the $K_m^{\text{N}_2}$ is half way between these two extremes, can be used to fit the values of three unknown rate constants k_{+8} , k_{+10} and k_{+11} . Firstly, at high $[\text{Kp2}]$ the principal reaction responsible for N_2 binding is k_{+11} , and the $K_m^{\text{N}_2}$ is a result of competition between $k_{+11}[\text{N}_2]$ and k_{+9} , $k_{-3}[\text{Kp2}_{\text{ox}}]$ and $k_{-3}[\text{Kp2}_i]$. Since both k_{+9} and k_{+11} are unknown, we have chosen $k_{+9} = 400 \text{ s}^{-1}$ (see below in the section on H_2 -release rates). Secondly, at low $[\text{Kp2}]$, k_{+10} is the main route for N_2 binding and the $K_m^{\text{N}_2}$ is a function of $k_{+10}[\text{N}_2]$, k_{+8} , $k_{-3}[\text{Kp2}_{\text{ox}}]$, $k_{-3}[\text{Kp2}_i]$ and $k_{+1}[\text{Kp2}_{\text{red}}]$. Finally, the shape of the curve at intermediate $[\text{Kp2}]$ is a result of increasing values of $k_{+1}[\text{Kp2}_{\text{red}}]$ changing the dominant N_2 -binding reaction from k_{+10} to k_{+11} . Since these dependences are not simple, the variations in the simulated curve for Fig. 2 with k_{+8} , k_{+10} and k_{+11} were used to estimate the values of these three rate constants. It may be noted that Scheme 1 predicts that the $K_m^{\text{N}_2}$ depends, not only

Scheme 1. N_2 - and H_2 -binding reactions of transient forms of the MoFe protein

Only free MoFe protein species are shown. In addition to the reactions involving active Kp2, each species can also bind Kp2_i with rate constants that have been assumed to be the same as those for oxidized active protein (k_{+3} and k_{-3}).

Fig. 3. Dependence of the apparent $K_m^{N_2}$ for NH_3 formation on H_2 partial pressure

The assay procedure, the concentrations of components, data analysis and computer simulation of the line through the data points were as described in the legend to Fig. 2. (a) $0.57 \mu M$ -Kp1 and $0.57 \mu M$ -Kp2. (b) $0.57 \mu M$ -Kp1 and $5.7 \mu M$ -Kp2.

on the $[Kp2]/[Kp1]$ ratio, but also on the specific activities of both proteins, their absolute concentrations, the extent of reduction of the Fe protein and the supply of MgATP.

H_2 inhibition of N_2 reduction

The dependence of the apparent $K_m^{N_2}$ on H_2 partial pressure [0 to 70 kPa (0.7 atm)] was determined under conditions of low ($[Kp2]/[Kp1]$ ratio 1:1; Fig. 3a) and high electron flux ($[Kp2]/[Kp1]$

ratio 10:1; Fig. 3b). At both high and low electron flux, V_{max} for NH_3 formation was independent of H_2 partial pressure. These data confirm that H_2 behaves as a competitive inhibitor of N_2 reduction (Hwang *et al.*, 1973) and allow the rate constants k_{-10} and k_{-11} in Scheme 1 to be determined.

The equations that describe the inhibition are not those of a classical Michaelis-Menten treatment because the enzyme-inhibitor complexes (E_3H_3 and E_4H_4) break down to give the product

H_2 . In addition, the substrate (N_2) does not bind to free enzyme but displaces the inhibitor (H_2). The values of k_{-10} and k_{-11} were therefore estimated from the best-fit curves to the data in Fig. 3. It is not possible to obtain curves approaching the data in these Figures by using Scheme 2 of Lowe & Thorneley (1984) unless irreversibility is introduced, such that N_2 bound via k_{+10} cannot be subsequently lost via k_{-11} ; this has been done in our computer model. We are unable to say precisely where this irreversible step occurs, although one might speculate that it is the irreversible protonation of the bound N_2 as shown in Scheme 1.

H_2 evolution, $^1H^2H$ formation and the nature of E_nH_n intermediates

At neutral pH, N_2 -independent H_2 evolution occurs by three reactions in Scheme 2 of Lowe & Thorneley (1984), and it is the rates of these reactions (k_{+7} , k_{+8} and k_{+9}) that control the quantitative response of the system to increasing electron flux through the MoFe protein.

The increased proportion of electrons diverted into H_2 evolution (under saturating N_2) at low electron flux is determined by k_{+7} , i.e. the rate of H_2 evolution from E_2H_2 . Hence k_{+7} was estimated from the distribution of products H_2 and NH_3 in the steady state under the conditions used for Fig. 3 of Lowe & Thorneley (1984). Unfortunately, the value obtained comes from differences between total electron flow, total H_2 evolved and N_2 -dependent H_2 evolution, all three of which are subject to experimental error. The value of k_{+7} is therefore subject to large errors, which are increased because of errors in the determination of free $[Kp2_{red}]$, the multiplier of k_{+1} in the reaction competing with k_{+7} . We therefore consider the accuracy of the values of k_{+7} to be of the order of $\pm 50\%$.

k_{+8} was determined above (see the section on the variation of $K_m^{N_2}$ with $[Kp2]/[Kp1]$ ratio). This value also depends on small differences between large numbers, and is also probably subject to similar errors. k_{+9} was chosen to be 400, and only affects the value of k_{+11} . The only restriction on k_{+9} is that it must be much greater than k_{-3} (the rate-limiting step in turnover), so that no detectable inhibition occurs at high electron flux; values less than 400 produce significant deviations from linear plots at times greater than 2 s in simulations such as Figs. 2 and 3 of Lowe & Thorneley (1984).

Evidence on the possible nature of the species E_nH_n and of the H_2 -evolution reactions comes from two crucial experiments, described by Burgess *et al.* (1981) and Burgess *et al.* (1984), concerned with the evolution of $^1H^2H$ by nitrogenase fixing N_2 in H_2O under an atmosphere of N_2 and 2H_2 . In the

first experiment, which studied this reaction under an atmosphere of 3H_2 and N_2 , the amount of $^3H^+$ incorporated into the aqueous phase is small (less than 3% of the $^1H^3H$ formed). In the second experiment, performed under an atmosphere of $^1H^2H$ plus N_2 , the amount of 2H_2 evolved was less than the background for the measurement. It is difficult to reconcile these results with any model in which E_3H_3 and E_4H_4 contain more than 2 H atoms, bound to the N_2 -binding site, that can react via k_{+10} and k_{-10} or k_{+11} and k_{-11} . Thus the maximum number of hydrides that is not exchangeable with water protons is two, and the additional proton(s) must be bound at other site(s), such as an adjacent aminoacyl side chain. This description of the N_2 -binding site is that indicated in Scheme 1, and expanded for the E_3 reduction level of $Kp1^\dagger$ in Scheme 2.

Scheme 2 implies that H_2 is evolved when one of the adjacent bound protons attacks a bound hydride. Only the bound dihydride can exchange with N_2 or 2H_2 . Any 3H_2 bound via k_{-10} or k_{-11} is completely displaced when further molecules of N_2 bind, and so cannot become incorporated into the aqueous phase as $^3H^+$. Also, if $^1H^2H$ binds via k_{-10} or k_{-11} , protic attack can only give 1H_2 or $^1H^2H$, since one half of the evolved molecule is always a solvent proton.

It is not clear whether nitrogenase catalyses the formation of $^1H^2H$ from 2H_2 and H_2O in the absence of N_2 , since there are conflicting reports in the literature [cf. Li & Burris (1983) and Wherland *et al.* (1981)]. If this reaction does occur, then it is presumably the result of 2H_2 replacing 1H_2 directly on $E_3-H_2(H^+)$ or $E_4-H_2(2H^+)$ followed by protic attack on the bound di-deuteride. The rate of direct $^1H_2/^2H_2$ exchange must be considerably slower than the $^1H_2/N_2/^2H_2$ exchange, since even Wherland *et al.* (1981) indicate that stimulation of $^1H^2H$ formation occurs in the presence of N_2 .

Is our solution unique?

The only rate constant values derived in the present paper that are crucial to all the simulations presented in this series of papers are those of k_{+1} and k_{+7} . Of the others, the values of k_{+10} , k_{-10} , k_{+11} , k_{-11} and k_{+8} are important largely because they show that Scheme 2 of Lowe & Thorneley (1984) is capable of simulating the variations in $K_m^{N_2}$ shown in Figs. 2 and 3. The only external check on their validity comes from the precision of the simulation of the time course for the N_2H_4 -yielding intermediate under an atmosphere of N_2 and H_2 (broken line in Fig. 1 of Thorneley & Lowe, 1984a). Indeed, if an irreversible protonation step following N_2 binding, as described above, is shown to occur, then the calculated values of k_{-10} and k_{-11} will change. Improvement to the fits in Figs.

Scheme 2. Proposed mechanism of N_2 binding and $^1H^2H$ formation at the E_3 reduction level of $Kp1^\dagger$

3(a) and 3(b) would be obtained, because such additional irreversible steps would provide an extra degree of freedom. We do not consider that their inclusion at this stage of the development of the mechanism is justified.

We are conscious of the criticism that 17 rate constants, which may have various values, allow great flexibility in the fitting of data. We therefore emphasize that there are severe constraints on their values, as follows.

(1) Two (k_{+9} and k_{+11}) were chosen arbitrarily within ranges in which they were known to lie and over which they were shown to affect the simulations by less than 5%.

(2) Five (k_{+10} , k_{-10} , k_{+11} , k_{-11} and k_{+8}) only affect the $K_m^{N_2}$ (Figs. 2 and 3), from which they are derived.

(3) Two (k_{+5} and k_{-5}) have no effect under the conditions of excess MgATP that we have used.

(4) Six (k_{+2} , k_{+3} , k_{-3} , k_{+4} , k_{+6} and k_{-6}) were determined independently of Scheme 2 of Lowe & Thorneley (1984).

(5) One (k_{+1}) was determined, as described in the present paper, from an experiment that depends only on it and the independently known values of k_{+2} , k_{+3} and k_{-3} .

(6) The values for the rate constants for $Kp2_i$ associating and dissociating from $Kp1^\dagger$ must be essentially the same as those for $Kp2_{ox} \cdot (MgADP)_2$ (k_{+3} and k_{-3}), which have been experimentally determined (Thorneley & Lowe, 1983), because all the simulations in this series of papers (Lowe & Thorneley, 1984; Thorneley & Lowe, 1984a,b) are sensitive to these values. The concentration of $Kp2_i$ was estimated from the measured specific

activity of the Fe protein used ($Kp2 + Kp1_i$) at 30°C under the standard conditions given by Eady *et al.* (1972). The maximum specific activity of $Kp2$ at 30°C can be calculated, from the specific activity for $Kp1$ containing 2 Mo atoms per molecule (Hawkes *et al.*, 1984), to be 4000 ± 800 nmol of H_2 /min per mg of $Kp2$ (Thorneley & Lowe, 1984b). The standard assay procedure of Eady *et al.* (1972) underestimates the true specific activity of $Kp2$ by about 10% because it uses a $S_2O_4^{2-}$ concentration (30 mM) that is not saturating [see Thorneley & Lowe (1984b) for the explanation of the shape of $Kp2$ titration curves]. Thus to calculate the percentage of $Kp2$ and $Kp2_i$ we have used the ratio of the measured specific activity under the conditions given by Eady *et al.* (1972) (1500 nmol of H_2 /min per mg of $Kp2$) to an apparent maximum specific activity of 3500 nmol of H_2 /min per mg of $Kp2$.

The constraints listed above leave the value of k_{+7} unconstrained. This only determines the distribution of reducing equivalents between H_2 and NH_3 for all simulations when N_2 is present. The value of k_{+7} was determined from the steady-state product distribution.

The rate constants in Table 1 of Lowe & Thorneley (1984) affect the simulations in this series of papers to varying degrees. The system is 'coarse-tuned' by k_{-3} . When reductant is limiting, the reactions described by k_{+4} and k_{+3} cause inhibition. At very low protein concentrations ($<1 \mu M$) k_{+1} becomes rate-limiting. At high protein concentrations ($>10 \mu M$) k_{+1} , k_{-3} and k_{+4} fine-tune the system. The product distribution between H_2 and NH_3 at saturating N_2 concentrations is

determined by k_{+7} in competition with k_{+1} . Clearly very large changes in the value of any one of the rate constants, such that it approaches the value of k_{-3} , will produce significant effects.

Conclusions

The outline scheme for nitrogenase action presented in the first paper of this series (Scheme 2 of Lowe & Thorneley, 1984), together with Table 1 of the same paper, has allowed us to simulate the pre-steady-state and steady-state kinetic data that led us to derive both the qualitative and quantitative aspects of the model (Lowe & Thorneley, 1984; Thorneley & Lowe, 1984a; and the present paper). In addition, as we show in the following paper in this series, we have been able to understand quantitatively some of the unusual behaviour shown by the enzyme, such as the forms of component protein and dithionite titration curves at various absolute protein concentrations (Thorneley & Lowe, 1984b). It is also possible to predict the results of experiments that are very difficult to perform; for example, it is possible to study the effect of changing the various rate constants on the activity of nitrogenase. This may be important before experiments such as site-directed codon mutagenesis, designed to increase the efficiency of nitrogenase, are undertaken.

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