Nitrogenase of *Klebsiella pneumoniae*

Reversibility of the reductant-independent MgATP-cleavage reaction is shown by MgADP-catalysed phosphate/water oxygen exchange

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The steady-state kinetics of reductant-independent ATP hydrolysis by *Klebsiella pneumoniae* nitrogenase at 23 °C at pH 7.4 were determined as a function of component protein ratio (optimal at an oxidized Fe protein/MoFe protein ratio of 3:1) and MgATP concentration (K_m 400 μ M). Competitive inhibition was observed for MgADP (K_i 145 μ M), [β -methylene]ATP (Mgp[CH₂]ppA) (K_i 115 μ M), [β -monofluoromethylene]ATP (Mgp[CHF]ppA) (K_i 53 μ M) and [β -difluoromethylene]ATP (Mgp[CF₂]ppA) (K_i 160 μ M). The tighter binding of MgADP to free oxidized Fe protein (K_D < 10 μ M) than to the oxidized Fe protein–MoFe protein complex (K_i 145 μ M) is proposed as the driving force that induces rate-limiting protein dissociation in the catalytic cycle of nitrogenase. The reversible nature of the reductant-independent MgATP-cleavage reaction was demonstrated by an MgADP-induced enhancement of the rate of the phosphate/water oxygen exchange reaction with ¹⁸O-labelled phosphate ion. This enhancement, like the reductant-independent ATPase reaction, only occurred with the complex formed by oxidized Fe protein and MoFe protein and not with the individual proteins. The results are discussed in terms of the mechanism of ATP hydrolysis by nitrogenase and other systems involving protein–protein interactions.

INTRODUCTION

Klebsiella pneumoniae nitrogenase comprises two metallo-proteins, the MoFe protein (Kp1; M_r 220 000) and the Fe protein (Kp2; M_r 67 000). Nitrogenase catalyses the reduction of N₂ to 2NH₃ with concomitant evolution of H₂ and the hydrolysis of MgATP to MgADP and P_i. Under optimum conditions there is a stoichiometry of 2 molecules of MgATP hydrolysed for each electron transferred to reducible substrate (either H⁺ or N₂). See Lowe *et al.* (1985), Burgess (1985), Thorneley & Lowe (1985) and Smith *et al.* (1987) for reviews on the structure and mechanism of nitrogenase. Two putative ATP-binding sites on the *Azotobacter vinelandii* Fe protein have been identified by X-ray crystallography following a structure determination of a molybdate derivative, assuming that molybdate binds at the same site as the terminal phosphate group of ATP (Georgiadis *et al.*, 1990). Electron transfer is uncoupled from MgATP hydrolysis at pH < 6.4 or > 8.5 (Jeng *et al.*, 1970; Iman & Eady, 1980), at temperatures < 20 °C or > 30 °C (Watt *et al.*, 1975) or at low electron flux (i.e. [Fe protein] < [MoFe protein]) (Hageman & Burris, 1978; Eady & Postgate, 1974). The nitrogenase-catalysed hydrolysis of MgATP in the absence of an electron source such as Na₂S₂O₄, when the Fe protein is in the oxidized state, shows that electron transfer from Fe protein to MoFe protein is not required for ATPase activity (Jeng *et al.*, 1970; Iman & Eady, 1980; Cordewener *et al.*, 1987). This ‘reductant-independent’ ATPase activity can explain why the limiting stoichiometry of 2 molecules of MgATP hydrolysed for each electron transferred to substrate is not maintained under all conditions. Reactions 1, 2, 3, 4, 5 and 6 of Scheme 1 comprise a cycle (A) that accounts for the limiting stoichiometry of 2 molecules of MgATP hydrolysed

for each electron transferred from the Fe protein to the MoFe protein (Thorneley & Lowe, 1983; Lowe & Thorneley, 1984). Reductant-independent ATPase activity is shown to occur by reactions 7 and 8 in cycle B of Scheme 1. These reactions occur only on the oxidized Fe protein–MoFe protein (Kp2_{ox}, Kp1) complex and not on the isolated proteins (Iman & Eady, 1980).

In the present study we have characterized the reductant-independent ATPase reaction and compare a number of parameters with those for the reductant-dependent reaction, in order to ascertain whether these reactions occur at the same sites on the protein complex as is implied by Scheme 1. In addition, we have used the kinetics of phosphate/water oxygen exchange to probe the nature of the ATP-binding site and to determine the extent to which reductant-independent ATP cleavage is reversible. The present experiments complement previous studies in which the reductant-dependent ATP cleavage and subsequent electron transfer (reactions 2 and 3 of Scheme 1) were resolved by stopped-flow calorimetry (Thorneley *et al.*, 1989). These studies showed that ATP cleavage on nitrogenase and myosin have similar kinetic and thermodynamic parameters. The [¹⁸O₄]P_i exchange technique was originally developed to study the actomyosin system (Koshland & Clarke, 1953; Levy & Koshland, 1959; Hibberd *et al.*, 1985; Webb *et al.*, 1986).

The mechanism by which GTP and ATP hydrolysis induces protein association and dissociations and related conformation changes is of considerable current interest in the context of G-proteins such as p21^{ras} (Neal *et al.*, 1990) and the muscle actomyosin system (Millar *et al.*, 1987). Although these proteins have diverse functions, they may have mechanisms of energy transduction in common with those of nitrogenase.

Abbreviations used: Kp1 and Kp2, MoFe protein and Fe protein components respectively of *Klebsiella pneumoniae* nitrogenase; p[CH₂]ppA, [β -methylene]ATP; p[CHF]ppA, [β -monofluoromethylene]ATP; p[CF₂]ppA, [β -difluoromethylene]ATP; Ap₆A, P¹P⁶-bis(adenosine-5')-pentaphosphate.

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Scheme 1. Oxidation-reduction cycle for Kp2

Kp1t represents one of two independently functioning halves of the tetrameric ($\alpha_2\beta_2$) structure of Kp1. Each Kp1t ($\alpha\beta$) is assumed to contain one Mo-substrate-binding site and one Kp2-binding site. Cycle A comprises steps (1) complex-formation, (2) ATP cleavage, (3) electron transfer, (4) rate-limiting complex dissociation, (5) reduction of Kp2_{ox} by Na₂S₂O₄ and (6) replacement of 2MgADP by 2MgATP is involved in substrate reduction, which is assumed to occur at the FeMo cofactor bound to Kp1t. Cycle B does not involve dissociation of the protein complex and comprises the two composite reactions (7) ATP cleavage and (8) replacement of 2(MgADP + P_i) by 2MgATP. The present paper is concerned with the reductant-independent ATPase described by cycle B and the extent to which ATP cleavage is reversible.

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 had specific activities at 30 °C of 1600 and 1150 nmol of ethylene produced/min per mg of protein respectively. Kp1 contained 1.4 ± 0.1 g-atom of Mo/mol. Na₂S₂O₄ was removed from Kp1 by gel filtration on Bio-Gel P-6DG (Bio-Rad Laboratories) (1 cm × 16 cm column) equilibrated with 25 mM-Hepes/NaOH buffer, pH 7.4, containing 90 mM-MgCl₂. The high MgCl₂ concentration is required to stop precipitation of the Kp1 on the column. After elution and concentration to approx. 85 mg/ml, by using a Minicon B-15 concentrator, the protein was dialysed against 25 mM-Hepes/NaOH buffer, pH 7.4, to lower the MgCl₂ concentration to approx. 10 mM. Oxidized Fe protein (Kp2_{ox}) was prepared as described by Ashby & Thorneley (1987). Dithionite-free Kp1 and Kp2_{ox} proteins were always prepared and used in an anaerobic glove-box operating with less than 1 p.p.m. O₂.

Assays of reductant-independent ATP hydrolysis were performed with dithionite-free Kp1, with Kp2_{ox} at various concentrations, in 25 mM-Hepes/NaOH buffer, pH 7.4, containing 10 mM-MgCl₂ at 23 °C; 20 μM-P¹P⁶-bis(adenosine-5')-pentaphosphate (Ap₅A) was used in the assays to inhibit (K_i 2.5 nM; Lienhard & Secemski, 1973) any adenylate kinase present as an impurity in the nitrogenase component proteins. Assays (1 ml final volume) were initiated by the addition of ATP, and samples (100 μl), taken every 10 or 15 s, were quenched with 30% (w/v) trichloroacetic acid (100 μl) contained in an Eppendorf tube cooled on ice. After removal from the glove-box, the volume was made up to 600 μl with water, and the precipitated protein was spun down by centrifugation in a cold cabinet at 5 °C. The supernatant (2 × 250 μl) was analysed for phosphate by the method of Ottolenghi (1975).

Steady-state assays of phosphate/water oxygen exchange with [¹⁸O₄]P_i (97.56% enriched) were performed in a glove-box at 23 °C with dithionite-free Kp1 (approx. 33 μM) and Kp2_{ox}

(approx. 100 μM) in 25 mM-Hepes/NaOH buffer, pH 7.4, containing 10 mM-MgCl₂, 20 μM-Ap₅A, 5 mM-[¹⁸O₄]P_i and various concentrations of ADP and ATP analogues. Assays (100 μl final volume) were started by the addition of a Kp1/Kp2_{ox} protein mixture and terminated by the addition of 100 mg of Dowex AG 50W-X2 resin (H⁺ form). The resin was prepared by washing with 5 M-HCl followed by several volumes of water to neutral pH and then dried by suction. Water (900 μl) was added to each quenched assay, and after shaking and centrifugation for 10 min at 5 °C the clear supernatant was put into precooled Eppendorf tubes, and 50%-saturated Tris buffer (approx. 2.5 μl) was added to raise the pH to approx. 7.5. The tubes were then frozen for subsequent determination of the distribution of remaining ¹⁸O in the P_i by m.s. after treatment with diazoethane to yield triethyl phosphate (Hackney *et al.*, 1980; Hibberd *et al.*, 1985; Webb *et al.*, 1986). Duplicate determinations were performed on each sample.

Stopped-flow spectrophotometry (model SF-51 instrument; Hi-Tech Scientific, Salisbury, Wilts., U.K.) was performed inside the anaerobic glove-box as previously described (Ashby & Thorneley, 1987).

E.p.r. samples of Kp2 protein were prepared and frozen inside the anaerobic glove-box and run on a Bruker ER 200D spectrometer as described by Thorneley & Lowe (1983). The extent of oxidation was determined from the residual amplitude of the $g = 1.94$ feature, which is a characteristic of the reduced Kp2 protein.

All biochemicals were purchased from Sigma Chemical Co. with the exception of [β -methylene]ATP (p[CH₂]ppA), [β -monofluoromethylene]ATP (p[CHF]ppA) and [β -difluoromethylene]ATP (p[CF₂]ppA) (Blackburn *et al.*, 1984), which were a gift from Dr. G. M. Blackburn.

RESULTS AND DISCUSSION

Reductant-independent ATPase activity

Fig. 1 shows a linear time course for P_i release during reductant-independent ATP hydrolysis by a mixture of Kp1 (15 μM) and

Fig. 1. Steady-state phosphate formation during reductant-independent ATP hydrolysis at 23 °C at pH 7.4

The assay mixtures contained ATP (9 mM), Ap_5A (20 μM), Hepes (25 mM) and MgCl_2 (10 mM). The protein concentrations were 45 μM for Kp2_{ox} , and 15 μM for Kp1. The data shown are for assays containing Kp1 alone ($\blacktriangledown$), Kp2_{ox} alone ($\triangle$) and Kp1 + Kp2_{ox} ($\bullet$). Assays and P_i determination were performed as described in the Materials and methods section.

Fig. 2. Reductant-independent ATP hydrolysis: titration of Kp1 with Kp2_{ox} to determine the competence of Kp2_{ox} to effect this reaction at 23 °C at pH 7.4

The assay mixtures contained ATP (9 mM), Ap_5A (20 μM), Hepes (25 mM) and MgCl_2 (10 mM). The Kp1 concentration was kept constant at 15 μM and that of the Kp2_{ox} was varied from 0 to 120 μM . Assays and P_i determination were performed as described in the Materials and methods section.

Kp2_{ox} (45 μM). No P_i was released when either Kp1 or Kp2_{ox} alone was present in the assay, confirming that reductant-independent ATPase activity is a property of the protein complex formed between Kp1 and Kp2_{ox} . It is important to note that no detectable ATP hydrolysis occurs with these high concentrations of Kp1 and Kp2_{ox} alone under the conditions used for the $^{18}\text{O}\text{P}_i$ experiments described below.

Fig. 2 shows the dependence of the rate of P_i formation from reductant-independent ATPase activity as a function of increasing Kp2_{ox} concentration (0 to 120 μM) at a constant Kp1 concentration (15 μM). The 'break-point' occurs at a $\text{Kp2}_{\text{ox}}/\text{Kp1}$ molar ratio of 3:1, corresponding to $\text{Kp2}_{\text{ox}}/\text{Mo}$ ratio of 2.2:1 (i.e. each mole of Kp1 contains 1.4 g-atom of Mo). Ashby & Thorneley (1987) obtained a titration curve (their Fig. 9) identical in form with that shown in Fig. 2 when they monitored the extent of $\text{Kp2}(\text{MgATP})_2$ oxidation by Kp1 using stopped-flow spectrophotometry. Their argument for Kp2 protein with a specific

Fig. 3. Competitive inhibition by MgADP of reductant-independent ATP hydrolysis at 23 °C at pH 7.4

Each assay mixture contained Kp2_{ox} (24 μM), Kp1 (6 μM), Hepes (25 mM) and MgCl_2 (10 mM). The ATP concentration was varied in the range 0.25 to 5 mM at a series of constant ADP concentrations, 0 mM ($\blacktriangle$), 0.21 mM ($\circ$), 0.5 mM (∇) and 1 mM ($\bullet$). The inset shows a secondary plot of the slopes of the lines in the main Figure versus ADP concentration and allows a K_i^{MgADP} value of 145 μM to be calculated from the abscissa intercept. A K_m^{MgATP} value of 400 μM was calculated from the data in the main Figure obtained in the absence of ADP. Assays and P_i determination were performed as described in the Materials and methods section.

activity of approx. 1500 nmol of H_2 produced/min per mg of protein being only 45 % active in the electron-transfer reaction can now be extended to the reductant-independent ATPase reaction.

Fig. 3 shows double-reciprocal plots for the rate of reductant-independent ATP hydrolysis as a function of MgATP concentration at various MgADP concentrations. The inset shows a secondary plot of the slopes of the graphs in the main Figure against MgADP concentration. The data show that MgADP is a competitive inhibitor (K_i^{MgADP} 145 μM) of reductant-independent ATP hydrolysis (K_m^{MgATP} 400 μM). The value of K_m^{MgATP} (400 μM) for binding to the Kp2_{ox} -Kp1 complex (involved in reductant-independent ATPase activity) is identical with that previously determined for MgATP binding to reduced Kp2Kp1 complex by monitoring the ATP-dependence of the electron transfer from Kp2 to Kp1 by stopped-flow spectrophotometry (Thorneley, 1975). Thus the oxidation level of the Fe protein does not affect the affinity of the Kp2Kp1 complex for MgATP. However, free Kp2_{ox} binds MgADP ($K_i^{\text{MgADP}} < 10 \mu\text{M}$; Ashby & Thorneley, 1987) much more tightly than does the Kp2_{ox} -Kp1 complex (K_i 145 μM ; data of Fig. 3). This suggests a mechanism by which the component proteins Kp2_{ox} and Kp1 are induced to dissociate in the rate-limiting step in the catalytic cycle (reaction 4 of Scheme 1). The driving force for this reaction is the tighter binding of MgADP to free Kp2_{ox} relative to its binding to the Kp2_{ox} -Kp1 complex. (i.e. MgADP stabilizes free Kp2_{ox}). This stabilization is reflected in the lower mid-point potential of $\text{Kp2}_{\text{ox}}(\text{MgADP})_2$ (E_m -350 mV) relative to that of Kp2_{ox} (E_m -200 mV) (Thorneley & Deistung, 1988). The tightly bound MgADP is only released from Kp2_{ox} after reduction in the catalytic cycle (reactions 5 and 6 of Scheme 1; Ashby & Thorneley, 1987).

Similar competitive inhibition data to those shown in Fig. 3 for MgADP were obtained with the ATP analogues $\text{p}[\text{CH}_2]\text{ppA}$ (K_i 115 $\pm$ 10 μM), $\text{p}[\text{CHF}]\text{ppA}$ (K_i 53 $\pm$ 5 μM) and $\text{p}[\text{CF}_2]\text{ppA}$ (K_i 160 $\pm$ 10 μM). In the case of the chiral $\text{p}[\text{CHF}]\text{ppA}$ analogue,

Fig. 4. Stopped-flow traces of the reduction of Kp2_{ox} by dithionite ion in the presence of p[CH₂]ppA at 23 °C at pH 7.4

The absorbance change was monitored at 430 nm when Kp2_{ox} (45 μM) plus p[CH₂]ppA (1 mM) from syringe A was mixed with Na₂S₂O₄ (2 mM) from syringe B (trace A). The slow biphasic reduction is characteristic of Kp2_{ox}, that has undergone a conformation change that lowers its redox potential by approx. 150 mV. In the absence of p[CH₂]ppA or ADP the reaction is complete within the mixing time (approx. 1 ms) (see Ashby & Thorneley, 1987). Trace B shows a similar rapid reduction obtained when the order of mixing was changed but with the same final concentrations as used for trace A, i.e. when Kp2_{ox} (45 μM) from syringe A was mixed with Na₂S₂O₄ (2 mM) plus p[CH₂]ppA (1 mM) from syringe B. The reduction is complete either before the binding of p[CH₂]ppA to Kp2_{ox}, or more likely the subsequent conformation change has occurred. Comparison with data obtained by Ashby & Thorneley (1987) shows that p[CH₂]ppA must bind and/or induce a conformation change in Kp2_{ox}, more slowly than does ADP.

a racemic mixture was used and, since it is not known whether only one or both dia stereoisomers bind, no correction has been made to the value of K_i . The demonstration that both MgADP and Mg p[CH₂]ppA are competitive inhibitors of reductant-independent ATPase activity is relevant to the analysis of the [¹⁸O]₄P_i isotope-exchange data presented below. The K_i values for the fluorinated ATP analogues will be useful in the design of ¹⁹F-ENDOR (electron nuclear double resonance) experiments to investigate possible interactions between bound nucleotides and paramagnetic metal ion centres in the protein complex.

Binding of p[CH₂]ppA to Kp2_{ox}.

The ATP analogue p[CH₂]ppA was used to inhibit the P_i/water oxygen exchange reaction catalysed by both Kp2_{ox} and the Kp2_{ox}-Kp1 complex (see below). The demonstration that p[CH₂]ppA is a competitive inhibitor of reductant-independent ATP hydrolysis (see above) demonstrates its binding at the ATP-binding site(s) on the Kp2_{ox}-Kp1 complex. The binding of p[CH₂]ppA to Kp2_{ox} alone was shown by its ability to induce a 'slow' phase when the reduction of Kp2_{ox} by Na₂S₂O₄ was monitored by stopped-flow spectrophotometry (Fig. 4). This slow reduction is diagnostic of a nucleotide-induced conformation change in Kp2_{ox}. In the absence of ADP or p[CH₂]ppA

the reduction of Kp2_{ox} is complete within the 1 ms dead time of the instrument, and a trace similar to that shown in Fig. 4 (trace B) is obtained (see Ashby & Thorneley, 1987). When the Kp2_{ox} was preincubated with p[CH₂]ppA before the mixing with 2 mM-Na₂S₂O₄, a slow biphasic reduction occurred (Fig. 4, trace A) with k_{obs} values of 5 s⁻¹ and 0.55 s⁻¹ with amplitudes in the ratio 2:1 respectively. Under these conditions ADP induces a single-phase reduction with a k_{obs} value of 5 s⁻¹ (Ashby & Thorneley, 1987).

When the same concentrations of reactants were used, but with the p[CH₂]ppA initially in the syringe containing the Na₂S₂O₄, the reduction was complete within the mixing time of 1 ms (Fig. 4, trace B). Ashby & Thorneley (1987) performed a similar 'order-of-mixing' experiment with ADP under the same conditions. When ADP was initially present in the dithionite syringe, 70% of the total absorbance change remained in the slow phase, with only 30% complete within the mixing time. We conclude that p[CH₂]ppA induces a similar conformation change in Kp2_{ox} to that induced by ADP, but that either the binding of p[CH₂]ppA or the subsequent conformation change occurs at a significantly lower rate than that with ADP.

[¹⁸O]₄P_i/water oxygen exchange

The nature of the ADP/ATP-binding site(s) involved in reductant-independent ATPase activity and the extent to which ATP cleavage is reversible under these conditions have been investigated by measuring the rate of exchange of ¹⁸O between [¹⁸O]₄P_i and water. McKenna *et al.* (1989) used ¹⁸O-labelled ATP to show that the nitrogenase catalyses the cleavage of the ATP P_(γ)-¹⁸OP_(β) bond. Mortenson *et al.* (1985) showed that the hydrolysis is via direct attack of a water oxygen atom on the γ-phosphate, not via a phosphorylated intermediate such as a phospho-enzyme. Scheme 2 shows that, if P_i can bind to the ADP-bound protein and ATP cleavage is reversible, an ¹⁸O atom will be lost from [¹⁸O]₄P_i (as H₂¹⁸O) on formation of the β-γ-phosphate bond (¹⁶O-bridge originating from the β-phosphate) to give bound ATP. The protein-bound ATP will then be hydrolysed, and P_i will re-form containing one oxygen atom from water (¹⁶O) (direct attack of ¹⁶OH⁻ on the γ-phosphorus atom with the bridging ¹⁶O being retained by the β-phosphorus atom). In this way ADP and protein catalyse the exchange of oxygen atoms in the P_i. In this reaction an oxygen atom of the β-phosphate group of ADP is acting as a nucleophile.

The site(s) at which the phosphate group binds on either Kp2_{ox} or Kp1 alone or on the Kp2_{ox}-Kp1 complex may contain other nucleophilic groups X [where X may be a cysteinyl -S-, a serinyl -OH or a tyrosyl -O(H) group] capable of catalysing ¹⁸O exchange from [¹⁸O]₄P_i in the absence of ADP. In addition, strong hydrogen-bonding of phosphate in the enzyme could promote loss of ¹⁸O by general acid catalysis. We have therefore measured rates of ¹⁸O exchange from [¹⁸O]₄P_i with Kp2_{ox} and Kp1 alone and in combination in the presence and in the absence of ADP and with the ATP analogue p[CH₂]ppA. The conditions under which reductant-independent ATPase activity occurs (Kp2_{ox} with Kp1 in the absence of Na₂S₂O₄) were chosen so that assays could be run for up to 60 min without complications due to the exhaustion of Na₂S₂O₄ and a change from reductant-dependent to reductant-independent ATPase activities. Anaerobic glove-box facilities ensured that no loss of activity due to adventitious damage by O₂ occurred over these long assay periods.

A component protein Kp2_{ox}/Kp1 ratio of 3:1 was used, since this ratio ensured a saturation of Kp1 by Kp2_{ox}, as judged by a maximum rate of reductant-independent ATPase activity (see Fig. 2). The high concentrations of Kp1 (33 μM) and Kp2_{ox} (100 μM), which are typical of estimates of intracellular concen-

Scheme 2. Mechanism for ADP-catalysed P_i /water oxygen exchange occurring on the $Kp2_{ox}$ - $Kp1$ complex

E represents the $Kp2_{ox}$ - $Kp1$ complex, which catalyses the reductant-independent hydrolysis of ATP (cycle B in Scheme 1). Although this reaction may occur at two sites formed or activated when the two proteins form a complex, only one is shown here for reasons of clarity. The black circles represent ^{18}O and the white circles ^{16}O . The site is initially occupied by $MgADP$ (represented as E-ADP) and in reaction 1 reversibly binds the other product of ATP hydrolysis, $[^{18}O_4]P_i$. Reaction 2 is the transient formation of ATP. The elimination of $H_2^{18}O$ is shown as irreversible owing to dilution of the $H_2^{18}O$ by the solvent, $H_2^{16}O$. ATP hydrolysis occurs in step 3 by direct attack of $H_2^{16}O$ (or $^{16}OH^-$). Step 4 is the release of $[^{18}O_3^{16}O]P_i$. Successive reactions of $[^{18}O_{4-x}^{16}O_x]P_i$ (where $x = 1, 2, 3$ or 4) give final isotope distributions of ^{18}O in the P_i similar to those in Fig. 5. No attempt has been made to show the extent of protonation of the phosphate groups or the binding of Mg^{2+} , both of which may change during the reaction sequence.

Fig. 5. ^{18}O distribution in P_i after ADP-catalysed phosphate/water oxygen exchange occurring on the $Kp2_{ox}$ - $Kp1$ complex

The histograms show the ^{18}O distribution in P_i after incubation of the $Kp2_{ox}$ - $Kp1$ complex with $[^{18}O_4]P_i$ and either ADP or $p[CH_2]ppA$ under the conditions described in the Materials and methods section and in the heading of Table 1. ADP catalyses the loss of ^{18}O from $[^{18}O_4]P_i$ and produces an isotope distribution pattern characteristic of a rate of phosphate release that is rapid compared with that of ATP formation. This results in only one ^{18}O atom being lost while both ADP and P_i remain bound to the protein complex. The control experiment with $p[CH_2]ppA$ shows a small 'background' exchange of ^{18}O with the major product being unchanged $[^{18}O_4]P_i$. These data were also used in the calculation of the exchange velocities given in Table 1.

Table 1. ^{18}O exchange from $[^{18}O_4]P_i$ catalysed by nitrogenase in the presence and in the absence of ADP and the analogue $p[CH_2]ppA$ at 23 °C at pH 7.4

The assay mixtures contained $[^{18}O]P_i$ (5 mM), Ap_5A (20 μM), Hepes (25 mM), $MgCl_2$ (10 mM) and ADP (250 μM) or $p[CH_2]ppA$ (250 μM or 1 mM). The protein concentrations were $[Kp1] = 33 \mu M$ and $[Kp2] = 100 \mu M$. Replicate determinations are given.

Sample	Velocity of exchange ($\mu M \cdot s^{-1}$)
$Kp2_{ox} + Kp1 + ADP$ (250 μM)	48.1, 48.9
$Kp2_{ox} + ADP$ (250 μM)	12.7, 15.5
$Kp1 + ADP$ (250 μM)	5.8, 6.3
$Kp2_{ox} + Kp1$	25.3, 23.4
$Kp2_{ox}$	13.5, 13.2
$Kp1$	4.7, 4.9
$Kp2_{ox} + Kp1 + p[CH_2]ppA$ (1 mM)	15.3, 16.0
$Kp2_{ox} + p[CH_2]ppA$ (1 mM)	8.8, 8.7
$Kp1 + p[CH_2]ppA$ (1 mM)	4.5, 4.5
$Kp2_{ox} + Kp1 + ADP$ (250 μM) + $p[CH_2]ppA$ (1 mM)	17.9, 17.8
$Kp2_{ox} + Kp1 + ADP$ (250 μM) + $p[CH_2]ppA$ (250 μM)	26.5, 26.2

sum of the rates for the individual proteins. In order to determine whether this ^{18}O exchange was associated with the ATP/ADP-binding site(s) on the individual proteins or at a site formed or modified when formation of the protein complex occurs, the ATP analogue $p[CH_2]ppA$ was used. $p[CH_2]ppA$ has the advantage over ATP of not being hydrolysed, and thereby complications arising from the generation of ADP and $[^{18}O]P_i$ during the course of the assay were avoided. Table 1 shows that $p[CH_2]ppA$ inhibited ^{18}O exchange on both the complexed proteins and on the individual proteins. In the case of the individual proteins this inhibition is associated with $Kp2_{ox}$, with no significant effect on $Kp1$.

trations of nitrogenase component proteins, were necessary to obtain significant ^{18}O loss from $[^{18}O_4]P_i$ at assay times of 10, 20, 30 and 60 min. In all experiments linear time courses for the extent of ^{18}O exchange were obtained. The assay conditions, 25 mM-Hepes/NaOH buffer, pH 7.4, containing 10 mM- $MgCl_2$ at 23 °C, were as used in previous kinetic experiments from this laboratory.

Table 1 summarizes the ^{18}O -exchange data. In the absence of ADP and $p[CH_2]ppA$, only a small increase in the ^{18}O -exchange rate occurs when the proteins are complexed compared with the

ADP (250 μM) did not significantly change the ^{18}O -exchange rate for the isolated proteins. However, a more than 2-fold increase occurred for the complexed proteins. This enhancement by MgADP increases to more than 3-fold if the rates for $\text{Kp2}_{\text{ox}}(\text{MgADP})_2\text{Kp1}$ complex are compared with those for the $\text{Kp2}_{\text{ox}}(\text{Mgp}[\text{CH}_2]\text{ppA})_2$ complex. These data are also shown in Fig. 5 in the form of a histogram, which shows a clear difference in the ^{18}O distributions in the P_i after incubation of $\text{Kp2}_{\text{ox}}\text{Kp1}$ with ADP or $\text{p}[\text{CH}_2]\text{ppA}$.

The extent of ^{18}O exchange depends on the velocity of the reaction in Scheme 2 (formation and breakdown of bound ATP from and to P_i and ADP) and on whether more than one oxygen atom can be lost from the phosphate group once it is bound to the protein. In the extreme case, each phosphate group would lose all its ^{18}O before release, as has been shown to occur with myosin subfragment 1 (Webb *et al.*, 1978). The mechanism in Scheme 2 can be used to model the distribution of ^{18}O and ^{16}O in the P_i by an iterative calculation, in terms of the velocity of the exchange reaction and the ratio of the rate constants (R_p) for release of phosphate from $\text{E} \cdot \text{ADP} \cdot \text{P}_i$ and the formation of $\text{E} \cdot \text{ATP}$. This modelling indicates that $R_p > 2.5$ for all the experiments detailed in Table 1. Therefore there is little loss of a second ^{18}O atom before phosphate release. Thus, in order for the phosphate to lose all four ^{18}O atoms, it must bind and go through the exchange reaction at least four times. The large value of R_p is consistent with the results on positional isotope exchange reported by McKenna *et al.* (1985). They showed that ATP labelled with ^{18}O in the $\beta\gamma$ -bridge position was recovered unchanged within the (5%) limit of the ^{31}P -n.m.r. detection method used for assays containing functional *A. vinelandii* nitrogenase. McKenna *et al.* (1985) concluded that the forward reaction (ATP cleavage) must be considerably more rapid than the reverse step (reaction of ADP with P_i and release of ATP), unless $\text{P}_{(\alpha)}\text{--O--P}_{(\beta)}$ rotation in the bound ADP fragment is unusually restricted. Further comment on these data requires knowledge in greater detail of the experimental conditions than was provided in their preliminary publication. It would be of particular interest to repeat the experiments on positional isotope exchange at low temperature (6 $^{\circ}\text{C}$), where the data of Thorneley *et al.* (1989) suggest the equilibrium constant for ATP cleavage is close to unity, and also to use the $\text{Kp2}_{\text{ox}}\text{Kp1}$ complex at the high concentration used in the present experiments to study reductant-independent ATPase activity.

Table 1 also shows that $\text{p}[\text{CH}_2]\text{ppA}$ (250 μM and 1 mM) inhibited the stimulation induced by ADP (250 μM). These rates were in reasonable agreement with values calculated by assuming that $\text{p}[\text{CH}_2]\text{ppA}$ is a competitive inhibitor (K_i 115 μM) of ADP (K_m 145 μM) and that the rate for the complexed proteins with $\text{p}[\text{CH}_2]\text{ppA}$ (1 mM) is due to reaction at another site. We cannot exclude the possibility that this 'background' rate is due to contaminating proteins that are not inhibited by Ap_5A . However, the pronounced effect of ADP must be associated with the nitrogenase protein complex, since it is extremely unlikely that the enhanced ^{18}O exchange could result from contaminating proteins present in the individual nitrogenase component protein solutions. These contaminants would have to interact or be activated when the stock solutions of Kp2_{ox} and Kp1 were mixed together.

We have interpreted the $^{18}\text{O}_4\text{P}_i$ /water oxygen exchange data in terms of a site formed or activated when Kp2_{ox} and Kp1 form a complex. This site is also responsible for reductant-independent ATPase activity. The site has a group X that is capable of catalysing a relatively low rate of ^{18}O exchange from $^{18}\text{O}_4\text{P}_i$ in the absence of ADP. Group X may be located on either Kp1 or Kp2_{ox} . Two pieces of evidence suggest that group X is not acting as a nucleophile. Mortenson *et al.* (1985) used $^{17}\text{O}^{18}\text{O}$ -labelled [γ -

thio]ATP to show that hydrolysis by nitrogenase (at a rate approx. 1% of that for ATP) results in inversion at the chiral terminal thiophosphate group. This strongly implies a one-step phosphotransfer to water without the involvement of a nucleophilic group X on the enzyme. It does not, of course, exclude group X acting as a general acid/base catalyst or indeed X being a metal ion possibly present as part of the FeMo-cofactor. The second piece of evidence is the similarity between the kinetics and enthalpy changes for ATP cleavage occurring on nitrogenase and that on myosin (Thorneley *et al.*, 1989). These data suggest a similar mechanism, which in the case of myosin is known to involve direct attack of water at the terminal phosphorus atom without the involvement of a group X on the protein (Webb & Trentham, 1980). $\text{p}[\text{CH}_2]\text{ppA}$ is assumed to bind to the protein complex and to inhibit ^{18}O exchange catalysed by group X by preventing the binding of $^{18}\text{O}_4\text{P}_i$. However, it must be possible for ADP and P_i to be bound, at least transiently, at adjacent sites, since ATP hydrolysis generates these species. The reversible nature of this reaction (shown in Scheme 2) is proposed to account for the main observation of this study, the ADP-induced enhancement of the ^{18}O exchange from $^{18}\text{O}_4\text{P}_i$. The ADP-enhanced exchange reaction, like ATP hydrolysis, only occurs on the $\text{Kp2}_{\text{ox}}\text{Kp1}$ complex, not on the isolated component proteins. This mechanism only requires a transient reversible formation of ATP on the enzyme from ADP plus P_i and does not require ATP to be released from the enzyme.

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