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A transient-kinetic study of the nitrogenase of *Klebsiella pneumoniae* by stopped-flow calorimetry

Comparison with the myosin ATPase

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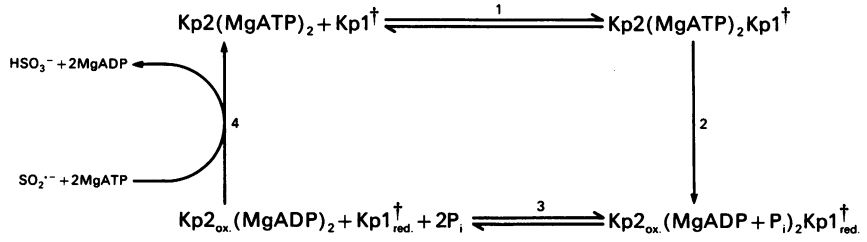
The pre-steady-state kinetics of MgATP hydrolysis by nitrogenase from *Klebsiella pneumoniae* were studied by stopped-flow calorimetry at 6 °C and at pH 7.0. An endothermic reaction ($\Delta H_{obs.} = +36$ kJ·mol of ATP^{-1}; $k_{obs.} = 9.4$ s^{-1}) in which 0.5 proton·mol of ATP^{-1} was released, has been assigned to the on-enzyme cleavage of MgATP to yield bound MgADP+P_i. The assignment is based on the similarity of these parameters to those of the corresponding reaction that occurs with rabbit muscle myosin subfragment-1 ($\Delta H_{obs.} = +32$ kJ·mol of ATP^{-1}; $k_{obs.} = 7.1$ s^{-1}; 0.2 proton released·mol of ATP^{-1}) [Millar, Howarth & Gutfreund (1987) Biochem. J. **248**, 683–690]. MgATP-dependent electron transfer from the nitrogenase Fe-protein to the MoFe-protein was monitored by stopped-flow spectrophotometry at 430 nm and occurred with a $k_{obs.}$ value of 3.0 s^{-1} at 6 °C. Thus, under these conditions, hydrolysis of MgATP precedes electron transfer within the protein complex. Evidence is presented that suggests that MgATP cleavage and subsequent electron transfer are reversible at 6 °C with an overall equilibrium constant close to unity, but that, at 23 °C, the reactions are essentially irreversible, with an overall equilibrium constant $\geqslant 10$.

INTRODUCTION

The nitrogenase of *Klebsiella pneumoniae* comprises two metalloproteins, the MoFe protein (Kp1; M_r 220000) and the Fe protein (Kp2; M_r 67000). In the presence of MgATP and a reductant (flavodoxin *in vivo*, sodium dithionite *in vitro*), nitrogenase catalyses the reduction of N_2 to $2NH_3$. Under optimal conditions, the reduction of N_2 is accompanied by the stoichiometric reduction of $2H^+$ to H_2 and the hydrolysis of 16MgATP to 16MgADP+16P_i [see Burgess (1985), Lowe *et al.* (1985), Orme-Johnson (1985), Thorneley & Lowe (1985) and Smith *et al.* (1987) for reviews on the structure and mechanism of nitrogenase].

Thorneley & Lowe (1983) determined the rate constants that are sufficient to describe a single electron-transfer cycle in which two equivalents of ATP are hydrolysed (Scheme 1). The coupling of eight of these cycles formed the basis for their comprehensive mechanism of nitrogenase action (Lowe & Thorneley, 1984*a*,*b*; Thorneley & Lowe, 1984*a*,*b*). Ashby & Thorneley (1987) showed that, in step 4 of Scheme 1, reduction of $Kp2_{ox.}$ $(MgADP)_2$ preceded replacement of 2MgADP by 2MgATP. Eady *et al.* (1978) and Hageman *et al.* (1980) used the rapid acid-quench technique to show that, in step 2, the transfer of one electron from Fe protein to MoFe protein is coupled to the hydrolysis of two equivalents of MgATP, a stoichiometry confirmed by comparison of the steady-state rates of substrate reduction and P_i formation under optimum conditions [Burgess (1985) and references cited therein]. However, electron transfer is uncoupled from MgATP hydrolysis at pH < 6.4 or > 8.5 (Jeng *et al.*, 1970; Imam & Eady, 1980), at temperatures < 20 °C or > 30 °C (Watt *et al.*, 1975) and at low electron flux (i.e. [Fe protein] < [MoFe protein]) (Eady & Postgate, 1974; Hageman & Burris, 1978).

MgATP hydrolysis is a property of the Fe-protein–



Scheme 1. Nitrogenase electron-transfer and MgATP-hydrolysis cycle

Kp1† represents one of two independently functioning halves of the tetrameric ($\alpha_2\beta_2$) structure of the MoFe protein. Each Kp1† is assumed to contain one Mo substrate-binding site and one Fe protein (Kp2)-binding site. The electron-transfer reaction from $Kp2(MgATP)_2$ to Kp1† is shown in step 2 coupled to the hydrolysis of 2MgATP. The results described in the present paper show that, at 6 °C, MgATP hydrolysis is uncoupled from, and precedes electron transfer within, the protein complex.

Abbreviations used: Kp1 and Kp2, the MoFe- and Fe-proteins respectively of *Klebsiella pneumoniae* nitrogenase; ATP[S], adenosine 5′-[γ-thio]triphosphate.

MoFe-protein complex, since neither protein on its own hydrolyses MgATP (Imam & Eady, 1980). The hydrolysis of MgATP in the absence of $Na_2S_2O_4$, when the Fe protein is in the oxidized state, showed that electron transfer from Fe protein to MoFe protein is not required for this reaction to occur (Jeng *et al.*, 1970; Imam & Eady, 1980; Cordewener *et al.*, 1987). This 'reductant-independent' ATPase activity can explain why the limiting stoichiometry of 2MgATP hydrolysed for each electron transferred to substrate is not maintained under all conditions.

Little is known about the detailed mechanism of MgATP hydrolysis and associated energy-transduction processes with nitrogenase [see Mortenson (1987) for a review of available data]. However, Mortenson *et al.* (1985) have used ^{18}O-labelled adenosine 5'-[γ-thio]triphosphate (ATP[S]) to show that the hydrolysis of this ATP analogue proceeded with inversion of the terminal phosphate.

In the present paper we have investigated the mechanism of MgATP hydrolysis by nitrogenase using stopped-flow calorimetry. This technique has previously been used to study the enthalpy changes associated with the hydrolysis of MgATP by myosin subfragment 1 (Millar *et al.*, 1987). At 5 °C the enthalpy changes of the individual steps of the myosin ATPase could be resolved and, of particular interest in the present context, the on-enzyme ATP-cleavage step was found to be endothermic ($\Delta H = +64$ kJ·mol^{-1}). The release and uptake of protons during the myosin ATPase reaction were also investigated by carrying out the reaction in two buffer systems with different heats of ionization. This application of the stopped-flow calorimeter is of particular importance with nitrogenase, since protons are involved in both ATPase and substrate-reduction reactions. Conventional methods of studying proton release in the presence of sodium dithionite are hampered by the bleaching of pH indicators at low potentials. Calorimetry also has the ability to reveal reaction steps that cannot be monitored by other techniques.

Although nitrogenase reactions have previously been investigated by steady-state calorimetry (Watt *et al.*, 1975), this paper reports the first calorimetric transient-kinetic study of this enzyme. We have used the experimental conditions of Millar *et al.* (1987) at 5 °C in order to bring the time constant for step 2 (Scheme 1) into the experimentally accessible range ($\tau >$ about 50 ms), and also to allow a direct comparison of the myosin and nitrogenase ATPase reactions.

MATERIALS AND METHODS

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 1750 and 1500 nmol of ethylene produced·min^{-1}·mg of protein^{-1} respectively. Kp1 contained 1.4±0.1 g-atom of Mo·mol^{-1}. The purified nitrogenase component proteins did not contain bound adenine nucleotides, as judged by the fluorimetric method of Yuki *et al.* (1972). All reactions were carried out at 5.5±0.5 °C in 50 mM-imidazole/HCl or 50 mM-cacodylate/HCl buffer, pH 7.0, containing 10 mM-$MgCl_2$, 5 mM-ATP and 10 mM-$Na_2S_2O_4$. All biochemicals were purchased from Sigma, Poole, Dorset, U.K., and salts from BDH, Poole, Dorset, U.K.

Stopped-flow spectrophotometry was performed with a Hi-tech SF-51 apparatus (Salisbury, Wilts, U.K.) and stopped-flow calorimetry using the apparatus essentially as described in Howarth *et al.* (1987). Enthalpy changes were calculated by using the relationship:

$$\Delta H = \Delta T \cdot C_p / C_1$$

where ΔT is the observed temperature change (mK), C_p is the heat capacity of the solution (4.2 kJ·l^{-1}·K^{-1}), C_1 is the concentration of the reactant limiting the extent of the reaction (mM) and ΔH is in kJ·mol^{-1}. The enthalpies of ionization of the buffers used were 36 kJ·mol^{-1} for imidazole and zero for cacodylate. Stopped-flow data were digitized and stored in a microcomputer for averaging and analysis using standard non-linear fitting procedures.

RESULTS AND DISCUSSION

Fig. 1 shows the calorimetric records after the mixing of nitrogenase with MgATP in two different buffers. After a rapid rise in temperature, there is an endothermic phase, followed by the onset of heating associated with

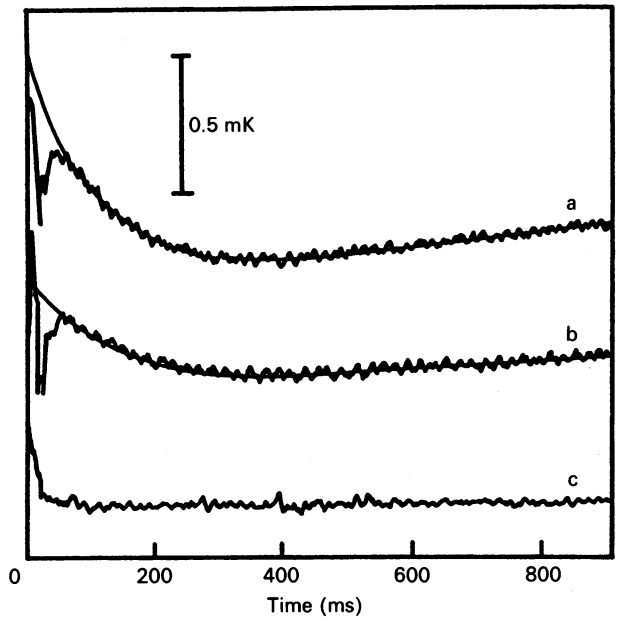


Fig. 1. Stopped-flow calorimetry of MgATP cleavage on nitrogenase

The traces were obtained when pre-equilibrated nitrogenase component proteins from *Klebsiella pneumoniae* [Kp1,76 μM (110 μM-Mo); Kp2, 250 μM] were mixed with MgATP (10 mM) or MgADP (10 mM) at 6 °C, pH 7.0. The small temperature change ($\sim 5 \times 10^{-4}$ °C) associated with on-enzyme, pre-steady-state, endothermic cleavage of MgATP to yield MgADP+P_i is shown as a function of time. The larger amplitude for trace a obtained in 50 mM-cacodylate buffer (ΔH^0 for buffer ionization ~ 0) compared with that for trace b in 50 mM-imidazole buffer (ΔH^0 for buffer ionization $= -36$ kJ·mol^{-1}) shows that MgATP cleavage is associated with the release of 0.5 proton. The curves drawn through the data of traces a and b yield values of $k = 9.4$ s^{-1} and 8.2 s^{-1} respectively associated with MgATP cleavage. The temperature rise after about 0.5 s is associated with the approach to the steady state. Trace c is a control in which MgADP replaced MgATP.

the steady state. The first 40 ms of observation are distorted by an artefact caused by pressure relaxation when the flow stops (Howarth *et al.*, 1987). However the records after 45 ms are fitted well by a single exponential cooling followed by a linear heating:

$$A = A_0 + A_1 \exp(-k_1 t) + A_2 k_2 t$$

where A is the signal amplitude, A_0 the baseline, A_1 the exponential amplitude and A_2 the steady-state slope. This gives rate constants for the endothermic phase of 9.4 s^{-1} in cacodylate buffer and 8.2 s^{-1} in imidazole. The observed enthalpy change (ΔH) for the endothermic process can be calculated from the observed amplitude after extrapolation back to zero time ($\Delta T = 0.96$ mK) in cacodylate and 0.49 mK in imidazole), and by assuming that the concentration of sites hydrolysing MgATP is 110 μM (i.e. two MgATP sites per Mo atom; see the legends to Scheme 1 and Fig. 1). In cacodylate this gives $\Delta H = +36$ kJ·mol^{-1}, and in imidazole $\Delta H = +18$kJ·mol^{-1}. The ΔH value in cacodylate represents the actual enthalpy change for this process, since the heat due to ionization of the buffer is negligible, whereas the difference between this value and the value in imidazole (18 kJ·mol^{-1}) represents the enthalpy change due to proton release (Howarth *et al.*, 1987). From the known enthalpy of ionization of imidazole (36 kJ·mol^{-1}) the stoichiometry of proton release is calculated as 0.5 mol of H^+ per mol of active site (i.e. per mol of ATP bound).

These calorimetric results can be compared with those previously obtained for the myosin ATPase under identical conditions (Millar *et al.*, 1987). After the mixing of myosin subfragment-1 with MgATP, an endothermic phase is seen with a rate of 7.1 s^{-1}, an observed enthalpy change of 32 kJ·mol^{-1}, and a release of 0.2 mol of H^+ per mol of MgATP bound. The endothermic reaction resulting in a large temperature-dependence for the rate constant points to the similarity of the ATP-cleavage step of nitrogenase and rabbit muscle myosin. In the case of myosin this endothermic process was correlated with the on-enzyme ATP-cleavage step, since the rate was similar to the rate of the 'phosphate burst' measured by rapid quenching (Sleep & Taylor, 1976). Since MgADP is a competitive inhibitor of MgATP-dependent nitrogenase activity (Thorneley & Cornish-Bowden, 1976), the failure of MgADP to induce any measureable enthalpy changes with nitrogenase (Fig. 1, trace c) supports the conclusion that the endothermic phase is associated with the MgATP cleavage step.

At 10 °C pH 7.4, Eady *et al.* (1978) found that the rate of a 'phosphate burst' with nitrogenase was the same as the rate of oxidation of Kp2 as measured by the absorbance change at 420 nm, and so they concluded that electron transfer from Kp2 to Kp1 was coupled to the ATP-cleavage step (as indicated in Scheme 1). Fig. 2(*a*) shows a record of the absorbance change at 430 nm under the conditions used for the calorimetric experiments. The rate of the oxidation of Kp2 is only 3.0 s^{-1} and is significantly lower than the endothermic process observed by calorimetry. A 20-fold dilution of the protein did not significantly change the rate of oxidation of Kp2 ($k = 2.6$ s^{-1} with [Kp2] = 6.5 μM, [Kp1] = 2 μM after mixing) indicating rapid, tight binding in step 1 of Scheme 1). These results contrast with the observation at 10 °C (Eady *et al.*, 1978) and indicate that ATP cleavage precedes electron transfer under these conditions at 6 °C.

The results of experiments on myosin ATPase (Millar *et al.*, 1987), demonstrating the endothermic character of the ATP-cleavage step, are used as a diagnostic to identify the same step on the nitrogenase pathway. The myosin ATP-cleavage step has an equilibrium constant (K_1) of 1 at 5 °C, so only 50% of the ATP is cleaved during

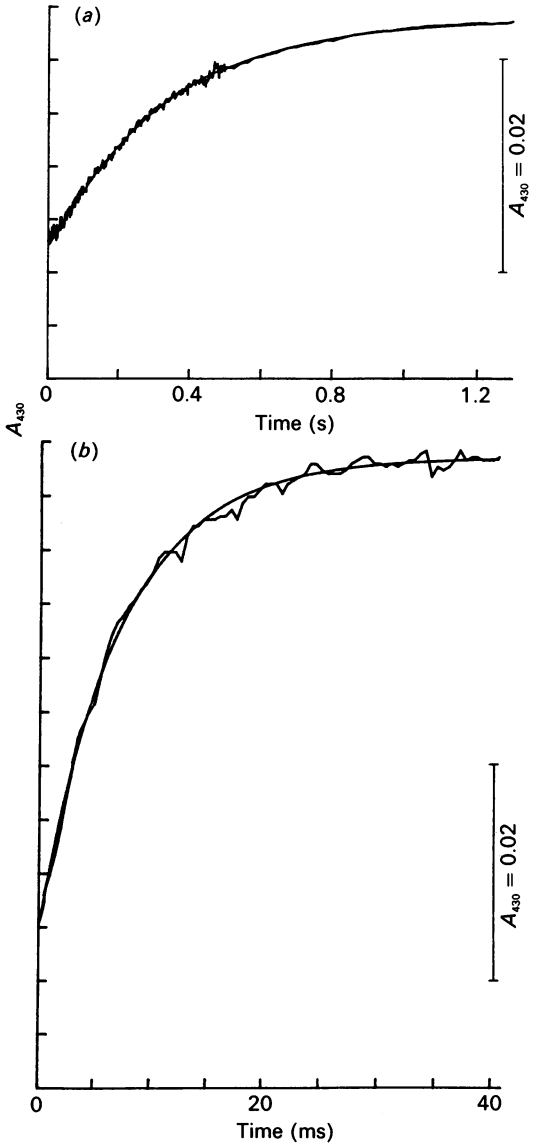
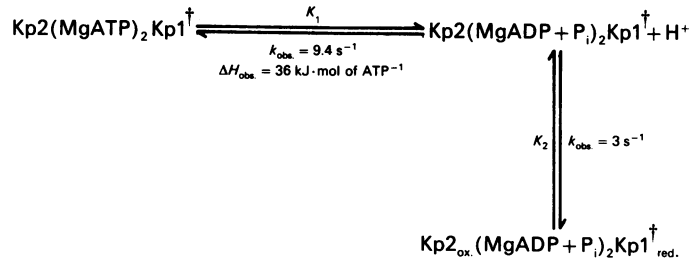


Fig. 2. MgATP-dependent electron transfer from the Fe protein to the MoFe protein of nitrogenase at 6 °C and 23 °C

The traces were obtained by stopped-flow spectrophotometry (430 nm; 2 mm path length) when pre-equilibrated nitrogenase component proteins (Kp1, 75 μM, 105 μM-Mo sites; Kp2, 250 μM) were mixed with MgATP (10 mM) in imidazole buffer (50 mM), pH 7.0. The trace in (*a*) was obtained at 6 °C and that in (*b*) at 23 °C. The decrease in noise level at 500 ms in the trace in (*a*) is due to an increase in the time constant of the digital filtering by the data-acquisition system. On increasing the temperature the amplitude increases from 0.021 to 0.043, and the observed first-order rate constant increases from 3 to 140 s^{-1}. The lines drawn through the data are single exponentials with these amplitudes and rate constants. A temperature-induced increase in the equilibrium constant for MgATP cleavage to yield MgADP+P_i from unity at 6 °C to greater than 10 at 23 °C is proposed to account for the increase in amplitude.



Scheme 2. Cleavage of MgATP to give MgADP + P_i on the complex formed between the Fe protein (Kp2) and MoFe protein (Kp1) is reversible, coupled to the release of 1.0 proton and precedes electron transfer from Kp2 to Kp1

The product of the equilibrium constants for the two steps (K_1K_2) is close to unity at 6 °C but increases to $\geqslant 10$ at 23 °C.

the rapid endothermic phase and the true enthalpy change for the cleavage step is twice that observed, i.e. $\Delta H = 64$ kJ·mol of ATP^{-1} and a proton yield of 0.4 mol/mol. The value of the corresponding equilibrium constant for nitrogenase is not known, and previously the MgATP-cleavage step has been assumed to be irreversible. However, a consideration of the amplitude of the absorbance change at 430 nm for the oxidation of Kp2(MgATP)$_2$ by Kp1 (Fig. 2) suggests that, at 6 °C, the cleavage step on nitrogenase is also reversible and that the extent of electron transfer within the Kp2–Kp1 protein complex depends on the value of the equilibrium constant (K_1).

The amplitudes of traces similar to that shown in Fig. 2(*a*) gives $\Delta\epsilon_{430} = 2.1 \pm 0.2$ mM^{-1}·cm^{-1} (on the basis of Mo active-site concentration). However, when the temperature was increased from 6 °C to 23 °C, the amplitudes increased to give $\Delta\epsilon_{430} = 4.3 \pm 0.2$ mM^{-1}·cm^{-1} with an increase in the rate constant to a value of 140 s^{-1} (Fig. 2*b*), which is close to the value of 120 s^{-1} reported by Thorneley (1975) under slightly different conditions (pH 7.4, 25 mM-Hepes, 23 °C). At 10 °C a value of $\Delta\epsilon_{430} = 3.4$ mM^{-1}·cm^{-1} was determined. This indicates that there is a significant increase in the value of the overall equilibrium constant (Scheme 2) on increasing the temperature from 6°C to 10 °C. This is consistent with the explanation given below for our failure to detect a 'phosphate burst' at 6 °C on acid quenching. In order to check that the Mo sites on Kp1 were saturated with Kp2(MgATP)$_2$, the protein solution described in the legend to Fig. 2 was diluted with an equal volume of Kp2 (403 μM) to yield a Kp2/Kp1 molar ratio of 8.6:1. On reaction with MgATP, this solution yielded $\Delta\epsilon_{430} = 2.4 \pm 0.3$ and 5.2 ± 0.4 mM^{-1}·cm^{-1} at 6 °C and 23 °C respectively. Ashby & Thorneley (1987), in a detailed stopped-flow amplitude titration of Kp1 with Kp2 at 23 °C, reported a value of $\Delta\epsilon_{430} = 5$ mM^{-1}·cm^{-1} for the electron-transfer reaction and concluded that oxidation of Kp2(MgATP)$_2$ contributed 4 mM^{-1}·cm^{-1} and reduction of Kp1 1 mM^{-1}·cm^{-1} to the total change in absorption coefficient at 430 nm. These data show that, under the conditions used to obtain the traces shown in Figs. 1 and 2, the Mo sites on Kp1 were about 90% saturated with Kp2(MgATP)$_2$.

The decreased value of $\Delta\epsilon_{430} = 2.4 \pm 0.3$ mM^{-1}·cm^{-1} at 6 °C is not due to the effect of temperature, pH or buffer concentration on isolated Kp2 protein, since when dye-oxidized Kp2$_{ox.}$(MgADP)$_2$ was reduced by sodium dithionite in the absence of Kp1 under the conditions described in the legend to Fig. 2, a value of $\Delta\epsilon_{430} = 4$ mM^{-1}·cm^{-1} was obtained [see Ashby & Thorneley (1987) for details of the preparation of Kp2$_{ox.}$(MgADP)$_2$].

We conclude that, at 23 °C, electron transfer from Kp2(MgATP)$_2$ to Kp1 is essentially irreversible, but at 6 °C, the reaction proceeds to only 50% completion. Since we have shown that electron transfer at 6 °C occurs after the MgATP-cleavage step, we suggest that it is the on-enzyme equilibrium between MgATP and MgADP + P_i that determines the extent of electron transfer.

If this interpretation of the amplitude data is correct, then nitrogenase, like myosin, has a reversible ATP-cleavage step. In the case of the two coupled equilibria shown in Scheme 2, the signal for the faster step, in the present case heat uptake, should be biphasic. The absence of a second endothermic phase, must, in our experiments, be due to compensation by an exothermic process. This could be the heat of electron transfer or the associated phosphate release. Although the overall equilibrium constant $K_1K_2 = 1$ is firmly established by the spectrophotometric data, the values for the two individual constants can only be given an approximate estimate until the results from further experiments become available. The observed $\Delta H = 36$ kJ·mol^{-1} and release of 0.5 proton then require doubling to yield $\Delta H = 72$ kJ·mol of ATP^{-1}, with 1 proton released for the complete cleavage of MgATP on nitrogenase. These values are similar to the values reported by Millar *et al.* (1987) for ATP cleavage on myosin ($\Delta H = 64$ kJ·mol of ATP^{-1}, 0.4 protons released). The equilibrium constant for MgATP cleavage to yield MgADP + P_i on myosin increases from unity at 5 °C (Millar *et al.*, 1987) to 9 at 21 °C (Bagshaw & Trentham, 1973; Bagshaw *et al.*, 1974). A similar temperature-induced change in the value of the equilibrium constant for the same reaction occurring on nitrogenase is entirely consistent with the endothermic changes of Fig. 1 and with the amplitude data shown in Fig. 2. It is also noteworthy that the rate of the cleavage step on myosin at 21 °C is $k \geqslant 160$ s^{-1} (Lymn & Taylor, 1971) and on nitrogenase at 23 °C, $k \geqslant 140$ s^{-1} (Fig. 2*b*).

We did not attempt to perform the stopped-flow calorimetry at 23 °C because the time constant for electron transfer (7 ms) indicated that any effects would have been obscured by the 40 ms stopping artefact discussed above.

The generation of Kp2(MgADP + P_i)$_2$Kp1 before electron transfer may explain why the mid-point potential (E_m) of Kp2(MgADP)$_2$ ($E_m = -350$ mV) is more negative than that of Kp2(MgATP)$_2$ ($E_m = -320$ mV) (Thorneley & Ashby, 1989), i.e. it is the cleavage of

MgATP that generates the active reductant (Kp2) and oxidant (Kp1). The failure of Kp2(MgADP)$_2$ to act as an electron donor to Kp1 implies that the P_i generated when MgATP is cleaved and the associated free energy change play essential roles in promoting electron transfer. Scheme 2 shows the sequence of events at 6 °C which these results imply and allows step 2 of Scheme 1 to be resolved into at least two partial reactions: MgATP cleavage with proton release, followed by electron transfer to Kp1. A knowledge of the individual rate constants for both the forward and reverse partial reactions of Scheme 2 together with computer modelling will be necessary before more detailed mechanistic conclusions can be made.

We have attempted to measure the kinetics and amplitude of the ATP-cleavage step at 6 °C by the rapid acid-quench technique used successfully at 10 °C by Eady *et al.* (1978). We failed to detect a 'burst' phase of phosphate at 5 °C; this may be due to the equilibrium between MgATP and MgADP + P_i such that on quenching with H^+, ATP is reformed and no phosphate is released into solution.

The similarity of the kinetics, enthalpy change and proton release for the ATP-cleavage step by myosin subfragment-1 and molybdenum nitrogenase suggests a common mechanism. It is noteworthy that in both systems ATP cleavage is followed by a rate-limiting step. In the case of myosin this is exothermic P_i release, which is thought to be the energy-yielding step in muscle contraction (Hibberd & Trentham, 1986). For nitrogenase, it is the dissociation of the oxidized-iron-protein–$MgADP_2$–reduced-molybdenum-protein complex (step 3, Scheme 1) which may also be induced by P_i release. The similarity between ATPase action of myosin and nitrogenase is also shown by the study by Mortenson *et al.* (1985), who demonstrated that nitrogenase-catalysed MgATP[S] hydrolysis occurs with inversion of configuration of the terminal phosphate. A one-step process, similar to that proposed for myosin, in which direct attack of water (or OH^-) on bound MgATP[S] occurs is the most likely explanation for the inversion.

In the present paper we have demonstrated the application of a recently developed technique, stopped-flow calorimetry, in studying the mechanism of ATP hydrolysis, proton transfer and energy transduction by nitrogenase. The technique has also the potential to monitor, for the first time, partial reactions involved in substrate binding and reduction without the perturbations of quenching or rapid freezing.

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