Covalent modification of nitrogenase MoFe protein by ADP

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MgADP[−] reacted with the nitrogenase molybdenum–iron (MoFe) protein of *Klebsiella pneumoniae* (Kp1) over a period of 2 h to yield a stable, catalytically active conjugate. The isolated protein exhibited a new, broad ³¹P NMR resonance at −1 p.p.m. lacking phosphorus *J* coupling. The adenine ring of [8-¹⁴C]ADP remained associated with the conjugate. A covalently bound nucleotide was identified as AMP by NMR and TLC. Extended dialysis of Kp1 against MgADP[−] resulted in further AMP binding at the protein surface. ADP was initially bound tightly to Kp1 at a site distinct from the AMP sites. ATP did not replace ADP. The time course of the formation of the Kp1-AMP was altered by the nitrogenase iron protein (Kp2) and was dependent on redox potential. Kp1-AMP was stable to concentration and oxidation with ferricyanide ion at −350 mV. Slow hydrolysis of Kp1-AMP over a period of 6 h yielded AMP and unaltered Kp1. The adenine ring of ADP exchanged with adenine of MgATP^{2−}

during reductant-limited turnover of nitrogenase under N₂, indicating reversibility of ATP hydrolysis at 15 °C. [³²P]P_i exchanged with the terminal phosphate group of both ADP and ATP on incubation with Kp1. ³²P exchange and the catalytic activity of Kp1 were inhibited by a 20-fold molar excess of the lysine-modifying reagent, *o*-phthalaldehyde (OPT). Pre-incubation with MgADP[−] protected against OPT inactivation. Two potentially reactive lysine residues on the α chain of the MoFe protein near a putative hydrophobic docking site for the nitrogenase Fe protein are proposed as sites of OPT and nucleotide binding. *Azotobacter vinelandii* MoFe protein (Av1) also formed an AMP adduct but Kp2 did not. Catalase did not interact with ADP. The reactions of the nitrogenase MoFe protein with adenine nucleotides have no counterpart in known protein–nucleotide interactions.

INTRODUCTION

Nitrogenase, the enzyme system that catalyses biological nitrogen fixation, is a complex of two metalloproteins that together perform the ATP-dependent, multiple-electron reduction of dinitrogen to ammonia (reviewed in [1–4]). The three-dimensional structure of the nitrogenase proteins of *Azotobacter vinelandii* has been determined and recently reviewed [5–8]. The tetrameric ($\alpha_2\beta_2$) MoFe protein of nitrogenase contains two types of paramagnetic metal cluster: two MoFe₇S₉:homocitrate clusters (FeMoco) are bound approx. 10 Å (1 Å = 0.1 nm) below the surface of the α subunit and are the sites of substrate binding and reduction. Two iron–sulphur clusters (the P clusters) with the composition [Fe₈S_{7–8}] are located at the α/β interfaces of the MoFe protein and may supply electrons for reduction of substrates. During turnover, the homodimeric (γ_2) Fe protein, which contains a single [4Fe4S] cubane cluster, binds at a docking site on the MoFe protein. After ATP-dependent electron transfer, the complex dissociates and the oxidized Fe protein carries away MgADP[−] in the overall rate-limiting step [9,10]. ATP hydrolysis is not stoichiometrically coupled to the reduction of N₂ or other substrates. Hence the ratio of ATP hydrolysed per electron pair donated to substrates is 4 or greater under standard conditions of assay. The mechanism by which the free energy of hydrolysis of ATP is utilized to drive the nitrogenase reaction has not been determined.

The interaction of nucleotides with Fe proteins is well documented (reviewed in [7]). The X-ray structure of Av2 at 2.9 Å

resolution shows nucleotide-binding sites to be located at the subunit interface 20 Å away from the FeS centre [8]. The Fe protein can bind two MgATP^{2−} molecules and it is generally held that primary sites of ATP binding and hydrolysis are located on the Fe protein. The hydrolysis of ATP might induce switching between conformational states of the nitrogenase proteins during turnover [7]. In the absence of MoFe protein, the wild-type Fe protein, or Fe proteins isolated from several site-directed mutants, does not catalyse ATP hydrolysis [11]. Hence the question of additional interactions of adenine nucleotides with the MoFe protein during catalytic turnover remains open.

Studies of nucleotide interactions of the isolated proteins have suggested that MoFe proteins, as well as Fe proteins, possess binding sites for adenine nucleotides [4,12–15]. It is possible that a nucleotide hydrolytic site resides on the surface of the enzyme complex near the interface between the component proteins [16]. A second role for ATP hydrolysis might be protonation of the reduced products of substrate reduction at the FeMoco centres [17].

Although no consensus Walker-type nucleotide-binding motifs are present in the sequences of MoFe proteins, a nucleotide-binding site on the β subunit has been suggested [18]. We have reported that ATP binding to Kp1 was relatively weak with *K_d* ≥ 600 μ M and *n* = 4 [12]. ADP was bound tightly to MoFe proteins and to the equivalent VFe protein of the vanadium nitrogenase system of *Azotobacter chroococcum* [14]. Kp1, having oxidized P clusters, bound ADP in both rapid, reversible and slower, non-reversible reactions as determined by gel-permeation

Abbreviations used: FeMoco, MoFe₇S₉:homocitrate cluster; OPT, *o*-phthalaldehyde; PEI, polyethylimine ion-exchange medium. Nitrogenase proteins are abbreviated as the Fe protein and the MoFe protein. Indications of bacterial genus and species names of sources of these proteins are used according to the illustration: MoFe proteins of *Azotobacter vinelandii*, *Klebsiella pneumoniae*, *Azotobacter chroococcum*, etc., are Av1, Kp1, Ac1, etc. Fe proteins from the same organisms are Av2, Kp2, Ac2, etc.

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chromatography. Kp1 and Ac1 catalysed the slow exchange of [^{32}P]P_i with the terminal phosphates of both ATP and ADP [12]. This reaction was not catalysed by either the Fe protein or adenylate kinase.

Kimber et al. [19] showed that a Kp1–Mn²⁺–ATP ternary complex resulted in a decreased enhancement of proton relaxation rates compared with the cation plus nucleotide. This effect was proportional to the catalytic activity of Kp1, and four nucleotide-binding sites on Kp1 were inferred. ENDOR (electron nuclear double resonance) studies indicated that MnATP²⁺, MnADP⁺ and vanadyl nucleotides, VOATP²⁺ and VOADP⁺ bound to Kp1 and to Kp2 [20].

In the present work we have found non-dissociable binding of a radiolabelled nucleotide to Kp1. Exchange of the ring as well as the terminal phosphate groups of adenine nucleotides has also been observed. The effect of blocking putative Kp1 nucleotide-binding sites with *o*-phthalaldehyde (OPT) on catalytic activity and the ^{32}P exchange reactions has been determined.

MATERIALS AND METHODS

Nitrogenase components

Kp1 and Kp2 were purified from extracts of *Klebsiella pneumoniae* (oxytoca) N.C.I.B. 12204 and assayed as previously described [21]. Final purification of Kp1 was by FPLC chromatography on Q-Sepharose [22]. Kp1 fractions having a minimum specific activity of 2000 nmol of ethylene/min per mg were concentrated to 20 mg/ml in the presence of 2 mM sodium dithionite. Residual acetylene-reducing activity in the absence of added Kp2 was 1–2 nmol/min per mg. This background level of Kp2 activity could allow Kp1 (20 mg/ml) to hydrolyse 2.0 $\mu\text{mol}/\text{ml}$ MgATP²⁺ to MgADP⁺ over a 16 h period in the presence of a reductant (dithionite) or 0.27 $\mu\text{mol}/\text{ml}$ in the absence of reductant [23]. All operations with the isolated nitrogenase proteins were performed under N₂ in an anaerobic chamber at less than 1.5 p.p.m. O₂.

Nitrogenase proteins used in these experiments had no demonstrable adenylate kinase (EC 2.7.4.3) activity. Neither Kp1 nor Kp2 converted ADP to AMP and ATP over a period of 30 min when 1–6 mg was assayed under aerobic conditions in the standard glucose-6-phosphate dehydrogenase-linked spectrophotometric assay system of Sottocasa et al. [24].

Reaction with MgADP⁺

Preconcentrated Kp1 (2 ml, 60 mg/ml) was freed of dithionite by gel-permeation chromatography on a 1.2 cm $\times$ 18 cm column of Bio-Gel P-6 DG (Bio-Rad), which had been equilibrated with a buffer containing 50 mM Tris/HCl, pH 7.8, 100 mM NaCl and 5 mM MgCl₂. Phenosafranine was added to oxidize the P clusters [14]. The MoFe protein (238 μM) was then treated with 3.8 mM MgADP⁺ at 22 °C for up to 3 h. In some experiments, 6.5 μM [8- ^{14}C]ADP (NEN Research Products, Wilmington, DL, U.S.A.) was added.

Alternatively, Kp1 (100 mg/5 ml) was oxidized, then dialysed at 15 °C against 250 ml of Tris/HCl column buffer as described above containing 2 mM adenine nucleotide. In some experiments [8- ^{14}C]ADP (2 MBq) was introduced into the dialysis buffer. The protein was removed from dialysis bags after 16 h and reduced in volume to 2 ml in Minicon concentrators (Amicon Ltd.). $^2\text{H}_2\text{O}$ (10%, v/v) was added to all NMR samples in sealed 10 mm tubes under N₂.

The product of the slow reaction between Kp1 and ADP was separated from free nucleotides and inorganic phosphate by gel filtration of the incubation mixture. The amount of adenine ring-

labelled nucleotide bound tightly to Kp1 was determined by scintillation counting.

Separation and quantification of nucleotides

^{14}C -labelled adenine nucleotides formed during anaerobic incubation of ADP with Kp1 were separated by chromatography on methanol-washed polyester sheets coated with polyethylimine ion-exchange medium (Polygram CEL 300 PEI/UV₂₅₄). Protein was precipitated from the reaction mixtures before chromatography by treatment with 2% (v/v) perchloric acid for 5 min in an ice bath. The samples were then neutralized with 1 M KOH, methanol was added to 30% (v/v) and denatured protein was recovered by centrifugation at 5000 g. The protein was washed with aqueous methanol (50%, v/v), then redissolved in 0.1 M KOH and neutralized with HCl. Protein and soluble nucleotide fractions were applied to the origin of chromatograms. After sample application, PEI sheets were immersed in distilled water for 2 min to dissolve inorganic salts. The chromatograms were developed with 0.85 M NaH₂PO₄ at pH 3 for 1 h and dried. Storage phosphor plates (Fuji ST III) were exposed to the chromatograms for 1 h and the phosphorimages were quantified with a Fuji BAS1000 BioImaging Analyser. Radioactivity present in nucleotide spots was determined with Fuji MAC BAS image analysis software. A standard curve obtained for [8- ^{14}C]ADP was used to calculate the amounts of radiolabelled ATP, ADP and AMP derived from labelled ADP.

NMR and EPR Spectroscopy

^{31}P NMR spectra were recorded at 109.25 MHz with a JEOL GSX270 Spectrometer. A bilevel proton-decoupling pulse sequence was repeated 1000 or 2000 times with less than a 1 °C rise in sample temperature. Deuterated phosphoric acid (85% in $^2\text{H}_2\text{O}$, w/v) was used as a primary chemical shift standard. All spectra were plotted with the chemical shift of P_i at pH 7.8 in 5 mM MgCl₂ at +3 p.p.m. Spectra were recorded at 22 °C or at lower temperatures after equilibration for 5 min. EPR spectroscopy was employed to verify the redox state of the Kp1 metal clusters.

Treatment with OPT

OPT (Sigma) was stored under N₂ and dissolved in ethanol (160 mg/10 ml). Aliquots of a fresh solution were pipetted into serum vials and flushed with argon. Kp1 (60 nmol) and Tris/HCl buffer (100 mM, pH 7.5) containing 1 mM dithionite and 10 mM MgCl₂ were added. In some cases, 1.5 mM ADP or ATP was included. Inorganic Na[^{32}P]P_i was added (20000 d.p.m., carrier-free) and the mixture was incubated at 25 °C for 30 min after which aliquots (5 μl) were withdrawn for the assay of catalytic activity. ^{32}P -labelled adenine nucleotides were isolated and radioactivity was determined as previously reported [12].

RESULTS AND DISCUSSION

Reaction of Kp1 with MgADP⁺

In contrast with studies of nucleotide binding to isolated Fe proteins, Kp1 reactions with MgADP⁺ have been found to be complicated by progressive, non-equilibrium interactions. When Kp1 with phenosafranine-oxidized P clusters was passed through a gel-permeation column equilibrated with [8- ^{14}C]MgADP⁺, 4 mol of nucleotide was bound per mol of Kp1 [14]. Typically, 2 mol of ADP were not removed by passage through a second,

Figure 1 Effect of redox potential and Kp2 on the binding of nucleotide to Kp1

Dithionite-free Kp1 (60–100 μ M) was incubated anaerobically with a 6–8-fold molar excess of [8- 14 C]ADP at 18 $^{\circ}$ C for the indicated times (curve B). Curve A, $K_3Fe(CN)_6$ was added to give a higher stable potential (-464 mV against saturated calomel reference electrode). Curve C, Fe protein was added (3 μ M) at the start of the reaction. Kp1 was reisolated from aliquots of the reaction mixtures by gel filtration and the bound nucleotides determined as stated in the Materials and methods section.

unlabelled gel-filtration column. This result indicated that nucleotide binding to Kp1 occurred in two phases: a rapid binding of ADP followed by a slow reaction to yield a stable Kp1-nucleotide complex [13].

The time dependence of tight binding of [8- 14 C]adenine nucleotide is shown Figure 1. The amount of radioactivity bound to Kp1 having partly oxidized P clusters and reduced FeMoco centres increased with time up to 2 h and beyond. The course of the reaction was dependent on the initial redox potential of the medium (Figure 1, curves A and B) or on the presence of substoichiometric amounts of Kp2 (Figure 1, curve C). After 160 min 2.6 mol of an adenine compound was bound per Kp1 at -680 mV against saturated calomel reference electrode, and

Figure 2 Time-dependence of NMR spectra of Kp1 plus MgADP $^{-}$ reaction mixture

Spectrum A, 15 min after mixing; spectrum B, 36 min; spectrum C, 45 min; spectrum D, 65 min. Numbered peaks above spectrum C are: 1, MgAMP; 2, MgPi; 3, Kp1-AMP; 4, 5, MgADP $^{-}$. Inset: integrated NMR signals as a function of reaction time.

Figure 3 ^{31}P NMR spectra of Kp1 + MgADP $^{-}$ incubation mixtures and isolated reaction products

Spectrum A, Kp1 + MgADP $^{-}$ after 2.5 h at 22 $^{\circ}$ C; curve B, integrals of resonances of spectrum A; spectrum C, MgADP $^{-}$ and P_i separated from protein by gel filtration; spectrum D, isolated protein product of reaction; spectrum E, contents of dialysis bag after 18 h dialysis of Kp1 against MgADP $^{-}$. Identities of resonances: 1, MgAMP; 2, P_i ; 3, Kp1-AMP conjugate; 4, 5, MgADP $^{-}$.

nucleotide binding continued beyond this time. Increasing the redox potential of the medium to -464 mV accelerated the reaction and after 120 min 3.3 mol was bound with further increases indicated. In the presence of Kp2, the amount bound approached 1.6 mol but was relatively constant after 160 min.

Although ^{31}P NMR is applicable to the study of protein-nucleotide interactions [25], the relative insensitivity of this nucleus dictates nucleotide concentrations in the range 0.5–2 mM. Dye-oxidized Kp1 (100–200 μ M) was mixed with MgADP $^{-}$, and NMR spectra were recorded at times up to 2 h. Because of the limited sensitivity of the method, little change was detected initially; during the first 15 min of reaction only the unbroadened β and α ADP phosphate resonances were observed at -5.3 and -9.2 p.p.m. (Figure 2, spectrum A). However, incubation for a further 20 min resulted in the detection of new resonances near $+3$ and $+4.1$ p.p.m., corresponding to the chemical shifts of P_i and AMP standards respectively. In addition, the resonances arising from ADP became broadened with time owing to tight binding to Kp1 consistent with the isotopic experiment of Figure 1. The observed broadening resulted in the loss of the splitting arising from J coupling between the nuclear spins of the adjacent phosphorus nuclei of ADP. The intensity of the signals at $+4.1$ and $+3$ p.p.m. increased with further incubation (Figure 2, spectra C and D). An unidentified species with a broad resonance at -1 p.p.m. was detected after 35 min reaction time. The bound ADP β resonance was not shifted or decreased in magnitude compared with the α peak. The decline in the integrated intensity of the overlapping α and β resonances of free and bound ADP corresponded within 10% to the sum of the intensities of P_i , AMP and the new resonance at -1 p.p.m. after 2.5 h (Figure 2 inset; Figure 3, curve B). A similar reaction was observed with highly purified MoFe protein from *Azotobacter vinelandii*, Av1 (results not shown).

The specificity of the reaction of Kp1 and Av1 with ADP was tested by using comparable concentrations of highly purified bovine liver catalase (Sigma) as a control. Catalase was chosen because it is a 270 kDa tetramer bearing a surface lysine amino

Figure 4 Time course of the hydrolysis of Kp1-AMP in the presence or absence of Kp2

(A) Spontaneous decay of Kp1-AMP NMR and release of AMP. Kp1-AMP was made by dialysis at 15 °C and concentrated to 59 mg/ml. The mixture was equilibrated at 22 °C. NMR spectra were recorded every 30 min (2000 pulses) and resonances integrated. (B) P_i and ADP did not vary. (C) Hydrolysis of Kp1-AMP (35 mg/ml) in the presence of Kp2. Kp2 (5 mg) and dithionite (0.33 mM) were added after 5 min. (D) ADP decreased and P_i increased owing to turnover of Kp1-AMP.

group distribution resembling that of Kp1. Catalase did not broaden or otherwise alter the NMR spectrum of $MgADP^-$ and did not bind $[8-^{14}C]ADP$ as determined by the gel-permeation method. ADP bound to the known nucleotide-binding sites of Kp2 [8] resulted in broadening of the α -phosphate resonance of ADP and broadening and shift of the β resonance to -1.5 p.p.m., similar to that of the S-1 fragment of myosin (results not shown).

Identification of the products of the reaction of ADP with Kp1

Gel-permeation chromatography of incubation mixtures after 2.5 h of incubation gave a low-molecular-mass fraction containing ADP, AMP and P_i (identified by NMR) and a high-molecular-mass fraction containing Kp1. The broader resonance at -1 p.p.m. exhibiting no apparent J coupling remained with the protein (see Figure 3, spectrum D). Suggestive evidence that this signal arises from a Kp1-AMP conjugate was provided by time course data, where the P_i resonance and the -1 p.p.m. resonance associated with Kp1 appeared before significant accumulation of the free AMP (Figure 2, inset). Direct evidence for this species being AMP stably bound to Kp1 was obtained when the concentrated protein conjugate prepared by dialysis (see below) was incubated at 22 °C for 6 h. Over this period the time course of the decay of the -1 p.p.m. resonance was correlated with the appearance of a resonance with a chemical shift of $+4$ p.p.m., characteristic of free AMP (see Figure 4). The protein-nucleotide compound was putatively identified as Kp1-AMP because both the ^{14}C -labelled adenine ring and a single phosphate group were retained. The identity of free AMP as the major product spontaneously released from the conjugate was confirmed by comparison of the mobility of the compound with authentic AMP by TLC and phosphorimaging analysis on PEI plates.

Large-scale preparation and isolation of Kp1-AMP

Dialysis of Kp1 (90 μM) against $MgADP^-$ (2 mM) for 16–18 h produced a mixture of NMR-detectable species in which a resonance at -1 p.p.m. predominated after concentration of the protein to 58 mg/ml (Figure 3, spectrum E). In these experiments a resonance corresponding to free $MgAMP$ was also present in the dialysed, concentrated protein mixture, presumably formed as a hydrolysis product of the Kp1-AMP adduct. Quantification of total P bound per mol of Kp1 was complicated by potential paramagnetic broadening of the ADP resonances owing to the proximity of reduced FeMoco centres. Determination of the amount of non-dissociably bound nucleotide was therefore limited to the incorporation of radiolabelled nucleotides. When $[8-^{14}C]ADP$ was included in the dialysate, radioactivity in isolated Kp1-AMP indicated 8–10 mol of AMP bound, in contrast with the experiment of Figure 1 and previous binding experiments, where a maximum of 4 mol of nucleotide bound per mol of Kp1 was indicated [13]. Labilization of the terminal phosphate of tightly bound ADP might have resulted in additional surface binding of AMP during the long (18 h) dialysis period.

Stability of Kp1-AMP

To investigate the stability of the new species, ^{31}P NMR spectra of concentrated Kp1-AMP dialysis mixtures were recorded as a function of incubation time at 22 °C. Plots of integrals of the resonances from Kp1-AMP, $MgAMP$, $MgADP^-$ and P_i in Figures 4(A) and 4(B) indicated a linear net hydrolysis process under these conditions with an apparent zero-order rate of release of AMP of 6.2 nmol/min. The initial NMR spectrum of the mixture used to obtain the results in Figure 4 corresponded to that of Figure 3 (spectrum E). Figure 4(B) shows that P_i and $MgADP^-$ were relatively invariant within experimental error (± 0.05 nmol) over 500 min in this experiment.

Figure 5 Effect of sample temperature on Kp1-AMP NMR spectrum

Kp1-AMP was made by dialysis of Kp1 against 2 mM MgADP^- for 16 h. The protein was concentrated to 49 mg/ml and equilibrated at each indicated temperature for 5 min. All spectra were acquired within a 60 min period to minimize hydrolysis of Kp1-AMP. Numbered peaks represent: 1, MgAMP; 2, P_i ; 3, Kp1-AMP; 4, 5, MgADP^- . Inset: width of Kp1-AMP resonance at half height as a function of sample temperature.

Figures 4(C) and 4(D) show the effect of adding sub-stoichiometric amounts of Kp2 on the stability of Kp1-AMP to a dialysis reaction mixture. Kp1-AMP was concentrated to 160 μM and mixed with Kp2 and dithionite (0.33 mM) at a molar Kp1/Kp2 ratio of 5. In this case, the pseudo-zero-order increase in the integrated NMR resonance of free AMP increased to 8.0 nmol/min. The concentration of MgADP^- declined and that of P_i increased markedly (Figure 4D), indicating that Kp1-AMP was being formed from ADP as well as being hydrolysed under these conditions. This contrasts with the constant ADP and P_i concentrations in similar incubations lacking Kp2 (Figure 4B). Kp2 accelerated the rates of both formation and hydrolysis of Kp1-AMP.

Effect of temperature on NMR signals of Kp1-AMP

The effect of temperature on chemical shifts and line widths of NMR resonances of protein-bound nucleotides can be used to distinguish stably bound from exchangeable ligands. Decreased rates of exchange at temperatures near 0 °C resulted in an enhancement of resolution of the overlapping β resonances of ADP bound to myosin S-1 fragment [26].

Low-temperature-induced decreases in molecular motion of a ligand in the absence of rapid exchange would be expected to enhance spin-spin relaxation processes including those resulting from interaction of the ^{31}P nuclear spins with paramagnetic centres of Kp1. The resultant broadening of resonances at lower temperatures provides a gauge of the thermal mobility of non-dissociable nucleotide-binding sites.

The temperature dependence of the Kp1-AMP resonance showed that further broadening occurred at temperatures below 20 °C with no J coupling or change in chemical shift (Figure 5). Linewidth at half height (Figure 5, inset) was inversely dependent on temperature, consistent with the location of bound nucleotide at temperature-sensitive, mobile sites on the surface of Kp1. The observed temperature effect contrasts with that of nucleotide binding by myosin. The width of the resonances of free MgADP^- ,

Figure 6 NMR spectra of Kp1 plus nucleotide reaction mixtures

(A) Kp1 incubated with 4 mM ATP for 6 h; (B) Kp1 dialysed against 2 mM MgATP^{2-} for 16 h; (C) MgATP^{2-} dialysate; (D) Kp1 dialysed against 2 mM MgADP^- for 16 h and concentrated to 59 mg/ml; (E) MgADP^- dialysate. Numbered peaks are: 1, MgAMP; 2, MgP_i ; 3, Kp1-AMP; 4, 5, MgADP^- and MgATP^{2-} ; 6, β -phosphate of MgATP^{2-} .

MgAMP and MgP_i present in the dialysis mixture were narrower, and J coupling was more pronounced, at lower temperatures.

Specific requirement for ADP in the formation of Kp1-AMP

Kp1 was dialysed for 16 h against MgATP^{2-} and MgAMP to determine whether the formation of Kp1-AMP specifically required MgADP^- . MgAMP produced no new resonances and the linewidth of the ^{31}P NMR signal of free AMP in the protein sample was not altered.

During dialysis against MgATP^{2-} , a Kp1-AMP resonance appeared much more slowly than in a parallel experiment with MgADP^- (compare spectra B and D in Figure 6). MgATP^{2-} diffusing to the protein was hydrolysed as indicated by the appearance of P_i and α and β resonances of ADP in the protein sample recovered from the dialysis bag (Figure 6, spectrum B). The hydrolysis of ATP can be attributed to low, residual Kp2 activity in Kp1 with resultant ATPase activities as summarized in the Materials and methods section. However, an ATPase activity inherent to the Kp1 protein cannot be ruled out. The spectrum of the dialysate was dominated by signals arising from free MgATP^{2-} but also had a small phosphate peak and shoulders on the γ and α peaks of ATP owing to the formation of dialysable ADP and P_i (Figure 6, spectrum C).

Comparison of spectra A and B of Figure 6 with spectra A and E respectively of Figure 3 shows that levels of Kp1-AMP approaching that observed in the ADP dialysis do not appear until ATP is hydrolysed. It is thus unlikely that MgATP^{2-} reacted directly with Kp1 to give Kp1-AMP. MgADP^- formed by hydrolysis of ATP reacted with Kp1 to produce the protein-nucleotide adduct. Inorganic pyrophosphate was not detected in any of our experiments.

Apparent differences in the free nucleotide and P_i spectra between the dialysate and the contents of the dialysis bag can arise from three sources. First, the time required to concentrate

Table 1 Exchange of [$8\text{-}^{14}\text{C}$]MgADP $^{2-}$ into ATP catalysed by Kp1 plus Kp2 during dialysis

An equimolar mixture of Kp1 and Kp2 (280 nmol of each) were dialysed against 2 mM MgATP $^{2-}$ in 50 mM Tris/HCl buffer, pH 7.8, containing 100 mM NaCl, 5 mM MgCl $_2$ and 2.7 μM labelled ADP for 16 h at 15 °C. The protein and contents of the dialysis bag were separated from the free nucleotides by gel filtration, concentrated and reacted for 2 h in an NMR sample tube. During this period the ATP concentration declined to less than 0.1 mM and was not detectable by NMR. The unbound nucleotides and the redissolved protein were streaked at the origin of PEI chromatograms and chromatographed as detailed in the Materials and methods section. Autoradiograms were developed on phosphorimager plates, and labelled nucleotides were quantified. An autoradiogram standard curve was used to calculate total amounts of labelled nucleotides in the free nucleotide fraction and the denatured Kp1 protein fraction. Values are means $\pm$ S.E.M. for three analyses.

Sample	Radioactivity (nmol as [$8\text{-}^{14}\text{C}$]adenine)			
	Origin	ATP	ADP	AMP
Denatured protein	2.57 $\pm$ 0.23	0.0 $\pm$ 0.18	1.70 $\pm$ 0.23	4.66 $\pm$ 0
Free nucleotides	0.0 $\pm$ 0.18	12.8 $\pm$ 0.66	110.7 $\pm$ 1.9	1.20 $\pm$ 0
[^{14}C]ADP standard	0.0 $\pm$ 0.18	0.0 $\pm$ 0.18	10.0 $\pm$ 0.65	0.0 $\pm$ 0.18

the protein and acquire NMR spectra in this experiment (40 min) allowed ADP hydrolysis to continue in the sample. Secondly, the proteins were concentrated 2-fold over the freely diffusing nucleotides and P $_i$, which had been in equilibrium with the dialysis medium. Lastly, ADP in the protein mixture must be tightly bound at a site distinct from the bound AMP site, and NMR signals broadened to an extent that rendered them undetectable. This broadening could arise either as a consequence of binding close to a paramagnetic centre (presumably FeMoco) or at a non-mobile region of the Kp1 protein.

Reversibility of hydrolysis of MgADP $^{2-}$ and MgATP $^{2-}$

We have previously reported that Kp1 catalyses the slow exchange of [^{32}P]P $_i$ into the terminal phosphates of both ADP and ATP [12]. To determine whether the nucleotide adenine ring also exchanged during reductant-limited catalytic turnover, an equimolar mixture (80 μM) of Kp1 and Kp2 was dialysed for 18 h against 2 mM MgATP $^{2-}$ containing [$8\text{-}^{14}\text{C}$]ADP and 1 mM dithionite. ATP entering the dialysis bag was hydrolysed by nitrogenase turnover at 15 °C. The system became reductant-limited before isolation of the protein sample for analysis.

The reaction of Kp1 with ADP (formed during the initial turnover period) to give Kp1-AMP was confirmed by ^{31}P NMR (results not shown). The protein was isolated by gel filtration, precipitated with 2% (v/v) perchloric acid, centrifuged and redissolved. The protein fraction and the gel-filtration eluate containing free nucleotides were analysed by TLC and radio-phosphorimaging as indicated in the Materials and methods section, and the results are summarized in Table 1.

Significant amounts of ^{14}C -labelled nucleotide remained at the chromatogram origin of the protein sample as a result of tight nucleotide binding to Kp1. The labelled nucleotides released from the denatured protein during TLC at pH 3.5 contained no ATP but did contain both AMP and ADP in a 3:1 molar ratio. Therefore ADP as well as AMP remained associated with the denatured protein even though only AMP was detected in the NMR spectrum of intact Kp1 (Figure 6, spectrum D).

Table 1 shows that 10% of the total radioactivity in the free nucleotide fraction was present in the ATP chromatogram spot even though ATP was not detected by NMR. Because only 1% of the free nucleotide label was found in AMP (arising from the

breakdown of Kp1-AMP), adenine ring exchange into ATP cannot be due to conventional adenylate kinase activity, which would have resulted in equal incorporation of the ADP label into ATP and AMP. The exchange of the adenine nucleus into ATP can only have resulted from reversal of hydrolysis of ATP to ADP at 15 °C during the catalytic turnover period.

Previous results indicating the exchange of [^{32}P]P $_i$ into both ADP and ATP when Kp1 was incubated with ADP or ATP [12] showed that the hydrolysis of ADP to AMP in the presence of Kp1 is also reversible. Pre-steady-state kinetic studies have also indicated that ATP cleavage by nitrogenase components during turnover is reversible at 6 °C (but not at 23 °C) with an equilibrium constant near 1 [27].

Our finding of tight binding of ADP to MoFe protein provides a rationale for the exchange of the adenine ring into ATP through reversal of the hydrolysis reactions at 15 °C. Rapid separation by centrifugal gel filtration of an Av1-Av2 complex from a reaction mixture during catalytic turnover indicated previously that both P $_i$ and ADP formed from the hydrolysis of ATP remained associated with the proteins [28].

Catalytic significance of Kp1-AMP

To determine whether Kp1-AMP was formed during enzyme turnover, catalytic amounts of Kp1 (0.5 μM) plus 0.25 μM Kp2 were incubated with 1 mM [^{14}C]MgATP $^{2-}$ in the presence of 1 mM dithionite plus a reducible substrate (acetylene). In contrast with the standard assay, which contains an ATP-regenerating system, [^{14}C]MgADP $^{2-}$ was allowed to accumulate. Analysis of the nucleotides in the supernatant showed the expected formation of labelled ADP, but only insignificant levels of radiolabelled AMP were detected before acetylene reduction ceased owing to ADP accumulation and reductant limitation. However, 5% (0.25 mM) of the original ATP accumulated as [^{14}C]AMP during subsequent incubation for up to 2 h (results not shown). This indicates that MgATP $^{2-}$ does not inhibit the formation of Kp1-AMP, which might therefore occur *in vivo* under reductant-limited conditions when ATP/ADP ratios seldom exceed 1.2 [4].

Table 2 shows that the catalytic activity of Kp1-AMP in the reduction of acetylene or protons was not impaired compared with untreated Kp1. We have also demonstrated that the AMP residue remains bound to Kp1 during catalytic turnover by incubating Kp1-AMP with equimolar Kp2, S $_2$ O $_4^{2-}$ (0.8 mM) and MgATP $^{2-}$ (1.5 mM). The NMR spectrum after 15 min of incubation showed the complete disappearance of ATP but the persistence of Kp1-AMP (results not shown).

Possible involvement of lysine residues in binding nucleotides

The lysine-modifying reagent OPT inhibited Kp1 nitrogenase activity when preincubated with the protein as described in the Materials and methods section. Figure 7 shows the decrease in specific activity of Kp1 as a function of molar excess of OPT (curve 1). MgADP $^{2-}$ but not MgATP $^{2-}$ provided complete protection against the inhibitory effect of a 6-fold excess of OPT over Kp1 as shown by curves 2 and 3. The inset to Figure 7 shows that OPT also inhibited ^{32}P exchange into ADP. In the absence of OPT pretreatment, 7–9.5% of the total ^{32}P present in the Kp1 reaction mixture exchanged into ADP. Preincubation with a 30-fold molar excess of OPT eliminated this exchange reaction completely. The exchange reaction was not inhibited by an up to 20-fold excess of OPT in the presence of MgADP $^{2-}$. The observed protection of OPT reaction sites was much less pronounced if ATP replaced ADP in the OPT preincubation mixture.

Table 2 Catalytic activity of Kp1-AMP determined with acetylene and proton reduction assays

Kp1 was reacted with MgADP^- to form Kp1-AMP with 2–4 mol of AMP bound per mol of protein. Kp1-AMP was then concentrated and some samples were separated from unbound nucleotides by gel filtration. The protein was assayed immediately or subjected to controlled oxidation and/or NMR analysis. Samples were stored bead-frozen as indicated. Abbreviation: n.d., not determined.

Sample treatment	Specific activity (nmol of product/min per mg)	
	Acetylene reduction	Proton reduction
Kp1-AMP isolated and confirmed by NMR (2 h)	1949 $\pm$ 146 (253.5 $\pm$ 3.5)*	1976 $\pm$ 66.5
Kp1-AMP (from dialysis) oxidized to -350 mV, NMR (4 h), frozen	1102	n.d.
Kp1-AMP isolated, NMR (4 h), frozen	1396	n.d.
Kp1-AMP made by dialysis, Kp2 added, NMR (8 h), frozen	890	n.d.
Kp1, no treatment, frozen	2000 $\pm$ 100 (250 $\pm$ 10)*	1900 $\pm$ 90

* Hydrogen produced under an atmosphere of 8% C_2H_2 .

Figure 7 Effect of OPT preincubation on Kp1 specific activity and ^{32}P exchange into ADP

Symbols: ■, Kp1 preincubated with indicated levels of OPT for 30 min at 25 °C; ●, 1.25 mM ATP added before preincubation with OPT; ▲, 1.25 mM ADP added before OPT. Inset: percentage of total ^{32}P incorporated into ADP after incubation of Kp1 with P_i and OPT plus MgADP^- (△) or OPT plus MgATP^{2-} (□).

Conclusions

All of the present and previous results demonstrating nucleotide binding by nitrogenase MoFe proteins and exchange reactions catalysed by Kp1 can be explained by the following reaction sequence:

Reaction (1), detected by equilibrium gel-permeation experiments, is reversible with approximately equivalent rates in the forward and reverse reactions. Reaction (2), indicating tight, non-equilibrium binding of ADP to Kp1, is consistent with experiments where ^{14}C -labelled nucleotides remained associated with Kp1 after removal of exchangeable MgADP^- by gel permeation [13] and with retention of ADP in the denatured

protein fraction after incubation with radiolabelled ADP. Slow reversal of reaction (3) results in the incorporation of ^{32}P into ADP [12], which can then be released from the protein by the reversal of reaction (2). Adenylate kinase, a common contaminant of unpurified nitrogenase proteins [12], does not catalyse this exchange reaction because no free P_i participates in the adenylate kinase reaction where ADP, AMP and ATP remain bound to the enzyme's catalytic site during nucleotide interconversion. Reaction (4) seems to be irreversible because we were unable to detect Kp1-AMP after dialysis of Kp1 against an excess of MgAMP . The slow rate of hydrolysis and release of AMP from the isolated conjugate containing multiple AMP residues per Kp1 confirms that AMP is covalently bound in Kp1-AMP. No adenylate kinase was present in Kp1, and ADP was not hydrolysed to AMP unless Kp1 P clusters were oxidized, indicating that contaminating phosphatase activity was not responsible for the hydrolysis of ADP. Furthermore incubation of Kp1-AMP in a dialysis mixture containing ADP did not result in an increase in inorganic phosphate over a 4 h period unless Kp2 was present (Figure 4).

Kp1-AMP was formed when Kp1 was dialysed against MgATP^{2-} or MgADP^- but ATP did not react directly with Kp1 to give Kp1-AMP. Incorporation of $[8\text{-}^{14}\text{C}]\text{ADP}$ into ATP during dialysis of an equimolar mixture of Kp1 and Kp2 against 2 mM MgATP^{2-} containing a small amount of labelled ADP shows that ATP hydrolysis by the nitrogenase system is reversible at 15 °C. Because nitrogen fixation can take place at temperatures between 5 and 20 °C [29], reversibility of ATP hydrolysis might occur under the temperate zone field conditions normally encountered by diazotrophic bacteria.

Reductant-independent hydrolysis of ATP by mixtures of heterologous MoFe and Fe proteins (Av1 plus Cp2 and Cp1 plus Av2) have been reported to hydrolyse MgADP^- to AMP and free adenosine during slow catalytic turnover with MgATP^{2-} [30]. By analogy with the present results, the formation of AMP would be expected to occur through intermediate steps of tight ADP binding followed by terminal phosphate labilization, covalent binding of AMP and subsequent release of AMP.

Although the reported interactions of the nitrogenase MoFe proteins with ADP seem to be unique, a direct role has not been established for the Kp1-ADP complex or Kp1-AMP during nitrogenase catalytic turnover. Formation of the AMP conjugate does not result in loss or known modification of the catalytic activity of Kp1. However, under physiological conditions where

intracellular ADP concentrations are significant and energy charge values near 0.6 prevail [29], Kp1-AMP could be formed.

OPT readily blocks lysine primary amine groups and this reaction could be responsible for the inhibition of both catalytic activity and the exchange of [^{32}P]P_i with the terminal phosphates of ADP and ATP. Because MgADP⁻ protects Kp1 against both inactivation and OPT-induced inhibition of P_i exchange, surface lysine groups are indicated as initial sites of ADP binding to Kp1. In the crystal structure of Av1 [5] there are four highly conserved [31] surface lysine amino groups, α -50 and α -26, located 10 Å above the buried FeMoco centres, which could react readily with OPT. Tight binding of ADP at these conserved sites of Kp1 could account for paramagnetic broadening of ADP phosphorus NMR resonances. These residues are located 20 Å away from phenylalanine residues α - and β -125, which are hydrophobic components of a postulated docking surface for the Fe protein located near the interface of the MoFe protein α and β subunits [7]. If these or other nearby lysine amino groups were blocked by OPT, an inhibitory effect on Fe protein binding during turnover might be expected.

The demonstrated effects of Kp2 and redox state of the MoFe protein on the formation of Kp1-AMP suggest the need for further investigation of the role(s) of the tightly bound nucleotide species in processes requiring nucleotides and the Fe protein, including catalytic cycles under adverse conditions or cofactor insertion [32]. Tight binding of ADP and the formation of the Kp1-AMP adduct under certain physiological conditions are indicated by the results reported here. It is probable that tight binding of ADP labilizes a terminal phosphorus—oxygen bond leading to the release of P_i and the covalent bonding of AMP at multiple surface lysine sites. The exchange of both the adenine ring and the terminal phosphate groups of ADP and ATP requires the presence of either the isolated MoFe protein or a complex of Kp1 and Kp2.

We gratefully acknowledge financial support from BBSRC and from NATO for Collaborative Research Grant no. 890112.

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