Klebsiella pneumoniae nitrogenase

The pre-steady-state kinetics of MoFe-protein reduction and hydrogen evolution under conditions of limiting electron flux show that the rates of association with the Fe-protein and electron transfer are independent of the oxidation level of the MoFe-protein

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The pre-steady-state kinetics of H_2 evolution from *Klebsiella pneumoniae* nitrogenase functioning at 23 °C, pH 7.4, under conditions of extremely low electron flux through the MoFe-protein exhibited a lag phase of several minutes duration. The approach to a steady-state rate of H_2 evolution was accompanied by a 50 % decrease in the amplitude of the MoFe-protein e.p.r. signal. These kinetics have been simulated using our published kinetic model for nitrogenase [Lowe & Thorneley (1984) *Biochem. J.* **224**, 877–886], which was developed using data obtained with nitrogenase functioning at high electron fluxes. The e.p.r. data showed that the rate of complex-formation between reduced Fe-protein and the MoFe-protein ($k_{+1} = 5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) is the same for the resting (E_0) and one-electron-reduced (E_1H) states of the MoFe-protein. Stopped-flow spectrophotometry also showed that electron transfer from the Fe-protein to the MoFe-protein in states E_0 and E_1H occurs at the same rate ($k_{\text{obs.}} = 140 \text{ s}^{-1}$). These data support our previous assumption that the rate constants that define the 'Fe-protein cycle' are independent of the level of reduction of the MoFe-protein.

INTRODUCTION

Klebsiella pneumoniae nitrogenase catalyses the reduction of N_2 to $2NH_3$ with the concomitant evolution of H_2 and hydrolysis of MgATP to MgADP and P_i . It comprises two metalloproteins, the MoFe-protein (Kp1; M_r 220 000) and the Fe-protein (Kp2; M_r 67 000) [see Thorneley & Lowe (1985), Orme-Johnson (1985), Burgess (1985), Smith (1990), Thorneley (1990) and Lowe (1991) for reviews on the structure and mechanism of nitrogenase]. Although a flavodoxin (often substituted by a ferredoxin in other organisms) acts as the electron donor to Kp nitrogenase *in vivo* (Hill & Kavanagh, 1980; Nieva-Gomez *et al.*, 1980), sodium dithionite ($Na_2S_2O_4$) is most commonly used for kinetic studies *in vitro*. Our current understanding of the complex kinetics and mechanism of nitrogenase is still largely based on five papers that describe the partial reactions that define the Fe-protein (Kp2) cycle (Scheme 1) (Thorneley & Lowe, 1983) and the subsequent combining of eight of these Fe-protein cycles into a single MoFe-protein (Kp1) cycle in which N_2 is reduced and H_2 evolved (Lowe & Thorneley, 1984a,b; Thorneley & Lowe, 1984a,b). However, under conditions of low electron flux through the MoFe-protein, induced in the present studies by using an Fe-protein/MoFe-protein ratio of 1:100, only the first two of the eight consecutive Fe-protein cycles need to be considered. These are shown in

Scheme 2, which is a subcycle of the complete MoFe-protein cycle (Scheme 2 of Lowe & Thorneley, 1984a). This subcycle only involves the first three (E_0 , E_1H and E_2H_2) of the eight intermediate states of Kp1 defined by the Lowe-Thorneley model. It is only concerned with proton reduction to H_2 since the N_2 -binding states of Kp1 (E_3 and E_4) are predicted by the complete model not to form to any significant extent under the conditions of low electron flux used. This is consistent with the failure of nitrogenase to reduce N_2 under these conditions (Eady & Postgate, 1974; Hageman & Burris, 1978; Wherland *et al.*, 1981).

A key assumption of our model is that the rate constants that define the Fe-protein cycle (Scheme 1) are independent of the oxidation level of the MoFe-protein, i.e. Kp1 in states E_0 , E_1H , E_2H_2 etc. react with $Kp2(MgATP)_2$ at the same rate. The values for the rate constants of Scheme 1 used in the successful simulations of the pre-steady-state and steady-state kinetics of H_2 evolution and N_2 reduction were determined only for Kp1 in state E_0 (Thorneley & Lowe, 1983). In the present paper we describe the measurement of the rate constants for protein complex-formation (k_{+1}) and subsequent electron transfer (k_{+2}) for Kp1 in state E_1H during the second Fe-protein cycle. In order to do this, we exploited the conditions of low electron flux originally used by Hageman & Burris (1979) with *Azotobacter vinelandii* nitrogenase (Av1 and Av2) to demonstrate a lag phase

Scheme 1. Oxidation–reduction cycle for the Fe-protein

Kp1 $\uparrow$ represents one of two independently functioning halves of the tetrameric ($\alpha_2\beta_2$ structure) Kp1. Each Kp1 $\uparrow$ is assumed to contain one Mo substrate-binding site and one Kp2-binding site. Table 1 contains the values of all the rate constants in Scheme 1 together with the value of k_{+7} , the rate of H_2 release from species E_2H_2 in Scheme 2.

Scheme 2. MoFe-protein cycle involved in H_2 evolution at low electron flux

Species E represents Kp1† as defined in the legend to Scheme 1. The subscript refers to the number of times Kp1† has completed the electron-transfer Fe-protein cycle shown in Scheme 1. The subscript therefore indicates the number of electron equivalents by which Kp1† has been reduced relative to resting E_0 , which is that of Kp1 as isolated in the presence of $Na_2S_2O_4$. Species E_0 has an e.p.r. signal (g values 4.3, 3.7, 2.01), while species E_1H and E_2H_2 are e.p.r.-silent. The values of the rate constants k_{+1} , k_{-1} , k_{+2} , k_{-2} , k_{+3} , k_{-3} and k_{+7} are given in Table 1 and the partial reactions with which they are associated are shown in Scheme 1.

of several minutes before H_2 evolution attained a steady-state rate. They also monitored an approx. 50 % bleaching of the Av1 e.p.r. signal that occurred with kinetics very similar to those of the H_2 evolution lag phase. These results are best understood in terms of Schemes 1 and 2 for *K. pneumoniae* nitrogenase with a slow increase in the concentration of the H_2 -evolving state of Kp1 (E_2H_2) during the lag phase until in the steady-state approx. 50 % of the Kp1 is in state E_0 and 50 % in E_1H (only a very small proportion of the total Kp1 is predicted by the model to be in other states). This procedure not only allowed us to prepare Kp1 in state E_1H , which could be used in electron-transfer studies, but also gave us two new accurate time courses (H_2 evolution and Kp1 e.p.r. signal intensity), which we could attempt to simulate. These new data, obtained under extreme conditions of low electron flux, have provided a further rigorous test of our kinetic model. The original data used in the development of the model were pre-steady-state and steady-state data obtained under conditions of high electron flux. It was not possible to use the low-electron-flux data of Hageman & Burris (1979) since these were obtained with Av nitrogenase under different assay conditions for which the rate constants for the partial reactions defining our model have not been determined.

MATERIALS AND METHODS

Nitrogenase proteins

The nitrogenase component proteins from *Klebsiella pneumoniae* (oxytoca) N.C.I.B. 12204 were purified and assayed as previously described (Thorneley & Lowe, 1983). Kp1 and Kp2 proteins had specific activities at 30 °C of 2000 and 1400 nmol of ethylene produced/min per mg of protein respectively. Kp1 contained 1.4 ± 0.1 g-atom of Mo/mol of protein

Reagents

All salts were purchased from BDH Chemicals; biochemicals were from Sigma Chemical Co., Poole, Dorset, U.K.

Assay procedure

The steady-state rates of H_2 formation at 23 °C and pH 7.4 were determined as previously described (Lowe & Thorneley, 1984b) in a medium containing 9 mM-ATP, 10 mM-MgCl₂,

25 mM-Hepes buffer and 10 mM- $Na_2S_2O_4$. Each assay also contained 18 mM-creatine phosphate and 60 µg of creatine kinase to prevent the accumulation of inhibitory concentrations of MgADP.

Assays were performed under an atmosphere of N_2 , in 7.8 ml serum bottles, fitted with rubber Suba-Seal closures, thermostatically maintained at 23 °C in a shaking water bath.

Assays were initiated by the syringe addition of premixed nitrogenase component proteins. The duration of the assays was varied from 0 to 20 min, and terminated by the addition of 0.1 ml of 30 % (w/v) trichloroacetic acid.

Computer simulations of kinetic data

Computer simulations of the time courses for H_2 evolution and the concentrations of Kp1 at various oxidation levels (E_0 , E_1H etc.) were performed as described by Lowe & Thorneley (1984a) using the scheme and rate constants therein.

Stopped-flow spectrophotometry

Stopped-flow spectrophotometry at 430 nm was performed at 23 °C with a Hi-Tech SF-51 instrument (Hi-Tech Scientific, Salisbury SP2 7PU, Wilts., U.K.) installed with a Techne-C400 thermostat (Techne Ltd., Duxford, Cambridge, U.K.) inside an anaerobic glove box operating with recirculating N_2 containing less than 1 p.p.m. of O_2 . Data acquisition and curve fitting were done as previously described (Ashby & Thorneley, 1987). The apparatus was modified by addition of a small Perspex premixer unit comprising two female Luer inlet ports, a T-mixer and male Luer exit port. The inlet ports were fitted with two 1 ml syringes which could be removed and separately filled with, in one case, a protein mix (Kp1, 40 µM; Kp2, 0.4 µM) and in the other case ATP (18 mM), creatine phosphate (18 mM) and creatine kinase (60 µg). After the two 1 ml syringes had been filled and fitted into the premixer block, the male Luer exit port was inserted into one of the two valve blocks of the stopped-flow apparatus. The two 1 ml syringes were manually driven in tandem to fill rapidly one of the two stopped-flow drive syringes with 2 ml of reacting Kp1 (20 µM), Kp2 (0.2 µM) and ATP (9 mM). The other drive syringe contained Kp2 (80 µM). After a pre-push to ensure that the tubes connecting the drive syringe to the mixing chamber were flushed, the first stopped-flow trace was obtained 15 s after the start of the premixing procedure with successive shots done at intervals up to 20 min. The final mixed concentrations were Kp1 (10 µM), Kp2 (40 µM + 0.1 µM) and ATP (4.5 mM). All the solutions contained $Na_2S_2O_4$ (10 mM), MgCl₂ (10 mM) and Hepes buffer (25 mM) at pH 7.4.

Preparation and measurement of e.p.r. samples

The time course for the decrease in the intensity of the e.p.r. signal characteristic of dithionite-reduced Kp1 protein (state E_0), which occurs on further reduction by Kp2 protein (to yield Kp1 in states E_1H and E_2H_2) was obtained as follows. Equal volumes (1.5 ml) of two solutions, one with Kp1 (40 µM) and Kp2 (0.4 µM) and the other with ATP (18 mM), creatine phosphate (18 mM) and creatine kinase (90 µg), were mixed in a glass vial (7.8 ml volume) fitted with a rubber-seal closure under an atmosphere of N_2 . Both solutions were thermostatically maintained at 23 °C, and, after mixing, the resulting solution was maintained at this temperature by immersion of the vial in a shaking water bath. In addition to the reagents given above, both solutions contained $Na_2S_2O_4$ (10 mM), MgCl₂ (10 mM) and Hepes buffer (25 mM, pH 7.4). Portions (0.3 ml) of the reacting protein solution were transferred at intervals to quartz e.p.r. tubes using a gas-tight N_2 -flushed Hamilton syringe (1 ml) fitted with a 15 cm stainless-steel needle. The long needle was required to syringe the sample into the bottom of the e.p.r. tube, which was positioned inside a

Schlenk tube, fitted with a Suba-Seal closure and attached via a side-arm to a vacuum/N₂ manifold. The solution transfer, removal of the Suba-Seal closure and then the e.p.r. tube from the Schlenk tube followed by freezing in isopentane, cooled in liquid N₂ to approx. -140 °C, took approx. 10 s.

E.p.r. spectra were recorded on a Bruker ER200D spectrometer at 10 K at 100 mW power at 9.47 GHz, by using 2.1 mT field modulation at 100 kHz. The relative amplitudes of the $g = 3.7$ feature, after correction for variation in the diameters of the quartz e.p.r. tubes, were determined and compared with the simulated time course for the decrease in Kp1 (state E₀) concentration.

RESULTS AND DISCUSSION

Simulation of the time course for H₂ evolution and Kp1 e.p.r. signal intensity under conditions of low electron flux

Fig. 1 shows a time course for H₂ evolution for nitrogenase functioning under conditions of low electron flux. This was achieved by using a Kp1/Kp2 molar ratio of 100:1. The concentrations of other reactants (i.e. Na₂S₂O₄, MgATP) and conditions (pH 7.4, 23 °C) were those used previously to obtain the data used in the development of our model of nitrogenase action (Lowe & Thorneley, 1984a). The rate of H₂ evolution increases over a period of minutes until a steady-state rate is achieved after approx. 15 min. The solid line through the data points is a simulation (not a fitted curve) using the rate constants, kinetic model and computing procedures previously described (Lowe & Thorneley, 1984a). This model was originally developed under very different conditions of relatively high electron flux (Kp1/Kp2 molar ratio 1:4) when nitrogenase not only evolves H₂ but also reduces N₂ to 2NH₃; despite small systematic differences (< 1 nmol of H₂ in a 1 ml assay) observable in Fig. 1, we regard the closeness of the simulation to the data as a further confirmation of our original model which defined eight states of Kp1 (E₀ to E₇) in the substrate-reduction cycle. Under conditions of low electron flux used here, the simulations predict that the Kp1 is predominantly in just two states, E₀ and E₁H, after the steady state has been attained. Initially all the Kp1 is in state E₀,

after 15 s, E₀ = 97 % with E₁H = 3 %, after 12 min E₀ = 55 % with E₁H = 45 %, at times greater than 20 min, E₀ is approx. 50 % with E₁H approx. 50 %. At all times, species other than E₀ and E₁H comprise less than 0.02 % of the total Kp1.

The decrease in E₀ concentration from 100 % at time zero to approx. 50 % of the Kp1 concentration in the steady state was monitored by e.p.r. spectroscopy. Fig. 1 (open circles) shows the time course for the amplitude of the $g = 3.7$ feature, which is associated with dithionite-reduced Kp1 (E₀). Representative spectra obtained before the reaction was initiated by the addition of ATP and after 12 min reaction are shown in Fig. 2. The dashed line through the data points of Fig. 1 is a simulation of the time course of E₀ obtained as described above and in the Materials and methods section. The third simulation in Fig. 1 shows the corresponding increase in the concentration of E₁H, which is e.p.r.-silent and which we are unable to monitor by existing techniques.

Although E₂H₂ is the species that evolves H₂ and in doing so reverts to species E₀ (Scheme 2), its steady-state concentration is very low. This is because its rate of formation is limited by the rate of association of reduced Kp2(MgATP)₂ with Kp1 in the state E₁H ($k_{+1} = 5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$; Lowe & Thorneley, 1984a). Although the value of k_{+1} is close to the diffusion-controlled limit, the extremely low concentrations of reacting free reduced Fe-protein cause E₂H₂ to form with a pseudo-first-order rate constant of only approx. 0.01 s⁻¹. This is very slow compared with the rate of oxidation of E₂H₂ to yield E₀ on H₂ evolution (k_{+7} , approx. 250 s⁻¹; Lowe & Thorneley, 1984a) and causes the steady-state concentration of E₂H₂ to be < 0.02 % of the total Kp1.

The steady-state concentrations of E₀ and E₁H are determined under these conditions of low electron flux by the relative values of k_{+1} for the reaction of Kp2(MgATP)₂ with Kp1 in states E₀ and E₁H. The 50 % bleaching of the Kp1 e.p.r. signal in the steady state (Fig. 1) means that k_{+1} must have the same value (to within 10 %) in the first and second Fe-protein cycles.

Although Hageman & Burris (1979) reported a similar time course for an initial 53 % bleaching of the e.p.r. signal of Av1 in an experiment analogous to that shown in Fig. 1, they also

Fig. 1. Hydrogen evolution and e.p.r. amplitude data simulated by the Lowe-Thorneley model as Kp1 reacts with a limiting amount of Kp2 at 23 °C, pH 7.4

The total amount of H₂ produced when Kp1 (20 μM) is reduced by Kp2 (0.2 μM) in the presence of ATP (9 mM), creatine phosphate (9 mM), creatine kinase (60 μg/ml), Na₂S₂O₄ (10 mM) and MgCl₂ (10 mM) in Hepes buffer (25 mM) is shown as a function of time (●). The solid line through these data points is a simulation using the rate constants and assumptions of the Lowe & Thorneley (1984a) model for nitrogenase (see Schemes 1 and 2). The open circles are the amplitudes of the Kp1 $g = 3.7$ e.p.r. feature recorded after freezing the reacting solution (representative spectra shown in Fig. 2). The simulation through these data points is the calculated time course of the concentration of Kp1 in state E₀ (---). The third simulated line (····), for which no data points are available, represents the calculated time course for the appearance of the super-reduced e.p.r.-silent state of Kp1, designated 'E₁H' in Scheme 2.

Fig. 2. E.p.r. spectra of Kp1 at zero time and 12 min reaction with a limiting concentration of Kp2 (MgATP)₂ at 23 °C, pH 7.4

The e.p.r. samples were prepared and their spectra recorded as described in the Materials and methods section. Both samples contained Kp1 (20 μ M), Kp2 (0.2 μ M), ATP (9 mM), creatine phosphate (9 mM), creatine kinase (60 μ g/ml), Na₂S₂O₄ (10 mM), MgCl₂ (10 mM) and Hepes buffer (25 mM). Spectrum (a) was obtained on freezing the sample in the absence of ATP. Spectrum (b) was obtained on freezing the sample after 12 min reaction after the addition of ATP. The arrows indicate the amplitudes of the $g = 3.7$ feature, which were used together with data from similar spectra taken after different periods of reaction to construct the time course shown in Fig. 1.

Table 1. Rate constants that define the Fe-protein cycle and the release of H₂ from the MoFe-protein under conditions of low electron flux

Rate constant	Value	Reference
k_{+1}	$5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$	Lowe & Thorneley (1984a)
k_{-1}	15 s^{-1}	Lowe & Thorneley (1984a)
k_{+2}	200 s^{-1}	Thorneley (1975)
k_{+3}	$4.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$	Thorneley & Lowe (1983)
k_{-3}	6.4 s^{-1}	Thorneley & Lowe (1983)
k_{+4}	$3.0 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$	Thorneley & Lowe (1983)
k_{+7}	250 s^{-1}	Lowe & Thorneley (1984a)

reported a second slow phase, for which they offered no explanation. Our kinetic model does not predict a second phase, and the data of Fig. 1 show that with Kp nitrogenase none occurs.

The failure of nitrogenase to reduce N₂ under these conditions of low electron flux is entirely due to the inability of the low concentration of Kp2(MgATP)₂ to reduce Kp1 to any significant extent beyond the E₂H₂ state. A further reduction to E₃ is the minimum that is required for Kp1 to bind N₂ by displacing H₂ from the active site, presumed to be a metal centre within the FeMo-cofactor (see Thorneley & Lowe, 1984b; Hawkes *et al.*, 1984). Thus, under the conditions used in these studies, H₂ is essentially the only reduction product.

Kinetics of electron transfer from Kp2(MgATP)₂ to Kp1 at levels E₀ and E₁H

The time-courses of Fig. 1 show that after 12 min reaction, 55% of Kp1 is in state E₀ and 45% in state E₁H. Thus if this slowly reacting mixture of Kp1 and Kp2 (molar ratio 100:1) is mixed in the stopped-flow apparatus with an excess of

Fig. 3. Electron transfer from Kp2 (MgATP)₂ to Kp1 at oxidation levels E₀ and E₁H occurs at the same rate at 23 °C and pH 7.4

The traces were obtained by stopped-flow spectrophotometry by monitoring the absorbance changes occurring at 430 nm (2 mm path length). Syringe A contained a reacting solution of Kp1 (20 μ M), Kp2 (0.2 μ M), MgATP (9 mM), creatine phosphate (9 mM) and creatine kinase (60 μ g/ml) which was rapidly prepared by premixing as described in the Materials and methods section. Syringe B contained Kp2 (80 μ M). Both syringes contained Na₂S₂O₄ (10 mM), MgCl₂ (10 mM) and Hepes buffer (25 mM, pH 7.4). The contents of syringe A were allowed to react for 15 s (a) and 12 min (b) before being mixed with the excess Kp2 contained in syringe B. Traces (a) and (b) are essentially identical and are both best fitted by a single exponential function with an amplitude of 0.048 absorbance units and $k_{\text{obs}} = 140 \text{ s}^{-1}$. The dotted and broken lines shown in (b) (which do not fit the data as well as the solid line) were drawn assuming that E₀ and E₁H are reduced at different rates. A value of k_{obs} for E₀ of 140 s^{-1} , with an amplitude 55% of the total absorbance change, was used for both lines but with $k_{\text{obs}} = 160 \text{ s}^{-1}$ (----) or 120 s^{-1} (....) for the reduction of E₁H (45% of the total amplitude). The simulations of Fig. 1 and the e.p.r. spectra in Fig. 2 show that, for trace (a) on mixing with Kp2, the Kp1 was 97% in state E₀ and 3% in state E₁H whereas for trace (b) it was 55% in state E₀ and 45% in state E₁H. It is concluded that Kp1 in states E₀ and E₁H is reduced by Kp2(MgATP)₂ at the same rate.

Kp2(MgATP)₂ after 12 min reaction, then electron transfer should occur from the Kp2(MgATP)₂ to the Kp1 present as 55% E₀ and 45% E₁H. If these reactions occur at different rates, a two-exponential function for the absorbance change at 430 nm should be observed. The two phases would be expected to have approximately equal amplitudes since the major contribution is from the absorbance change that occurs in the Kp2(MgATP)₂ spectrum as it is oxidized ($\Delta\epsilon_{430} = 4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; Ashby & Thorneley, 1987). Fig. 3 shows that the same single-exponential

absorbance change ($k_{\text{obs.}} = 140 \text{ s}^{-1}$, amplitude, 0.048 absorbance units) was observed for the reduction of Kp1 present in 100% state E_0 (Fig. 3a) and for Kp1 present in 45% state E_0 and 55% E_1H (Fig. 3b). Assigning values of 120 s^{-1} and 160 s^{-1} to $k_{\text{obs.}}$ for the reaction of E_1H clearly produces less good fits to the data. We conclude that states E_0 and E_1H of Kp1 are reduced by Kp2(MgATP)_2 at the same rate. The difference between the value of $k_{\text{obs.}} = 140 \text{ s}^{-1}$ and that of $k_2 = 200 \text{ s}^{-1}$ (Table 1) is accounted for by the ATP-dependence of $k_{\text{obs.}}$ (Thorneley, 1975).

This is the first time that the rates of the partial reactions that comprise the Fe-protein cycle (Scheme 1) have been measured directly for the MoFe-protein that has already completed one reduction cycle, i.e. rate constants for a reaction taking place in the second of the eight Fe-protein cycles that occur sequentially and together comprise one MoFe-protein cycle under N_2 -reducing conditions (Lowe & Thorneley, 1984a). A basic assumption of our model is that the rate constants that define the Fe-protein cycle are independent of the oxidation level of the MoFe-protein. We have now demonstrated this experimentally for protein complex-formation (k_{+1}) and the subsequent electron transfer from Kp2(MgATP)_2 to Kp1 (k_{+2}). In addition, the steady-state rate of H_2 evolution, under conditions of high electron flux when protein complex dissociation (k_{-3}) is rate-limiting, shows that k_{-3} must have the same value for E_0 and E_1H . This was implied, but not explicitly stated, by Thorneley & Lowe (1984a).

Our conclusion that E_0 and E_1H are reduced by Kp2(MgATP)_2 at the same rate suggests that either electron transfer *per se* is not rate-limiting or that the site on Kp1 initially receiving the electron is at the same redox level in E_0 and E_1H . The first possibility is consistent with ATP hydrolysis limiting electron transfer at 23°C as discussed by Thorneley *et al.* (1989). The second possibility is consistent with electron transfer from Kp2(MgATP)_2 initially occurring to a site other than the FeMo-cofactor, the e.p.r. signal of which is ultimately bleached. The 'P' centres in Kp1 are obvious candidates, particularly since electron transfer, albeit in the opposite direction under oxidizing conditions, from the FeMo-cofactor to the 'P' clusters has been demonstrated (Smith *et al.*, 1983). However, without any evidence at present for transient reduction of these centres we do not wish to speculate further.

The conclusion that k_{+1} has the same value ($5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) for complex-formation between Kp2(MgATP)_2 and Kp1 in states E_0 and E_1H indicates that reduction of Kp1 does not cause any

significant change in protein conformation or charge density at the surface of Kp1 that docks with the Fe-protein. This is consistent with our representation of the reduced form of Kp1 as E_1H and E_2H_2 and the assumption that the negative charges of the electrons transferred to the FeMo-cofactor are neutralized on formation of metal hydrides before release of H_2 .

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REFERENCES

- Ashby, G. A. & Thorneley, R. N. F. (1987) *Biochem. J.* **246**, 455–465
 Burgess, B. K. (1985) in *Metal Ions in Biology Series*, vol. 7, Molybdenum Enzymes (Spiro, T. G., ed.), pp. 161–219, Wiley Interscience, New York
 Eady, R. R. & Postgate, J. R. (1974) *Nature (London)* **249**, 805–810
 Hageman, R. V. & Burris, R. H. (1978) *Biochemistry* **17**, 4117–4124
 Hageman, R. V. & Burris, R. H. (1979) *J. Biol. Chem.* **254**, 11189–11192
 Hawkes, T. R., McLean, P. A. & Smith, B. E. (1984) *Biochem. J.* **217**, 317–321
 Hill, S. & Kavanagh, E. P. (1980) *J. Bacteriol.* **141**, 470–475
 Lowe, D. J. (1991) in *Electron and Proton Transfer in Chemistry and Biology* (Müller, A., ed.), in the press
 Lowe, D. J. & Thorneley, R. N. F. (1984a) *Biochem. J.* **224**, 877–886
 Lowe, D. J. & Thorneley, R. N. F. (1984b) *Biochem. J.* **224**, 895–901
 Nieva-Gomez, D., Roberts, G. P., Klevickis, S. & Brill, W. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2555–2558
 Orme-Johnson, W. H. (1985) *Annu. Rev. Biophys. Biophys. Chem.* **14**, 419–549
 Smith, B. E. (1990) in *Nitrogen Fixation: Achievements and Objectives* (Gresshof, P. M., Roth, L. E., Stacey, G. & Newton, W. E., eds.), pp. 3–13, Chapman and Hall, New York and London
 Smith, B. E., Lowe, D. J., Chen, G.-X., O'Donnell, M. J. & Hawkes, T. R. (1983) *Biochem. J.* **209**, 207–213
 Thorneley, R. N. F. (1975) *Biochem. J.* **145**, 391–396
 Thorneley, R. N. F. (1990) in *Nitrogen Fixation: Achievements and Objectives* (Gresshof, P. M., Roth, L. E., Stacey, G. & Newton, W. E., eds.), pp. 103–109, Chapman and Hall, New York and London
 Thorneley, R. N. F. & Lowe, D. J. (1983) *Biochem. J.* **215**, 393–403
 Thorneley, R. N. F. & Lowe, D. J. (1984a) *Biochem. J.* **224**, 887–894
 Thorneley, R. N. F. & Lowe, D. J. (1984b) *Biochem. J.* **224**, 903–909
 Thorneley, R. N. F. & Lowe, D. J. (1985) in *Metal Ions in Biology Series*, vol. 7: Molybdenum Enzymes (Spiro, T. G., ed.), pp. 117–159, Wiley-Interscience, New York
 Thorneley, R. N. F., Ashby, G., Howarth, J. V., Millar, N. C. & Gutfreund, H. (1989) *Biochem. J.* **264**, 657–661
 Wherland, S., Burgess, B. K., Stiefel, E. I. & Newton, W. E. (1981) *Biochemistry* **20**, 5132–5140

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