Nitrogenase of *Klebsiella pneumoniae*

Kinetics of the dissociation of oxidized iron protein from molybdenum–iron protein: Identification of the rate-limiting step for substrate reduction

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(Received 3 May 1983/Accepted 21 July 1983)

Stopped-flow spectrophotometry and e.p.r. spectroscopy were used to study the kinetics of reduction by dithionite of the oxidized Fe protein of nitrogenase from *Klebsiella pneumoniae* (Kp2_{ox}) in the presence of MgADP at 23°C at pH 7.4. The active reductant, SO₂^{•−}, produced by the predissociation of S₂O₄^{2−} = 2SO₂^{•−}, reacts with Kp2_{ox}.(MgADP)₂ with $k_4 = 3.0 \times 10^6 \pm 0.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. The inhibition of this reaction by the Mo–Fe protein (Kp1) has enabled the rate of dissociation of Kp2_{ox}.(MgADP)₂ from Kp1⁺ (the Kp2-binding site on Kp1) to be measured ($k_{-3} = 6.4 \pm 0.8 \text{ s}^{-1}$). Comparison with the steady-state rate of substrate reduction shows that the dissociation (k_{-3}) of the complex Kp2_{ox}.(MgADP)₂–Kp1⁺, which is formed after MgATP-induced electron transfer from Kp2 to Kp1⁺, is the rate-limiting step in the catalytic cycle for substrate reduction.

Klebsiella pneumoniae nitrogenase consists of two metallothioproteins: the Mo–Fe protein (Kp1) has M_r 218 000 and contains 32Fe and 2Mo atoms, and the Fe protein (Kp2) with M_r 68 000 contains 4Fe atoms. Nitrogenase catalyses the reduction of N₂ to NH₃ in a reaction that is coupled to the hydrolysis of MgATP to MgADP + P_i (Mortenson & Thorneley, 1979; Lowe *et al.*, 1983). In the absence of N₂, the enzyme functions as an ATP-dependent hydrogenase and reduces protons to H₂. The hydrogenase activity is incompletely suppressed by N₂ such that a limiting stoichiometry (eqn. 1) is reached with 1 atm of N₂:

The source of electrons *in vivo* is probably a flavodoxin or a ferredoxin (Bothe *et al.*, 1980; Yates, 1980), and *in vitro* Na₂S₂O₄ is normally used. E.p.r. (Smith *et al.*, 1972; Orme-Johnson *et al.*, 1972; Zumft *et al.*, 1974) and Mössbauer (Smith & Lang, 1974) studies showed that the sequence of electron transfers is as shown in Scheme 1. A knowledge of

the rate constants for these partial reactions, which include reversible complex-formation between the Fe and Mo–Fe proteins, is essential for the understanding of pre-steady-state kinetic data for H₂, N₂H₄ (derived from an enzyme-bound intermediate) and NH₃ production and the consequent formulation of a mechanism for nitrogenase.

We report in the present paper the results obtained by using stopped-flow kinetic spectroscopy to study the first two partial reactions of Scheme 1. These two steps are part of a cycle (Scheme 2) in which one electron is transferred from Kp2 to Kp1⁺ with the concomitant hydrolysis of 2MgATP to 2MgADP + 2P_i (Eady *et al.*, 1978a; Hageman *et al.*, 1980). In Scheme 2 Kp1⁺ represents one of two independently functioning halves of the tetrameric (α₂β₂ structure) Kp1. Each Kp1⁺ is assumed to contain one Mo substrate-binding site and one Kp2-binding site.

The present paper is concerned with the determination of k_{+3} , k_{-3} and k_{+4} and shows that the dissociation of Kp2_{ox} from Kp1 protein (k_{-3}) is the rate-limiting reaction in the catalytic cycle for

Scheme 1

Abbreviations used: the nitrogenase components of the various organisms are denoted by a capital letter indicating the genus and a lower case letter indicating the species, and the number 1 indicates the Mo–Fe-containing protein and the number 2 the Fe-containing protein: Kp, *Klebsiella pneumoniae*; Av, *Azotobacter vinelandii*; Cp, *Clostridium pasteurianum*.

Scheme 2

nitrogenase at the concentrations of component proteins reported to be present *in vivo* under N_2 -fixing conditions.

Materials and methods

Enzyme preparation

The nitrogenase component proteins were purified as described by Eady *et al.* (1972), with an additional DEAE-cellulose chromatography (Smith *et al.*, 1976). Gel filtration on Sephadex G-25 (medium grade) was used to prepare stock solutions of Kp1 (21 mg/ml) and Kp2 (21 mg/ml) in 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 7.4, containing 20 mM-MgCl₂ and 1 mM-Na₂S₂O₄. Kp1 and Kp2 proteins had specific activities of 1550 and 1600 nmol of ethylene reduced/min per mg of protein respectively when assayed at 30°C under the standard conditions described by Eady *et al.* (1972). The method of Clarke & Axley (1955) was used to determine a molybdenum content of 1.3 ± 0.1 g-atom of Mo/mol of Kp1. Atomic absorption was used to determine the Fe content of Kp1 protein (20 ± 2 g-atoms of Fe/mol) and Kp2 protein (3.5 ± 0.3 g-atoms of Fe/mol). In the analysis of the data, the concentration of active site ($\text{Kp1}^{\dagger}$) was assumed to be the product of the Mo content/mol and the Kp1 concentration. No correction was made to the Kp2 concentration. A minor contaminant, demolybdo-Kp1 (cf. demolybdo-Cp1; Zumft *et al.*, 1974) was present and comprised 6% of the total Kp1 (estimated by integration of the $g_{\text{av.}} = 1.95$ e.p.r. signal).

All biochemicals were purchased from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K., and salts from BDH Chemicals, Poole, Dorset, U.K.

Instrumentation and data analysis

Stopped-flow spectroscopy was performed with a commercially available Aminco-Morrow apparatus modified as previously described (Thorneley, 1974) to improve the thermostatic control of the observation cell (9.2 mm light-path). The apparatus was

installed in an anaerobic glove box (type 4B; Faircrest Engineering, Croydon, Surrey, U.K.) through which N_2 gas was circulated. Inside the glove box, the O_2 concentration was less than 1 p.p.m., as monitored by a coulometer (Chandos Intercontinental, Stockport, Cheshire, U.K.). The glove box was fitted with a cooling system to dissipate the heat from the 200 W lamp of the stopped-flow apparatus and to maintain an ambient temperature close to 23°C. The stopped-flow apparatus was thermostatically maintained at $23.0 \pm 0.2^\circ\text{C}$ by circulation of water from a bath situated outside the glove box. A Tektronix type 549 storage oscilloscope and lamp power supply were sited outside the glove box and connected to the output-signal amplifier and 200 W quartz-iodide lamp inside the glove box by sealed electrical breakthroughs. Stored oscilloscope traces were photographed and subsequently projected on to graph paper for tracing and digitalization before data processing on a PDP1134A computer.

Reactions were monitored at 430 nm, since this wavelength provided the maximum ΔA for $\text{Kp2}_{\text{ox.}}$ reduction while ensuring that at the high protein concentrations used the absolute absorbance was not greater than 2.0 (upper limit for the stopped-flow spectrophotometer).

The fitting procedure used minimized the sum of the squares of the differences between the experimental and simulated absorbance-versus-time curves by using a quasi-Newton algorithm (NAG Subroutine D02ABF). Local searches were made at intervals to avoid saddle points. The simulated curves were produced by solving the differential equations describing the appropriate scheme by using a Runge-Kutta method (NAG Subroutine E04JBF), assuming that the only change in absorbance was produced by reduction of $\text{Kp2}_{\text{ox.}}$.

Protein samples for e.p.r. spectroscopy were prepared inside the glove box by freezing in tubes immersed in methanol at -25°C . The methanol was contained in a slot in a hollow copper block through which cold N_2 gas was passed via a closed loop from outside the glove box. The N_2 gas was cooled by passage through a coil of copper pipe immersed in liquid N_2 .

Preparation of oxidized Kp2 protein

Reduced Kp2 protein was mixed with the requisite volume of Kp1 protein inside the glove box to give a total protein solution in 25 mM-Hepes buffer, pH 7.4, containing 20 mM-MgCl₂ and 1 mM-Na₂S₂O₄. A sample (0.2 ml) of this solution was frozen in an e.p.r. tube for subsequent analysis. ATP was added to give a final concentration between 2 and 4.5 mM in order to initiate the reduction of N₂ and protons. Since dithionite ion is limiting, when the reductant is exhausted, the solution contains Kp2_{ox.} (e.p.r.-silent), Kp1 (e.p.r.-active) and a mixture of ATP and ADP (Smith *et al.*, 1973). After 40 min another protein sample (0.2 ml) was frozen in an e.p.r. tube and the spectrum was compared with that of the sample taken before the addition of ATP. The decrease in amplitude of the e.p.r. signal at $g = 1.94$ (run at 11.9 K, 2.06 mW), which is associated with Kp2 protein, indicated the degree of oxidation of this protein (typically >80%).

The concentration of ATP in the e.p.r. samples was determined by using a commercial luciferase assay (LKB Instruments, South Croydon, Surrey, U.K.) based on the method of Thore (1979). The extent of ATP hydrolysis over 40 min depended on the ratio and concentrations of Kp2 and Kp1 proteins. Typically, with $[Kp2] \approx [Kp1] \approx 80 \mu\text{M}$, more than 99% of the ATP was hydrolysed. However, when $[Kp2] = 70 \mu\text{M}$, $[Kp1] = 6 \mu\text{M}$, only 70% of the ATP was hydrolysed. This is because both reductant-dependent electron transfer from Kp2 to Kp1 (Eady *et al.*, 1978a) and reductant-independent (Imam & Eady, 1980) ATPase activity are associated with a complex of Kp2 with Kp1. This oxidative procedure for Kp2 has the advantage that it uses the enzymically active oxidizing agent (Kp1). Dyes and chemical oxidants may damage or modify the Kp2, may oxidize it to a state that does not occur during turnover, or may bind to the protein causing a change in reactivity. In addition, the removal of the dye or oxidant by gel filtration or dialysis increases the risk of inactivation and leads to a dilution of the protein. This latter point is particularly important for these studies, which require the concentration of protein stock solutions to be >100 μM . However, physiological oxidation of Kp2 by Kp1 has the disadvantage that it is not easy to control the concentration of ATP and ADP. We have overcome this problem by the use of control experiments in which the $[ADP]/[ATP]$ ratio was changed 10-fold with no measurable effect on the kinetic parameters (see Fig. 1). In other experiments, when the $[ADP]/[ATP]$ ratio was approx. 100:1 (see Fig. 4), we have considered that ADP will compete effectively with ATP at the nucleotide-binding sites on the proteins.

One drive syringe of the stopped-flow apparatus

contained Kp2_{ox.}, Kp1, ADP, ATP and MgCl₂ at various concentrations in 25 mM-Hepes buffer, pH 7.4. The other drive syringe contained Na₂S₂O₄, Na₂SO₄, ADP and reduced Kp2 protein, at various concentrations, in 25 mM-Hepes buffer, pH 7.4. The concentrations of all the components are given in the text or legends to the Figures.

Preparation of Kp1 with the 'P' centres oxidized and 'M' centres reduced

Kp1 was oxidized by Fe(CN)₆³⁻ by using dye mediators as described by Dutton (1970) and O'Donnell & Smith (1978). A potential of -290 mV (standard hydrogen electrode) was maintained for 15 min before the protein solution (5 ml; 15 mg of Kp1/ml) was removed from the potentiostat cell and run down a column comprised of 1:1 mixture of Dowex AG1-X2 and AG50W-X2 resins (1 cm × 1 cm) on top of Sephadex G-25 (20 cm × 1 cm) in order to remove the dye mediators, Fe(CN)₆³⁻, Fe(CN)₆⁴⁻ and dithionite oxidation products. The column was pre-equilibrated and run with 25 mM-Hepes buffer, pH 7.4, containing 10 mM-MgCl₂. The complete retention of the $g = 3.7$ feature of the Kp1 e.p.r. spectrum confirmed that the Fe-Mo cofactors ('M' centres) had not been oxidized. This signal is slightly broadened on oxidation, presumably owing to a magnetic interaction with the 'P' centres (probably four [4Fe-4S]⁰ clusters; Zimmerman *et al.*, 1978) or a protein conformation change consequent on the oxidation of the 'P' centres [mid-point potential of -340 mV at pH 8.7 (standard hydrogen electrode) (O'Donnell & Smith, 1978)]. The signal at $g = 1.95$ due to demolybdo-Kp1 was absent from the oxidized protein, indicating that this species has also been oxidized at a potential of -290 mV (standard hydrogen electrode).

Results

Reduction of Kp2_{ox.} (ADP)₂ by dithionite

Kp2 was oxidized by the addition of Kp1 and MgATP as described above. A low concentration of Kp1 was used in order to minimize the extent of complex-formation between Kp1 and Kp2_{ox.}- (MgADP)₂ (see below). Thus one syringe of the stopped-flow apparatus contained 73 μM -Kp2_{ox.}, 4 μM -Kp2, 3.6 μM -Kp1, 2.8 mM-(ATP + ADP), 20 mM-MgCl₂ and 25 mM-Hepes buffer, pH 7.4. For one experiment the Kp2_{ox.}, Kp2, Kp1 and ATP concentrations were decreased 10-fold while the ADP, MgCl₂ and Hepes concentrations were maintained as above. The other syringe contained various concentrations of Na₂S₂O₄ (2–160 mM) together with 2.2 mM-ADP and 25 mM-Hepes buffer, pH 7.4. For one experiment with 2 mM-Na₂S₂O₄, 100 mM-Na₂SO₄ was also present in order to simulate the

Fig. 1. Reduction of $Kp2_{ox}(MgADP)_2$ by $Na_2S_2O_4$ at $23^\circ C$ at pH 7.4

The data were obtained by using the stopped-flow technique, with monitoring of absorbance changes at 430 nm as described in the Materials and methods section. (a) Syringe A contained: $72 \mu M$ - $Kp2_{ox}$, $5 \mu M$ - $Kp2_{red}$, $3.6 \mu M$ - $Kp1$, $2.8 mM$ -ADP, $20 mM$ - $MgCl_2$, $25 mM$ -Hepes. Syringe B contained: $20 mM$ - $Na_2S_2O_4$, $2.2 mM$ -ADP. (b) Syringe A contained: $82 \mu M$ - $Kp2_{ox}$, $67 \mu M$ - $Kp1$, $4.5 mM$ -ADP, less than $50 \mu M$ -ATP, $17 mM$ - $MgCl_2$, $25 mM$ -Hepes. Syringe B contained: $20 mM$ - $Na_2S_2O_4$, $235 \mu M$ - $Kp2_{red}$, $20 mM$ - $MgCl_2$, $25 mM$ -Hepes. (c) Syringe A contained: $82 \mu M$ - $Kp2_{ox}$, $67 \mu M$ - $Kp1$, $3.8 mM$ -ADP, $50 \mu M$ -ATP, $20 mM$ - $MgCl_2$, $25 mM$ -Hepes. Syringe B contained: $100 mM$ - $Na_2S_2O_4$, $1.5 mM$ -ADP, $25 mM$ -Hepes. (d) Syringe A contained: $5.3 \mu M$ - $Kp2_{ox}$, $4.5 \mu M$ - $Kp1$, $4.0 mM$ -ADP, $20 mM$ - $MgCl_2$, $25 mM$ -Hepes. Syringe B contained: $100 mM$ - $Na_2S_2O_4$, $1.5 mM$ -ADP, $25 mM$ -Hepes. (e) Syringe A contained: $82 \mu M$ - $Kp2_{ox}$, $67 \mu M$ - $Kp1$, $4.5 mM$ -ADP, less than $50 \mu M$ -ATP, $17 mM$ - $MgCl_2$, $25 mM$ -Hepes. Syringe B contained: $20 mM$ - $Na_2S_2O_4$, $20 mM$ - $MgCl_2$, $25 mM$ -Hepes. (f) Syringe A contained: $82 \mu M$ - $Kp2_{ox}$, $67 \mu M$ - $Kp1$, $4.5 mM$ -ADP, less than $50 \mu M$ -ATP, $17 mM$ - $MgCl_2$, $25 mM$ -Hepes. Syringe B contained: $20 mM$ - $Na_2S_2O_4$, $180 mM$ - Na_2SO_4 .

effect of ionic strength at high dithionite concentrations. The concentrations of the reagents in the drive syringes of the stopped-flow apparatus are given in the legends to Figs. 1 and 2, since the kinetics of this system are affected by (a) the

pre-mixing of components and (b) the 2-fold dilution of the protein components that occurs on mixing in a stopped-flow experiment.

A typical oscilloscope trace is shown in Fig. 1(a). The absorbance change occurring up to a time of 1 s

Fig. 2. Reduction of $Kp2_{ox.}(MgADP)_2$ by $Na_2S_2O_4$ at 23°C at pH 7.4

The data were obtained by using the stopped-flow technique and with the kinetic analysis described in the Materials and methods section. O, Dithionite-dependence. Syringe A contained: 73 μ M- $Kp2_{ox.}$, 4 μ M- $Kp2_{red.}$, 3.6 μ M- $Kp1$, 2.8 mM-(ATP + ADP), 20 mM- $MgCl_2$, 25 mM-Hepes. Syringe B contained: 2 mM- to 160 mM- $Na_2S_2O_4$, 2.2 mM-ADP, 25 mM-Hepes. □, Effect of dilution of the protein. Syringe A contained: 7.3 μ M- $Kp2_{ox.}$, 0.4 μ M- $Kp2_{red.}$, 0.36 μ M- $Kp1$, 2.8 mM-(ATP + ADP), 20 mM- $MgCl_2$, 25 mM-Hepes. Syringe B contained: 100 mM- $Na_2S_2O_4$, 2.2 mM-ADP, 25 mM-Hepes. △, Effect of salt. Syringe A contained: 73 μ M- $Kp2_{ox.}$, 4.0 μ M- $Kp2_{red.}$, 3.6 μ M- $Kp1$, 2.8 mM-(ATP + ADP), 20 mM- $MgCl_2$, 25 mM-Hepes. Syringe B contained: 2 mM- $Na_2S_2O_4$, 98 mM- Na_2SO_4 , 2.2 mM-ADP, 25 mM-Hepes.

is described by a single exponential decay with a first-order rate constant $k_{obs.} = 10.3 s^{-1}$ and an amplitude of $\Delta A_{430} = 0.126$ ($\Delta \epsilon = 3.8 \times 10^3 m^{-1} \cdot cm^{-1}$). Subsequent triggering of the oscilloscope showed that a further smaller decrease in absorption occurs with a long time constant ($\tau > 10 s$). Since this second process is slow compared with the turnover time of nitrogenase, we have not investigated it further. It may be associated with unreactive or damaged protein (Thorneley *et al.*, 1976).

The dependence of $k_{obs.}$ on $[S_2O_4^{2-}]^{1/2}$ is shown in Fig. 2. Dilution of the protein 10-fold caused no change in $k_{obs.}$ when the reductant was 50 mM- $Na_2S_2O_4$, and only the expected 10-fold decrease in amplitude was observed. The presence of 50 mM- Na_2SO_4 did not change $k_{obs.}$ when the reductant was 1 mM- $Na_2S_2O_4$. This is consistent with the linear dependence of $k_{obs.}$ on $[S_2O_4^{2-}]^{1/2}$ until the concentration of $S_2O_4^{2-}$ was greater than 50 mM. Thus this reaction does not exhibit a significant ionic-strength- or salt-dependence, in contrast with the protein dissociation reaction discussed below, which is accelerated by Na_2SO_4 .

These data allow the apparent rate constant k_{+4} of Scheme 2 to be calculated. We use the description 'apparent' because the detailed mechanism by which $Kp_{ox.}(MgADP)_2$ is reduced (i.e. whether $MgATP$ dissociates before reduction or whether $MgATP$ catalyses this reduction) is not important in this context, because the essential aim of the present paper is to use k_{+4} to determine the rates of protein dissociation (k_{-3}) and association (k_{+3}).

Kinetics involving dithionite ion as a reductant are complicated by the equilibrium shown by eqn. (2):

Both $S_2O_4^{2-}$ and $SO_2^{\cdot-}$ are active in the reduction of one- and two-electron-acceptor metalloproteins (Lambeth & Palmer, 1973; Creutz & Sutin, 1973). However, as is demonstrated below, $SO_2^{\cdot-}$ is the active reductant in this system (eqn. 3):

We have previously reported a value for $k_{+6} = 1.7 s^{-1}$ at 23°C (Thorneley *et al.*, 1976). The equilibrium constant for eqn. (2), $K_6 = k_{+6}/k_{-6}$, was calculated after using e.p.r. spectroscopy to measure the concentration of $SO_2^{\cdot-}$ radicals at a series of dithionite ion concentrations. 1,1-Diphenyl-2-picrylhydrazyl was used as a free-radical standard to calibrate the e.p.r. flat cell used for liquid samples. Fig. 3 shows a linear dependence of $[SO_2^{\cdot-}]^2$ on $[S_2O_4^{2-}]$, and the slope gives a value of $K_6 = 1.6 \times 10^{-9} \pm 0.2 \times 10^{-9} M$ at 23°C. This compares with a value of $K_6 = 1.4 \times 10^{-9} \pm 0.4 \times 10^{-9} M$ at 25°C reported by Lambeth & Palmer (1973). The inset to Fig. 3 shows that increasing the concentration of Na_2SO_4 from zero to 200 mM caused only a 7% decrease in the concentration of $SO_2^{\cdot-}$ in equilibrium with 10 mM- $S_2O_4^{2-}$. Thus perturbation of equilibrium (2) cannot account for the kinetic effects induced by Na_2SO_4 that are presented below.

The derivation of the rate expressions for the reduction of dye-oxidized $Ac2_{ox.}$ protein by $Na_2S_2O_4$ has been reported previously (Thorneley *et al.*, 1976). The appropriate expressions for $Kp2_{ox.}(MgADP)_2$ reduction with an excess of $Na_2S_2O_4$ is given in eqn. (4):

$$-\frac{d}{dt} [Kp2_{ox.}(MgADP)_2] = k_{+4} K_6^{1/2} [S_2O_4^{2-}]^{1/2} [Kp2_{ox.}(MgADP)_2] \quad (4)$$

The single exponential decay of $Kp2_{ox.}(MgADP)_2$ (Fig. 1a) and the linear dependence of $k_{obs.}$ on $[S_2O_4^{2-}]^{1/2}$ (Fig. 2) verifies this rate expression. The slope of the line of Fig. 2 together with $K_6 =$

Fig. 4. Reduction of $Kp2_{ox}(\text{MgADP})_2$ by $\text{Na}_2\text{S}_2\text{O}_4$ in the presence of $Kp1$ showing the effect of increasing the concentration of $Kp2_{red}(\text{MgADP})_2$ at 23°C at pH 7.4

The data were obtained by using the stopped-flow technique and fitted to Scheme 3 as described in the Materials and methods section. In all the experiments syringe A contained: $67\text{ }\mu\text{M}$ - $Kp1$, $82\text{ }\mu\text{M}$ - $Kp2_{ox}$, 4.5 mM -ADP, less than $50\text{ }\mu\text{M}$ -ATP, 17 mM - MgCl_2 and 25 mM -Hepes. Syringe B contained: 20 mM - $\text{Na}_2\text{S}_2\text{O}_4$, 20 mM - MgCl_2 , 25 mM -Hepes and various concentrations of $Kp2_{red}$, i.e. (a) $0.0\text{ }\mu\text{M}$, (b) $47\text{ }\mu\text{M}$, (c) $94\text{ }\mu\text{M}$, (d) $157\text{ }\mu\text{M}$ and (e) $235\text{ }\mu\text{M}$. The curves shown (—) for (a)–(e) were obtained by subtracting the absorption change due to $Kp1$ protein as described in the text. This procedure is illustrated in (f), which shows the original time course for experiment (e) (—) together with the calculated absorption change for $Kp1$ (-----). The subtraction of curve 1 from curve 2 generates the data line (—) in (e). A similar procedure was used for (a), (b), (c) and (d). The computed best-fit lines (—) are shown for (a)–(e) and the calculated rate constants for Scheme 3 are given in Table 1.

Fig. 1(f) relative to Fig. 1(e) is not important in the present paper, since high ionic strength has been avoided for kinetic reasons. We conclude that Na_2SO_4 perturbs the equilibrium between $Kp1^+$ and $Kp2_{ox}(\text{MgADP})_2$. These qualitative conclusions place constraints on the concentrations of component proteins, $\text{Na}_2\text{S}_2\text{O}_4$ and salts that can be used in experiments to determine the rate constants in Scheme 3.

Fig. 4 shows the absorbance–time curves together with the computer best fits for these data on the basis of Scheme 3. The experimental conditions given in the legend to Fig. 4 were required for the following reasons. (1) High concentrations of $Kp1$ and $Kp2_{ox}$ ($>50\text{ }\mu\text{M}$) are required such that most of the $Kp2_{ox}$ is complexed to $Kp1^+$. $Kp1$ and $Kp2_{ox}$ must be in the same drive syringe in the absence of $\text{Na}_2\text{S}_2\text{O}_4$. (2) The concentration of $\text{Na}_2\text{S}_2\text{O}_4$ (10 mM) was not varied, since, although increasing the concentration of $\text{Na}_2\text{S}_2\text{O}_4$ to 50 mM would have increased k_{obs} from 10.4 s^{-1} (Fig. 2), which is desirable for the resolution of the fast (free $Kp2_{ox}$)

and slow ($Kp2_{ox}$ complexed to $Kp1^+$) phases, the attendant salt effect on k_{+3} and k_{-3} cannot be easily accommodated in the analysis. In addition, since the pre-steady-state kinetics of substrate reduction were studied with 10 mM - $\text{Na}_2\text{S}_2\text{O}_4$, we are particularly interested in the values of k_{+3} and k_{-3} under these conditions. (3) Various concentrations of reduced $Kp2$ were included in the syringe containing a constant concentration of $\text{Na}_2\text{S}_2\text{O}_4$ (20 mM) in order to produce a series of progress curves. As the concentration of reduced $Kp2$ was increased from 0 to $235\text{ }\mu\text{M}$, the observed curves approach the single exponential defined by k_{-3} . Since reduced $Kp2$ is the product of the reduction of $Kp2_{ox}$, even if no reduced $Kp2$ is included initially, reactions k_{+5} and k_{-5} need to be considered in fitting the data. (4) The concentration of MgATP ($<40\text{ }\mu\text{M}$) and of MgADP (between 4.46 and 4.50 mM) in the syringe containing $Kp2_{ox}$ and $Kp1$ should have ensured saturation of nucleotide-binding sites with MgADP and the exclusion of MgATP . This assumption is based on (a) values of the constants determined for

Fig. 5. Reduction of $Kp1_{ox}$ by $Na_2S_2O_4$ and the effect of $Kp2(MgADP)_2$ on this reaction at $23^\circ C$ at pH 7.4 $Kp1_{ox}$ ('P' centres reduced, 'M' centres oxidized) was prepared as described in the text, and the traces were obtained with the stopped-flow technique as described in the Materials and methods section. (a) Single exponential $k_{obs.} = 0.75 s^{-1}$. Syringe A contained: $48 \mu M$ - $Kp1_{ox}$, $5 mM$ -ADP, $10 mM$ - $MgCl_2$, $25 mM$ -Hepes. Syringe B contained: $20 mM$ - $Na_2S_2O_4$, $10 mM$ - $MgCl_2$, $25 mM$ -Hepes. (b) Single exponential $k_{obs.} = 1.0 s^{-1}$. Conditions as for trace (a) except for the addition of $87 \mu M$ - $Kp2_{red}$ to syringe B.

Table 1. Measured rate constants for electron transfer and component protein association-dissociation at $23^\circ C$ at pH 7.4

The reactions are defined by Schemes 2 and 3 and eqn. (2).

Rate constant	Value	Reference
k_{+1}	$> 1 \times 10^7 M^{-1} \cdot s^{-1}$	Thorneley (1975)
k_{-1}	$< 50 s^{-1}$	Thorneley (1975)
k_{+2}	$2 \times 10^2 s^{-1}$	Thorneley (1975)
k_{+3}	$4.4 \times 10^6 \pm 0.5 \times 10^6 M^{-1} \cdot s^{-1}$	Present work
k_{-3}	$6.4 \pm 0.8 s^{-1}$	Present work
k_{+4}	$3.0 \times 10^6 \pm 0.4 \times 10^6 M^{-1} \cdot s^{-1}$	Present work
k_{+5}	$> 2 \times 10^6 M^{-1} \cdot s^{-1}$	Present work
k_{-5}/k_{+5}	$2.2 \pm 1.0 \mu M$	Present work
k_{+6}	$1.2 \times 10^3 M^{-1} \cdot s^{-1}$	Present work
k_{-6}	$1.7 s^{-1}$	Thorneley <i>et al.</i> (1976)

MgADP inhibition of MgATP-induced electron transfer from $Kp2$ to $Kp1$ (Thorneley, 1975; Thorneley & Cornish-Bowden, 1977) and from $Av2$ to $Av1$ (Hageman *et al.*, 1980) and (b) the binding constant for MgATP to isolated Mo-Fe proteins (Miller *et al.*, 1980).

The values for k_{+3} , k_{-3} , k_{+5} and k_{-5} given in Table 1 were obtained by simultaneously fitting all the stopped-flow curves shown in Fig. 4 and allowing these rate constants, the absorbance change associated with the reduction of $Kp2_{ox}$, and the baselines to vary independently. The points on the curves used in the fitting were taken more frequently at short time than at long times in order to weight changes in absorbance approximately equally.

Absorbance changes associated with $Kp1$

The reduction of $Kp2_{ox}(MgADP)_2$ by dithionite was associated with a $\Delta\epsilon_{430} = 3.8 \times 10^3 M^{-1} \cdot cm^{-1}$.

when $Kp1$ was essentially absent (Fig. 1a). However, when $Kp1$ was present at concentrations approximately equal to that of the $Kp2_{ox}(MgADP)_2$ [Figs. 1(b)–1(e) and Fig. 4], an apparent $\Delta\epsilon_{430} = 5.4 \times 10^3 \pm 0.3 \times 10^3 M^{-1} \cdot cm^{-1}$ was observed up to 5 s reaction time (assuming the absorbance change is only due to $Kp2_{ox}$ reduction). The difference between these values of $\Delta\epsilon_{430}$ indicates that a species other than $Kp2_{ox}$ is undergoing reduction. The magnitude of this difference suggests that this species is either $Kp1$ with oxidized 'P' centres or oxidized demolybdo- $Kp1$, which was present as a minor contaminant (see the Materials and methods section).

A time course for the reduction by dithionite of $Kp1$ ['P' centres oxidized by controlled potential oxidation at $-290 mV$ (standard hydrogen electrode)] is shown in Fig. 5(a). The curve up to 5 s is fitted by a single exponential ($k_{obs.} = 0.75 s^{-1}$) with an amplitude corresponding to $\Delta\epsilon_{430} = 1.4 \times 10^3 M^{-1} \cdot cm^{-1}$. Addition of $87 \mu M$ - $Kp2_{red}$ to the drive syringe containing the $Na_2S_2O_4$ (Fig. 5b) caused a small increase in the reduction rate ($k_{obs.} = 1.0 s^{-1}$) but no significant change in the amplitude. We have subtracted out a time course for $Kp1_{ox}$ reduction ($k_{obs.} = 1.0 s^{-1}$; $\Delta\epsilon_{430} = 1.4 \times 10^3 M^{-1} \cdot cm^{-1}$) from the original stopped-flow data to generate the curves shown in Fig. 4. An original stopped-flow trace is shown in Fig. 4(f), together with the exponential to be subtracted that is associated with $Kp1_{ox}$ reduction. The resulting curve for $Kp2_{ox}$ reduction is shown in Fig. 4(e).

Discussion

The $Kp1$ protein used in these studies exhibited an e.p.r. signal at $g = 1.95$, which is characteristic of an inactive demolybdo-protein (Zumft & Mortenson,

1973). The presence of this contaminating protein (6% of the total Kp1) makes the assignment of the origin of the absorbance change associated with the Kp1 difficult. The mid-point potential of the 'P' centres in Kp1 is -340 mV (standard hydrogen electrode) at pH 8.7 (Smith *et al.*, 1980) and the $g = 1.95$ e.p.r. signal of the demolybdo-protein is absent after oxidation at -290 mV (see the Materials and methods section). Thus both types of centre could become oxidized when dithionite is exhausted in a 'full fixing system' of nitrogenase. However, the $\Delta\epsilon = 1.4 \times 10^3\text{ M}^{-1}\cdot\text{cm}^{-1}$ associated with the reduction of oxidized Kp1 is probably too large to be attributed to the demolybdo-protein contaminant, since this would require a $\Delta\epsilon_{430} = 2.3 \times 10^4\text{ M}^{-1}\cdot\text{cm}^{-1}$ for demolybdo-protein. We therefore conclude that when dithionite is exhausted during substrate reduction the 'P' centres are probably oxidized.

The value of $k_{-3} = 6.4 \pm 0.8\text{ s}^{-1}$ (Table 1) causes us to conclude that the rate-limiting step in the catalytic cycle of nitrogenase is the dissociation of oxidized Fe protein from the Mo-Fe protein after MgATP-induced electron transfer has occurred. This conclusion follows from the equivalence of the value of the specific activity for Kp1 calculated by using $k_{-3} = 6.4 \pm 0.8\text{ s}^{-1}$ as the rate-limiting step to that measured with the standard steady-state assay for H_2 production. The calculation assumes that the concentration of active Kp2-binding sites on Kp1 is determined by the Mo content of the Kp1 ($1.3 \pm 0.1\text{ g-atom of Mo/mol of Kp1}$) and that Kp1 transfers one electron to an Mo centre during each cycle depicted in Scheme 2. The calculated value of $1140 \pm 150\text{ nmol/min per mg of Kp1 protein}$ is in good agreement with that measured by using the standard steady-state assay procedure for nitrogenase, which is $1160 \pm 50\text{ nmol of H}_2/\text{min per mg of Kp1 protein at } 23^\circ\text{C}$. The latter procedure uses an excess of Kp2 over Kp1 with high dithionite concentration (30 mM), which ensures that there is no inhibition due to the back-reaction of Kp2_{ox} with Kp1 (k_{+3}). We consider the value of $k_{-3} = 6.4 \pm 0.8\text{ s}^{-1}$ to be an improvement on a previous value of 5 s^{-1} (Thorneley & Lowe, 1982), since in our preliminary analysis we did not take into account the factors discussed above.

The assumption that Kp2 interacts independently with two sites on Kp1 is supported by two types of experimental evidence. Firstly, Ac2 (Thorneley *et al.*, 1976), Cp2 (Ljones & Burris, 1978) and Av2 (Hageman *et al.*, 1980) are reported to be one-electron-donor proteins. Secondly, the pre-steady-state kinetics of H_2 and ethylene formation require two slow steps (k_{-3}) before the two-electron-reduced product is detected (Thorneley & Lowe, 1981, 1982; Lowe *et al.*, 1983). Kp2 cannot interact with only one site on Kp1, since otherwise the steady-state rate

of H_2 or ethylene formation would be half of that observed.

We are unable to exclude an alternative model based on the conclusions of Braamska *et al.* (1982), who have proposed that Av2 is a two-electron donor. This model requires that two electrons are transferred to two sites on Kp1, with two dissociations of a 1:1 Kp2-Kp1 complex (two cycles of Scheme 2 completed) before two molecules of H_2 or ethylene are released. The fits to the stopped-flow data given in the present paper are the same for both models, but if the Fe protein is a two-electron donor the value of k_{+3} is approximately twice that given in Table 1. Hallenbeck (1983) has repeated the critical experiments of Braamska *et al.* (1982), and from similar data concluded that these provide no evidence for the Fe protein being a two-electron donor. Thus we do not consider the model with Kp2 as a two-electron donor likely.

Scheme 2 contains both first-order and second-order reactions. A second-order reaction can always be made slower than a first-order reaction by decreasing the concentrations of reactants. Thus, when $[\text{Kp1}^+]$ and $[\text{Kp2}]$ are $< 1\text{ }\mu\text{M}$, the second-order protein association reaction (k_{+1}) becomes rate-limiting and the protein 'dilution effect' is observed (Thorneley *et al.*, 1975). Similarly, when a high Mo-Fe protein/Fe protein ratio is used (Hageman & Burris, 1978a,b, 1979), the second-order association reaction between singly reduced Mo-Fe protein and reduced Fe protein is rate-limiting in the steady state. The protein dissociation reaction (k_{-3}) is rate-limiting for nitrogenase inasmuch as it determines the maximum rate of substrate reduction that can be obtained per Mo centre when all other reactants, including Fe protein, are saturating. The use of limiting dithionite ion or another reductant can also cause a change in rate-limiting step. The values of k_{+3} , k_{-3} and the dependence of k_{+4} on $[\text{S}_2\text{O}_4^{2-}]$ (Fig. 2) provide an explanation for the dependence of the apparent K_m for $\text{S}_2\text{O}_4^{2-}$ on the ratio Fe protein/Mo-Fe protein described by Hageman & Burris (1978b). Fig. 2 shows that $k_{+4} = k_{-3} = 6.4 \pm 0.8\text{ s}^{-1}$ when $[\text{S}_2\text{O}_4^{2-}] = 2\text{ mM}$. Thus 2 mM is an upper limit for the apparent K_m for $\text{S}_2\text{O}_4^{2-}$ at protein concentrations when inhibition due to the back-reaction k_{+3} is negligible (i.e. standard assay conditions). This apparent K_m will decrease as the Fe protein/MoFe protein ratio increases, since at high ratios a lower concentration of $\text{S}_2\text{O}_4^{2-}$ will be required to maintain an optimum steady-state concentration of reduced Fe protein. This is consistent with the decrease in apparent K_m with increasing Kp2 protein/Kp1 protein ratio observed in this laboratory. This explanation is one of the alternatives offered by Hageman & Burris (1978b) for similar effects observed with *Azotobacter vinelandii* nitrogenase.

We have proposed a mechanism for substrate reduction that involves the catalytic cycle of Scheme 2 being repeated eight times for N_2 reduction and concomitant H_2 evolution (Thorneley & Lowe, 1981, 1982; Lowe *et al.*, 1983). This mechanism was formulated in order to explain the pre-steady-state kinetics of H_2 and NH_3 formation as well as those of a dinitrogen hydride intermediate that produces hydrazine on quenching (Thorneley *et al.*, 1978). The mechanism required that the rate-limiting step in Scheme 2, which at that stage had not been identified, has a rate constant with a value close to $5s^{-1}$. We have now independently confirmed that Scheme 2 includes a reaction with a rate constant $k_{-3} = 6.4 \pm 0.8s^{-1}$ and have identified this reaction as the dissociation of $Kp2_{ox} \cdot (MgADP)_2$ from the complex with $Kp1^+$. Now all the rate constants in Scheme 2 are known, it is possible to set up a computer model for nitrogenase that can be tested with both pre-steady-state and steady-state kinetic data for product and intermediate formation during N_2 reduction.

Our results are complementary to and consistent with those of Hageman *et al.* (1980), who considered a cycle similar to that of Scheme 2 but with emphasis on the binding of MgATP and the modulation by MgATP of electron transfer from Av2 to Av1. We have not considered the detailed mechanism of MgATP or MgADP binding, since, under our conditions, these are rapid pre-equilibria compared with the protein dissociation reactions (Thorneley, 1975; Thorneley & Cornish-Bowden, 1977). Hageman *et al.* (1980) did not consider k_{+3} , k_{+5} and k_{-5} (our nomenclature). These rate constants are extremely important for the minimization of H_2 evolution (see below) under our conditions, which also approximate to the calculated concentration of nitrogenase *in vivo* (Eady *et al.*, 1978b; Roberts *et al.*, 1978; Hennecke & Shanmugam, 1979).

It has been suggested that *in vivo* nitrogenase activity is regulated by the $[MgATP]/[MgADP]$ ratio (Upchurch & Mortenson, 1980). Hageman *et al.* (1980) showed that, with dilute enzyme and saturating reductant, the binding constants for MgATP determined by monitoring H_2 evolution rate as a function of MgATP concentration are similar to those determined by measuring the MgATP-dependence of electron transfer from Av2 to Av1. Hence under these conditions, accepting that MgADP is a competitive inhibitor of MgATP (Thorneley & Cornish-Bowden, 1977), modulation of k_{+1} and k_{-1} accounts for MgADP inhibition of substrate reduction. However, at high protein concentrations, k_{+3} , k_{-3} , k_{+4} , k_{+5} and k_{-5} are also significant in the control of activity. The values of these rate constants (Table 1) indicate that the concentration and ratio of component proteins, the

value of k_{+4} (determined by the concentration and nature of reductant) and the $[MgATP]/[MgADP]$ ratio can all be important in determining the degree of inhibition of substrate reduction. A detailed analysis of these effects cannot be discussed without a comprehensive model for substrate reduction. This is beyond the scope of the present paper. However, it is noteworthy that the rate-limiting step (k_{-3}) is a protein dissociation reaction and is apparently not substrate reduction or ATP hydrolysis. We consider that protein dissociation is rate-limiting in order to minimize the evolution of H_2 concomitant with N_2 reduction (Thorneley & Lowe, 1982; Lowe *et al.*, 1983). This is thought to be a consequence of the need to generate a low-potential hydridic metal site at which N_2 will bind by H_2 displacement, but which is also capable of H_2 evolution by protic attack. Thus attempts to 'speed up' nitrogenase *in vivo* by modifying the structures of the proteins such that the value of k_{-3} increases may be at the expense of increasing H_2 evolution relative to N_2 reduction.

We thank Professor J. R. Postgate for comments on the manuscript, Mr. K. Baker and Miss L. Sones for growing *K. pneumoniae* cells, Mrs. G. A. Ashby and Mr. K. Fisher for technical assistance and Dr. J. R. Dilworth for advice during the development of the anaerobic glove-box facility for rapid-reaction kinetic studies.

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