Vanadium nitrogenase of *Azotobacter chroococcum*

MgATP-dependent electron transfer within the protein complex

Roger N. F. THORNELEY,* N. H. Jörgen BERGSTRÖM,† Robert R. EADY and David J. LOWE

A.F.R.C. Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, U.K.

The kinetics of MgATP-induced electron transfer from the Fe protein ($\text{Ac}2^{\text{V}}$) to the VFe protein ($\text{Ac}1^{\text{V}}$) of the vanadium-containing nitrogenase from *Azotobacter chroococcum* were studied by stopped-flow spectrophotometry at 23 °C at pH 7.2. They are very similar to those of the molybdenum nitrogenase of *Klebsiella pneumoniae* [Thorneley (1975) *Biochem. J.* **145**, 391–396]. Extrapolation of the dependence of k_{obs} on $[\text{MgATP}]$ to infinite MgATP concentration gave $k = 46 \text{ s}^{-1}$ for the first-order electron-transfer reaction that occurs with the $\text{Ac}2^{\text{V}}$ MgATP $\text{Ac}1^{\text{V}}$ complex. MgATP binds with an apparent $K_{\text{D}} = 230 \pm 10 \mu\text{M}$ and MgADP acts as a competitive inhibitor with $K_{\text{i}} = 30 \pm 5 \mu\text{M}$. The Fe protein and VFe protein associate with $k \geq 3 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. A comparison of the dependences of k_{obs} for electron transfer on protein concentrations for the vanadium nitrogenase from *A. chroococcum* with those for the molybdenum nitrogenase from *K. pneumoniae* [Lowe & Thorneley (1984) *Biochem. J.* **224**, 895–901] indicates that the proteins of the vanadium nitrogenase system form a weaker electron-transfer complex.

INTRODUCTION

The recently discovered vanadium-containing nitrogenases from *Azotobacter chroococcum* (Robson *et al.*, 1986; Eady *et al.*, 1987, 1988a) and *Azotobacter vinelandii* (Hales *et al.*, 1986a,b) catalyse the reduction of N_2 to NH_3 in a reaction coupled to the hydrolysis of MgATP and the evolution of H_2 . Although a detailed mechanism for the action of the intensively studied molybdenum-containing nitrogenase from *Klebsiella pneumoniae* has been proposed [see Thorneley & Lowe (1985) and Orme-Johnson (1985) for reviews], kinetic data for the purified vanadium nitrogenase have only been obtained under steady-state assay conditions with necessarily limited mechanistic conclusions (Hales *et al.*, 1986a,b; Eady *et al.*, 1987, 1988a; Dilworth *et al.*, 1987, 1988).

The vanadium nitrogenase systems in *A. chroococcum* and *A. vinelandii* are genetically distinct from the molybdenum nitrogenase systems that are normally expressed in these organisms when routinely grown on nitrogen-free medium containing molybdenum (Robson *et al.*, 1986; Bishop *et al.*, 1986; Hales *et al.*, 1986a). The ability of molybdenum to suppress the synthesis of the vanadium nitrogenase largely explains why it has been identified only recently.

The vanadium nitrogenase from *A. chroococcum* comprises two proteins. The VFe protein ($\text{Ac}1^{\text{V}}$), of M_r 210000, contains 2 V atoms, 23 Fe atoms and 20 acid-labile sulphide atoms per molecule (Eady *et al.*, 1987). It has been shown that, unlike the MoFe proteins, which have an $\alpha_2\beta_2$ subunit structure, $\text{Ac}1^{\text{V}}$ has an additional subunit of M_r 14000 and an $\alpha_2\beta_2\delta_2$ subunit structure

(Eady *et al.*, 1988b). The distribution of these metal and sulphide ions between the two types of subunit (M_r 50000 and 55000, analogous to those of MoFe proteins) is not known, although an iron- and vanadium-containing cofactor (FeVaco), analogous to the iron- and molybdenum-containing cofactor (FeMoco) of the MoFe proteins, has been isolated (Smith *et al.*, 1988). The oxidation level and ligand environment of the vanadium in the protein has been shown by V K-edge X-ray-absorption spectroscopy (Arber *et al.*, 1987) to be similar to that of octahedral V(III) in the cluster compound $[\text{Me}_4\text{N}][\text{VFe}_3\text{S}_4\text{Cl}_3(\text{DMF})_3]$ (where DMF represents dimethylformamide) first reported by Kovacs & Holm (1986). The dithionite-reduced protein exhibits an e.p.r. signal with features at $g = 5.60$, 4.35 and 3.77 characteristic of $S = \frac{3}{2}$ spin systems and by analogy with molybdenum nitrogenases is probably associated with FeVaco centres (Eady *et al.*, 1987; Smith *et al.*, 1988). A similar VFe protein has been characterized from the vanadium nitrogenase of *A. vinelandii* (Hales *et al.*, 1986a) and magnetic c.d. spectra are consistent with the presence of FeS clusters similar to the 'P' clusters of MoFe proteins (Morningstar *et al.*, 1987).

The Fe protein ($\text{Ac}2^{\text{V}}$, of M_r 60000) has been characterized and contains 4 Fe atoms with 4 acid-labile sulphide groups per molecule (Robson *et al.*, 1986; Eady *et al.*, 1988a). The dithionite-reduced protein exhibits a rhombic-type e.p.r. signal with $g_{\text{av.}} = 1.93$, which becomes more axial in the presence of MgATP or MgADP and which is similar to that of the Fe protein ($\text{Ac}2^{\text{Mo}}$) of the molybdenum nitrogenase (Yates & Planque, 1975). $\text{Ac}2^{\text{Mo}}$ (MgADP)₂ and $\text{Ac}2^{\text{V}}$ (MgADP)₂ have similar mid-point potentials (−450 mV and

Abbreviations used: we refer to the protein components as $\text{Ac}1^{\text{V}}$ for the vanadium-iron protein and $\text{Ac}2^{\text{V}}$ for the iron protein of the vanadium nitrogenase from *Azotobacter chroococcum*, in order to distinguish these proteins from the molybdenum nitrogenase components, $\text{Ac}1^{\text{Mo}}$ and $\text{Ac}2^{\text{Mo}}$; similarly, the components of the molybdenum nitrogenases of *Klebsiella pneumoniae* and *Azotobacter vinelandii* are designated as $\text{Kp}1^{\text{Mo}}$, $\text{Kp}2^{\text{Mo}}$, $\text{Av}1^{\text{Mo}}$ and $\text{Av}2^{\text{Mo}}$.

* To whom correspondence should be addressed.

† Present address: Department of Biochemistry and Biophysics, University of Göteborg and Chalmers University of Technology, S 41296 Göteborg, Sweden.

−463 mV respectively) and are reduced by $\text{SO}_2^{\cdot-}$ (formed by pre-dissociation of $\text{S}_2\text{O}_4^{2-}$) at similar rates ($k = 4.7 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $3.2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively) (Bergström *et al.*, 1988). A similar Fe protein associated with the vanadium nitrogenase system in *A. vinelandii* has been characterized by Hales *et al.* (1986b).

The vanadium nitrogenase from *A. chroococcum*, like the molybdenum nitrogenases, requires MgATP and a source of low-potential electrons ($\text{Na}_2\text{S}_2\text{O}_4$ *in vitro*) to reduce substrates such as H^+ , N_2 and C_2H_2 . Steady-state assays with AcI^{V} or AcI^{Mo} in combination with Ac2^{V} or Ac2^{Mo} showed that, although the heterologous nitrogenases ($\text{AcI}^{\text{V}}\text{Ac2}^{\text{Mo}}$ and $\text{AcI}^{\text{Mo}}\text{Ac2}^{\text{V}}$) are active, it is the AcI^{V} or AcI^{Mo} that determines substrate specificity. AcI^{V} with either Ac2^{V} or Ac2^{Mo} evolves significantly more H_2 concomitant with C_2H_2 reduction than does AcI^{Mo} with Ac2^{Mo} or Ac2^{V} (Eady *et al.*, 1987). AcI^{V} reduces C_2H_2 to C_2H_4 plus C_2H_6 , whereas AcI^{Mo} only yields C_2H_4 , a reduction that can be used *in vivo* to distinguish between functioning molybdenum nitrogenases and vanadium nitrogenases (Dilworth *et al.*, 1988). Thus a detailed spectroscopic and kinetic investigation into the mechanism of action of vanadium nitrogenase from *A. chroococcum* is of interest, not only because it is representative of a new class of nitrogenases, but also because its reactivity towards reducible substrates is different from that of the better-understood molybdenum nitrogenases. In addition, comparisons between the vanadium nitrogenases and molybdenum nitrogenases are likely to lead to a greater understanding of both systems.

The one-electron oxidation/reduction cycle of the Fe protein of molybdenum nitrogenase from *K. pneumoniae* (Kp2^{Mo}) in which 2 MgATP are hydrolysed to 2 MgADP + 2 P_i involves several partial reactions that have been studied by stopped-flow spectrophotometry (Thorneley, 1975; Thorneley & Cornish-Bowden, 1977; Thorneley & Lowe, 1983; Ashby & Thorneley, 1987) and rapid chemical quench (Eady *et al.*, 1978). The kinetic description of this cycle enabled Lowe & Thorneley (1984a, and references cited therein) to couple together eight of these cycles to yield a comprehensive model for N_2 reduction and H_2 evolution by the molybdenum nitrogenase of *K. pneumoniae*. We have commenced a similar investigation with the vanadium nitrogenase from *A. chroococcum* and in the present paper report the kinetics of association of Ac2^{V} with AcI^{V} and of the subsequent MgATP-dependent electron transfer that occurs within the protein complex. Comparisons have been made with the kinetics of analogous reactions that occur with the molybdenum nitrogenase from *K. pneumoniae*.

MATERIALS AND METHODS

AcI^{V} and Ac2^{V} were purified from *Azotobacter chroococcum* (M.C.D. 1155) and assayed as previously described (Robson *et al.*, 1986; Eady *et al.*, 1987, 1988a). AcI^{V} and Ac2^{V} had specific activities of 1100–1400 and 1300–1700 nmol of H_2 produced/min per mg of protein respectively. AcI^{V} did not have a full complement of vanadium since it contained 1.1 ± 0.1 g-atoms of V and 19 ± 1 g-atoms of Fe/mol of protein. Eady *et al.* (1987) discuss the difficulty of separating AcI^{V} with a full metal complement from inactive AcI^{V} species. Similar problems have been encountered with molybdenum nitrogenases

from many sources, but the correlation of molybdenum content with activity is now established (Thorneley & Lowe, 1985). Ac2^{V} contained 3.7 ± 0.2 g-atoms of Fe/mol of protein.

All reactions were studied in 25 mM-Hepes buffer, pH 7.2 at 23 °C, containing 10 mM-MgCl₂ and 2 mM- $\text{Na}_2\text{S}_2\text{O}_4$ with 0.35 mM- Na_2SO_3 as impurity determined as described by Li *et al.* (1982).

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

Stopped-flow spectrophotometry was performed either with a Hi-Tech Scientific SF-51 instrument (1.0 cm pathlength) or with a modified Aminco apparatus (0.92 cm pathlength). The reservoirs, drive syringes and integral mixer-observation chamber of the Hi-Tech apparatus were kept anoxic by adding buffered $\text{Na}_2\text{S}_2\text{O}_4$ (final concentration approx. 1 mM in 50 mM-Tris/HCl buffer, pH 8.5) to the closed-loop thermostat system. The Aminco apparatus was installed in a high-integrity glove-box through which N_2 gas was circulated and an O_2 concentration less than 1 p.p.m. was maintained (Thorneley & Lowe, 1983).

The stopped-flow data were recorded on a BBC model B microcomputer interfaced to the stopped-flow spectrophotometer, and transferred for analysis to a Digital Equipment Co. PDP1134A computer. Rate constants were obtained by fitting the recorded data to exponential functions by using least-squares minimization as previously described (Thorneley & Lowe, 1983). The fits to Scheme 1 were done in a similar way on VAX750 and MicroVAX II computers.

RESULTS AND DISCUSSION

Kinetic data obtained on mixing pre-equilibrated solutions of Ac2^{V} and AcI^{V} with MgATP and MgADP at various concentrations in the presence of $\text{Na}_2\text{S}_2\text{O}_4$ in the stopped-flow spectrophotometer were analysed in terms of Scheme 1. An analogous scheme and rate expression (eqn. 1) were used by Thorneley (1975) and Thorneley & Cornish-Bowden (1977) with data obtained in similar experiments with the molybdenum nitrogenase from *K. pneumoniae*:

$$\tau = \frac{1}{k_{\text{obs}}} = \frac{K_{\text{D}}^{\text{MgATP}}}{k_{+2}[\text{MgATP}]} \left(1 + \frac{[\text{MgADP}]}{K_{\text{I}}^{\text{MgADP}}} \right) + \frac{1}{k_2} \quad (1)$$

where $K_{\text{D}}^{\text{MgATP}}$ and $K_{\text{I}}^{\text{MgADP}}$ are the apparent dissociation constants of MgATP and MgADP from the protein complexes $\text{Ac2}^{\text{V}}\text{AcI}^{\text{V}}$ and $(\text{Ac2}^{\text{V}})_2\text{AcI}^{\text{V}}$. The above rate expression is based on the following assumptions: (1) rapid pre-equilibrium binding of MgATP and MgADP to the protein(s) and of Ac2^{V} to AcI^{V} ; (2) electron transfer from Ac2^{V} (MgATP)₂ to AcI^{V} (k_{+2}) is irreversible; (3) electron transfer is rapid compared with the subsequent reduction by $\text{Na}_2\text{S}_2\text{O}_4$ of $\text{Ac2}^{\text{V}}(\text{MgADP})_2$ under the conditions used. Assumption (3) is reasonable because the $g_{\text{av.}} = 1.93$ e.p.r. signal of Ac2^{V} is bleached (i.e. oxidized) under steady-state conditions and because with $[\text{Na}_2\text{S}_2\text{O}_4] = 2 \text{ mM}$ $\text{Ac2}^{\text{V}}_{\text{ox.}}(\text{MgADP})_2$ is reduced with $k_{\text{obs.}} = 5 \text{ s}^{-1}$ (Bergström *et al.*, 1988).

The MgATP-dependent electron transfer from Ac2^{V} to AcI^{V} (k_{+2}) was associated with a net increase in absorbance at 420 nm. This absorbance change could be fitted to a single-exponential function with time constants

Scheme 1. MgATP-induced electron transfer from Ac2^V to Ac1^V and inhibition of this reaction by MgADP

(τ) in the range 5–200 ms depending on the MgATP concentration. The amplitude can be used to determine the competence of Ac2^V(MgATP)₂ to effect the reduction of Ac1^V and to estimate the $\Delta\epsilon_{420}$ for the oxidation of Ac2^V(MgATP)₂. Fig. 1 shows amplitude data for a titration of Ac1^V (5 μM) with Ac2^V when [MgATP] = 1 mM and [Na₂S₂O₄] = 2 mM. The inset shows a typical experimental trace with τ = 25 ms. The amplitudes of such traces increased to a maximum absorbance with $\Delta A = 0.036$ (corrected to 1 cm pathlength) at [Ac2^V] = 19 μM ; they remained constant at higher Ac2^V concentrations. The time constant (τ) was independent

Fig. 1. Electron transfer from Ac2^V(MgATP)₂ to Ac1^V at 23 °C at pH 7.2

Stopped-flow amplitude titration to determine the competence of Ac2^V to effect this reaction. The electron-transfer reaction was monitored at 420 nm. The amplitude of the single-exponential absorbance change associated with the oxidation of Ac2^V(MgATP)₂ to yield Ac2^V-(MgADP + P_i)₂ is plotted as a function of [Ac2^V(MgATP)₂] at a fixed [Ac1^V]. Syringe A contained [Ac1^V] = 10 μM and [Ac2^V] = 5–100 μM . Syringe B contained [ATP] = 2 mM. Both syringes contained [Na₂S₂O₄] = 2 mM with [Na₂SO₃] = 0.35 mM as impurity, [Hepes] = 25 mM and [MgCl₂] = 10 μM . The 'break point' occurs at a [Ac2^V]/[Ac1^V] molar ratio = 3.3, consistent with Ac2^V being only 30% active with respect to its competence at Ac1^V reduction. The inset shows a typical stopped-flow trace obtained with [Ac2^V] = 15 μM and [Ac1^V] = 5 μM after mixing. The line is a computer least-squares fit to a single-exponential function with $k_{\text{obs.}} = 44 \text{ s}^{-1}$.

of [Ac2^V], which is consistent with Scheme 1 and eqn. (1). Similar data have been obtained with Kp1^{Mo} and Kp2^{Mo} (Thorneley, 1975; Ashby & Thorneley, 1987). Lowe & Thorneley (1984a) and Ashby & Thorneley (1987) showed that the molybdenum content of a given preparation of Kp1^{Mo} determined the number of active sites on Kp1^{Mo} capable of accepting an electron from Kp2^{Mo}-(MgATP)₂. Assuming that the vanadium content of Ac1^V determines the number of comparable sites (i.e. 5 μM -Ac1^V containing 5.5 μg -atoms of V/l has 5.5 μM sites), then, since up to 19 μM -Ac2^V was rapidly oxidized by Ac1^V (5 μM) (Fig. 1), Ac2^V with a specific activity of 1350 nmol of H₂ produced/min per mg of Ac2^V is only 30% active with respect to electron transfer to Ac1^V. This leads us to predict a specific activity of 4500 nmol of H₂ produced/min per mg of Ac2^V for fully active Ac2^V. This value is very close to 4000 ± 800 nmol of H₂ produced/min per mg of Kp2^{Mo} predicted by Thorneley & Lowe (1984) for *K. pneumoniae* molybdenum nitrogenase. Thus dissociation of oxidized Fe protein with MgADP bound may be rate-determining for both the molybdenum and the vanadium enzymes. The high proportion of Fe protein that is incapable of electron transfer to both the VFe protein and MoFe protein is not thought to be due to inactivation during isolation, but most likely reflects a role for inactive Fe protein in optimizing N₂ reduction over H₂ evolution, as proposed for inactive Kp2^{Mo} by Ashby & Thorneley (1987).

The maximum amplitude in Fig. 1 of 0.036 *A* unit and the active-site concentration, determined by the vanadium content of Ac1^V (5.5 μM), gives $\Delta\epsilon_{420} = 6.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the oxidation of Ac2^V(MgATP)₂ by Ac1^V. This value is similar to that determined by Ashby & Thorneley (1987) with *K. pneumoniae* molybdenum nitrogenase components ($\Delta\epsilon_{430} = 5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) and includes the small change in the absorbance of Ac1^V that presumably occurs on reduction.

Rate of electron transfer within the complex Ac2^V(MgATP)₂ Ac1^V (k_{+2}) and the binding of MgATP (K_D^{MgATP})

The observed first-order rate constant ($k_{\text{obs.}} = 35 \text{ s}^{-1}$) for the oxidation of Ac2^V was independent of protein concentration in the range [Ac2^V] = 3.5–10 μM with an [Ac2^V]/[Ac1^V] = 3:1 molar ratio with [MgATP] = 1 mM. These data were obtained from experiments in which Ac2^V, Ac1^V, MgCl₂ and Na₂S₂O₄ were pre-equilibrated in one syringe of the stopped-flow apparatus, and the other syringe contained MgATP, MgCl₂ and Na₂S₂O₄.

Fig. 2. Dependence of $k_{\text{obs}}^{-1}(\tau)$ on $[\text{MgATP}]^{-1}$ for the oxidation of $\text{Ac}_2\text{V}(\text{MgATP})_2$ by Ac_1V at 23 °C at pH 7.2

The data were obtained from stopped-flow traces similar to those shown in the inset to Fig. 1. Syringe A contained $[\text{Ac}_2\text{V}] = 30$ or $40 \mu\text{M}$ and $[\text{Ac}_1\text{V}] = 10 \mu\text{M}$. Syringe B contained $[\text{MgATP}] = 0.13\text{--}18 \text{ mM}$. Both syringes contained $[\text{MgCl}_2] = 10 \text{ mM}$, $[\text{Hepes}] = 25 \text{ mM}$ and $[\text{Na}_2\text{S}_2\text{O}_4] = 2 \text{ mM}$ with $[\text{Na}_2\text{SO}_3] = 0.35 \text{ mM}$ impurity. k_{obs} was determined by fitting the absorbance changes at 420 nm to a single-exponential function as described in the Materials and methods section. The line is a least-squares best fit with an ordinate intercept giving $k_{+2} = 46 \pm 2 \text{ s}^{-1}$ and an abscissa intercept giving $K_D^{\text{MgATP}} = 230 \pm 10 \mu\text{M}$.

Pre-equilibration of Ac_2V with MgATP , when the other syringe contained Ac_1V , did not change k_{obs} . This justifies the assumption that at these protein and MgATP concentrations the formation of the complex $\text{Ac}_2\text{V}(\text{MgATP})_2\text{Ac}_1\text{V}$ is rapid compared with the electron-transfer reaction (k_2). At lower protein concentrations this is not the case (see below).

However, pre-equilibration of Ac_1V with MgATP , when the other syringe contained Ac_2V (both syringes contain MgCl_2 and $\text{Na}_2\text{S}_2\text{O}_4$), caused k_{obs} to decrease significantly. The extent of this decrease depended on the time of pre-equilibration (2 to 25 min), during which time significant amounts of P_i and MgADP accumulated. The detection of H_2 from samples of Ac_1V preincubated with MgATP and $\text{Na}_2\text{S}_2\text{O}_4$ showed that contamination by Ac_2V was responsible for the ATPase activity of the Ac_1V . An $\text{ATP}/2e$ ratio of 5.6:1 was calculated from these data, which compares with a previous value of 5.5:1 (Eady *et al.*, 1987) and with 4.3:1 for molybdenum nitrogenase from *K. pneumoniae* (Ashby *et al.*, 1987). These values suggest that Ac_2V , like $\text{Kp}2^{\text{Mo}}$, binds 2 mol of MgATP/mol , both of which are hydrolysed when one electron is transferred to Ac_1 (Eady *et al.*, 1978; Ashby & Thorneley, 1987). The data in Fig. 2 show a linear dependence of $k_{\text{obs}}^{-1}(\tau)$ on $[\text{MgATP}]^{-1}$, consistent with eqn. (1). The intercepts give $k_{+2} = 46 \pm 2 \text{ s}^{-1}$ and $K_D^{\text{MgATP}} = 0.23 \pm 0.01 \text{ mM}$. If two sites bind MgATP , then they must be non-co-operative with similar affinities.

Some deviation from linearity occurred at the highest MgATP concentration (9 mM), indicating 30 % inhibition of the electron-transfer rate. Increasing the $[\text{Mg}^{2+}]_{\text{total}}$ from 10 mM to 15 mM did not relieve this inhibition.

The rate of electron transfer ($k_{+2} = 46 \pm 2 \text{ s}^{-1}$) within the vanadium nitrogenase complex is significantly lower than that for the corresponding reaction of the molybdenum nitrogenases from *A. chroococcum* ($k_{+2} = 220 \text{ s}^{-1}$; Thorneley *et al.*, 1976) and *K. pneumoniae* ($k_{+2} = 200 \text{ s}^{-1}$; Thorneley, 1975). However, even with an electron-transfer rate of $k_{+2} = 46 \text{ s}^{-1}$ this reaction is still about 10 times faster than the rate-determining step in the catalytic cycle for substrate reduction. The affinity for MgATP of either Ac_2V or the complex $\text{Ac}_2\text{VAc}_1\text{V}$ (the kinetics cannot distinguish between these alternatives), $K_D^{\text{MgATP}} = 230 \pm 10 \mu\text{M}$, is higher than that of the molybdenum nitrogenases from *A. chroococcum* ($K_D^{\text{MgATP}} = 1 \text{ mM}$; Thorneley *et al.*, 1976) and *K. pneumoniae* ($K_D^{\text{MgATP}} = 400 \mu\text{M}$; Thorneley, 1975).

Inhibition of electron transfer by MgADP (K_i^{MgADP})

The observed first-order rate constant for the oxidation of Ac_2V (k_{obs}) decreased as the concentration of MgADP was increased (over the range 0.1–2.0 mM) at a fixed concentration of MgATP in the range 0.2–2 mM. The data are shown in Fig. 3 as plots of $k_{\text{obs}}^{-1}(\tau)$ against $[\text{MgADP}]$ at a series of MgATP concentrations (analogous to Dixon plots for steady-state kinetic data). At $[\text{MgATP}] = 2.0 \text{ mM}$ and 1.0 mM , linear plots over the whole range of MgADP concentrations confirm the appropriateness of Scheme 1 and eqn. (1) with MgADP as a competitive inhibitor ($K_i^{\text{MgADP}} = 30 \pm 5 \mu\text{M}$) of MgATP binding ($K_D^{\text{MgATP}} = 230 \pm 10 \mu\text{M}$). The ordinate value

Fig. 3. Dependence of $k_{\text{obs}}^{-1}(\tau)$ for the electron-transfer reaction from $\text{Ac}_2\text{V}(\text{MgATP})_2$ to Ac_1V on MgADP concentration at various MgATP concentrations at 23 °C at pH 7.2

The data were obtained from stopped-flow traces similar to those shown in the inset to Fig. 1. Syringe A contained $[\text{Ac}_2\text{V}] = 30$ or $40 \mu\text{M}$ and $[\text{Ac}_1\text{V}] = 10 \mu\text{M}$. Syringe B contained various concentrations of MgATP and MgADP . Both syringes contained $[\text{MgCl}_2] = 10 \text{ mM}$, $[\text{Hepes}] = 25 \text{ mM}$ and $[\text{Na}_2\text{S}_2\text{O}_4] = 2 \text{ mM}$ with $[\text{Na}_2\text{SO}_3] = 0.35 \text{ mM}$ as impurity. k_{obs} was determined by fitting the absorbance changes at 420 nm to a single-exponential function. The lines intersect at a point corresponding to $K_i^{\text{MgADP}} = 30 \mu\text{M}$. The concentrations of MgATP after mixing were: $\circ$, 0.2 mM; $\blacksquare$, 0.5 mM; $\bullet$, 1.0 mM; $\square$, 2.0 mM. $[\text{MgADP}]$ was varied from 0 to 2.0 mM (fixed concentration).

Fig. 4. Stopped-flow trace showing the partial oxidation of $\text{Ac}2^{\text{V}}$ by SO_3^{2-} ion induced by MgADP binding in the presence of $\text{S}_2\text{O}_4^{2-}$ ion at 23°C at pH 7.2

The reaction was monitored at 420 nm. Syringe A contained $[\text{Ac}2^{\text{V}}] = 50 \mu\text{M}$. Syringe B contained $[\text{MgADP}] = 4 \text{ mM}$. Both syringes contained $[\text{Na}_2\text{S}_2\text{O}_4] = 2 \text{ mM}$ with $[\text{Na}_2\text{SO}_3] = 0.35 \text{ mM}$ as impurity. The curve is a single exponential with $k_{\text{obs.}} = 9 \text{ s}^{-1}$ and an amplitude of 0.0048 A unit.

for the point of intersection in Fig. 3 ($\tau = 17 \pm 3 \text{ ms}$) is close to the value of τ at infinite $[\text{MgATP}]$ ($21 \pm 1 \text{ ms}$) obtained by extrapolation of the data points in Fig. 2. We do not feel justified in considering a mixed inhibition mechanism that requires these values of τ to differ significantly. However, with $[\text{MgATP}] = 0.5$ or 0.2 mM , deviations from linearity occur at high concentrations of MgADP, with τ values significantly smaller than those predicted by eqn. (1) using the values of $K_{\text{D}}^{\text{MgATP}}$ and $K_{\text{I}}^{\text{MgADP}}$ given above. We attribute this deviation in part to a competing reaction in which $\text{Ac}2^{\text{V}}\text{-(MgADP)}_2$ is partially oxidized by SO_3^{2-} ion, which was present as an impurity (approx. 15%) in the $\text{Na}_2\text{S}_2\text{O}_4$.

The reduction potential of a $2.0 \text{ mM-Na}_2\text{S}_2\text{O}_4$ solution containing $0.35 \text{ mM-Na}_2\text{SO}_3$ at pH 7.2 is -530 mV (Mayhew, 1978) and that of $\text{Ac}2^{\text{V}}\text{-(MgADP)}_2$ is -463 mV (Bergström *et al.*, 1988). Sulphite oxidation of $\text{Ac}2^{\text{V}}$, induced by MgADP binding, was observed in a stopped-flow experiment in which $\text{Ac}2^{\text{V}}$ ($50 \mu\text{M}$) plus $\text{Na}_2\text{S}_2\text{O}_4$ (2.0 mM) and Na_2SO_3 (0.35 mM) was mixed with MgADP (2 mM) plus $\text{Na}_2\text{S}_2\text{O}_4$ (2.0 mM) and Na_2SO_3 (0.35 mM). An increase in absorbance at 420 nm occurred with $\tau = 110 \text{ ms}$ (Fig. 4). This amplitude indicated that approx. 10% of the $\text{Ac}2^{\text{V}}\text{-(MgADP)}_2$ formed on mixing was oxidized. This degree of oxidation is consistent with the mid-point potential given above. In a similar experiment in which MgADP was replaced by MgATP no absorbance change was detected, indicating mid-point potential $> -470 \text{ mV}$ for the couple $\text{Ac}2^{\text{V}}\text{-(MgATP)}_2/\text{Ac}2^{\text{V}}\text{-(MgATP)}_2$. Watt (1985) has reported the mid-point potentials of -310 , -430 and -480 mV for $\text{Av}2^{\text{Mo}}$, $\text{Av}2^{\text{Mo}}\text{-(MgATP)}_2$ and $\text{Av}2^{\text{Mo}}\text{-(MgADP)}_2$, and similar values for the corresponding forms of $\text{Ac}2^{\text{V}}$ would be consistent with the observations in the present paper. The low mid-point potentials for the MgADP-bound forms of $\text{Av}2^{\text{Mo}}$ and $\text{Ac}2^{\text{V}}$ are likely to complicate that interpretation of data from all experiments involving these protein forms when commercially available $\text{Na}_2\text{S}_2\text{O}_4$ is used as a reductant, particularly if the $\text{Na}_2\text{S}_2\text{O}_4$ is further oxidized during sample preparation. The problem cannot be overcome by increasing the concentration of $\text{Na}_2\text{S}_2\text{O}_4$, since the mid-point potential of the $\frac{1}{2}\text{S}_2\text{O}_4^{2-}/\text{SO}_3^{2-}$ couple increases by 29 mV for each factor of 10 in concentration as a result of the predissociation of $\text{S}_2\text{O}_4^{2-}$ to yield the active reductant $\text{SO}_2^{\cdot-}$ (Mayhew, 1978). The problem does not arise with $\text{Kp}2^{\text{Mo}}\text{-(MgADP)}_2$, which has a mid-point potential of -350 mV (Thorneley & Deistung, 1988). Deviation from linearity in Fig. 3 may also be a consequence of assumptions (1) and (3) made above with respect to eqn. (1) and Scheme 1 not being valid at high MgADP concentration with the two lowest MgATP concentrations used.

Fig. 5. Dependence of $k_{\text{obs.}}$ for electron transfer from $\text{Ac}2^{\text{V}}\text{-(MgATP)}_2$ to $\text{Ac}1^{\text{V}}$ on protein concentration: determination of the rate of protein association (k_{+1})

(a) $[\text{Ac}1^{\text{V}}] = [\text{Ac}2^{\text{V}}] = 11.0 \mu\text{M}$; (b) $[\text{Ac}1^{\text{V}}] = [\text{Ac}2^{\text{V}}] = 2.2 \mu\text{M}$. The continuous lines are a computed best fit to Scheme 1 allowing the baseline, k_{+1} , and the absorption coefficient to vary. The broken lines are single-exponential fits with rate constants of (a) 42.8 s^{-1} and (b) 30.0 s^{-1} .

Rate of formation of the protein complex Ac^{2V}(MgATP)₂Ac^{1V} (k_{+1})

The observed first-order rate constant for electron transfer ($k_{\text{obs.}}$) decreased at concentrations of $[\text{Ac}^{2V}] = [\text{Ac}^{1V}] < 3 \mu\text{M}$ (Fig. 5). This is consistent with the protein association reaction (k_{+1} , Scheme 1) limiting the rate of electron transfer at low protein concentrations, i.e. $k_{+1}[\text{Ac}^{2V}(\text{MgATP})_2][\text{Ac}^{1V}] \leq (k_{-1} + k_{+2})[\text{Ac}^{2V}(\text{MgATP})_2\text{Ac}^{1V}]$. The continuous lines through the data are computed best fits obtained by simulation using $k_{+1} = 3.0 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{+2} = 46 \text{ s}^{-1}$, $K_D^{\text{MgATP}} = 230 \mu\text{M}$ and $[\text{MgATP}] = 2.0 \text{ mM}$. The broken lines show the best-fit single exponentials through the data. The closeness of these different fits justifies the use of single exponentials in the analysis described in the above sections. The value of k_{-1} does not significantly affect the form of the fits to Scheme 1 when it is less than 20 s^{-1} . If it is greater than this, similar fits can be obtained by increasing k_{+1} ; this analysis is similar to that described by Lowe & Thorneley (1984) for the Kp1^{Mo}Kp2^{Mo} system. The value of $k_{+1} \geq 3.0 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ compares with $k_{+1} \geq 5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ determined by Lowe & Thorneley (1984b) for the association of Kp2(MgATP)₂ with Kp1^{Mo}.

CONCLUSIONS

The pre-steady-state kinetics of the vanadium-containing nitrogenase from *A. chroococcum* reported here show that the first two steps in the catalytic cycle, the association of the component proteins and the MgATP-dependent electron transfer from Ac^{2V} to Ac^{1V}, occur at very similar rates to those previously reported for the molybdenum-containing nitrogenase from *K. pneumoniae* (Thorneley, 1975; Lowe & Thorneley, 1984a). These results suggest a similar overall mechanism for N₂ reduction by the two systems, which is not perhaps surprising considering their structural similarities. In the case of molybdenum nitrogenase, the reduction of N₂ to NH₃ and concomitant evolution of H₂ involves eight successive one-electron transfers from Fe protein to MoFe protein. Each Fe protein cycle is associated with the hydrolysis of 2 MgATP to 2 MgADP + 2 P_i. The differences in substrate specificity (Dilworth *et al.*, 1987, 1988), and in particular the high proportion of electron flux directed into H₂ evolution concomitant with N₂ reduction (Eady *et al.*, 1987), are presumably a consequence of the different reactivities of FeVaco and FeMoco centres in these enzymes.

We thank Mrs M. Eldridge for skilful technical assistance and Ms. Beryl Scutt for typing the manuscript. N.H.J.B. thanks the Swedish Natural Science Council (N.F.R.) for the post-doctoral fellowship awarded to him.

REFERENCES

- Arber, J. M., Dobson, B. R., Eady, R. R., Stevens, P., Hasnain, S. S., Garner, C. D. & Smith, B. E. (1987) *Nature* (London) **325**, 372–374
- Ashby, G. A. & Thorneley, R. N. F. (1987) *Biochem. J.* **246**, 455–465
- Ashby, G. A., Dilworth, M. J. & Thorneley, R. N. F. (1987) *Biochem. J.* **247**, 547–554
- Bergström, J., Eady, R. R. & Thorneley, R. N. F. (1988) *Biochem. J.* **251**, 165–169
- Bishop, P. E., Premakumar, R., Dean, D. R., Chisnell, J. R., Rizzo, T. M. & Kopczynski, J. (1986) *Science* **232**, 69–75
- Dilworth, M. J., Eady, R. R., Robson, R. L. & Miller, R. W. (1987) *Nature* (London) **327**, 167–168
- Dilworth, M. J., Eady, R. R. & Eldridge, M. E. (1988) *Biochem. J.* **249**, 745–751
- Eady, R. R., Lowe, D. J. & Thorneley, R. N. F. (1978) *FEBS Lett.* **95**, 211–213
- Eady, R. R., Robson, R. L., Richardson, T. H., Miller, R. W. & Hawkins, M. (1987) *Biochem. J.* **244**, 197–207
- Eady, R. R., Richardson, T. H., Miller, R. W., Hawkins, M. & Lowe, D. J. (1988a) *Biochem. J.* **256**, 189–196
- Eady, R. R., Robson, R. L., Pau, R. N., Woodley, P., Lowe, D. J., Miller, R. W., Thorneley, R. N. F., Smith, B. E., Gormal, C., Fisher, K., Eldridge, M. & Bergström, J. (1988b) *Proc. Int. Symp. Nitrogen Fixation 7th*, Cologne, 81–86
- Hales, J. B., Case, E. E., Morningstar, J. E., Dzeda, M. F. & Mauterer, L. A. (1986a) *Biochemistry* **25**, 7251–7255
- Hales, J. B., Langosch, D. J. & Case, E. E. (1986b) *J. Biol. Chem.* **261**, 15301–15306
- Kovacs, J. A. & Holm, R. H. (1986) *J. Am. Chem. Soc.* **108**, 340–341
- Li, J.-G., Burgess, B. K. & Corbin, J. L. (1982) *Biochemistry* **21**, 4343–4402
- 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
- Mayhew, S. G. (1978) *Eur. J. Biochem.* **85**, 535–547
- Morningstar, J. E., Johnson, M. K., Case, E. E. & Hales, B. J. (1987) *Biochemistry* **26**, 1795–1800
- Orme-Johnson, W. H. (1985) *Annu. Rev. Biophys. Chem.* **14**, 419–459
- Robson, R. L., Eady, R. R., Richardson, T. H., Miller, R. W., Hawkins, M. & Postgate, J. R. (1986) *Nature* (London) **322**, 388–390
- Smith, B. E., Eady, R. R., Lowe, D. J. & Gormal, C. (1988) *Biochem. J.* **250**, 299–302
- Thorneley, R. N. F. (1975) *Biochem. J.* **145**, 391–396
- Thorneley, R. N. F. & Cornish-Bowden, A. (1977) *Biochem. J.* **165**, 255–262
- Thorneley, R. N. F. & Deistung, J. (1988) *Biochem. J.* **253**, 587–595
- Thorneley, R. N. F. & Lowe, D. J. (1983) *Biochem. J.* **215**, 393–403
- Thorneley, R. N. F. & Lowe, D. J. (1984) *Biochem. J.* **224**, 903–909
- Thorneley, R. N. F. & Lowe, D. J. (1985) *Met. Ions Biol.* **7**, 221–284
- Thorneley, R. N. F., Yates, M. G. & Lowe, D. J. (1976) *Biochem. J.* **155**, 137–144
- Watt, G. D. (1985) in *Nitrogen Fixation Research Progress* (Evans, H. J., Bottomley, P. J. & Newton, W. E., eds.), pp. 585–590, Martinus Nijhoff, Dordrecht and Boston
- Yates, M. G. & Planque, K. (1975) *Eur. J. Biochem.* **60**, 467–476