Nucleotide binding by the nitrogenase Fe protein: a ^{31}P NMR study of ADP and ATP interactions with the Fe protein of *Klebsiella pneumoniae*

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Investigation of the interaction of MgADP^- and MgATP^{2-} with the Fe protein of *Klebsiella pneumoniae* nitrogenase by ^{31}P NMR showed that the adenine nucleotides are reversibly bound in slow exchange with free nucleotides. Dissociation of the MgADP^- -Fe protein complex was slow enough to enable its isolation by gel filtration, thus permitting the assignment of resonances to bound nucleotides. Spectra of ADP bound to Kp2 were similar to spectra of ADP bound to the myosin motor domain. Oxidative inactivation of a Kp2- MgADP^- complex with excess ferricyanide ion eliminated exchange between bound and free ADP, indicating that the intact iron sulphur cluster, located 20 Å from the binding sites, is required for the reversible binding of MgADP^- . A change in conformation on controlled oxidation of Kp2 with indigocarmine increased the chemical shift of the β phosphate resonance of bound MgADP^- . Both oxidized and reduced

conformers were observed transiently in the absence of dithionite. The ^{31}P resonances of both the β and γ phosphates of bound MgATP^{2-} indicated major changes in environment and labilization of both groups on binding to the Fe protein. Highly purified Kp2 slowly hydrolysed ATP, resulting in mixtures of bound nucleotides. Partial occupation of Kp2 MgATP^{2-} -binding sites ($N = 1.9 \pm 0.2$, $K_d = 145 \mu\text{M}$) in concentrated protein solutions was demonstrated by flow dialysis. Scatchard plots of data for bound and free ligand obtained after equilibration with Kp2 were linear and no co-operative interactions were detected. We conclude that MgADP^- stabilizes the oxidized Fe protein conformer and this conformation in turn triggers the dissociation of the Fe protein from the MoFe protein in the rate-limiting step of the overall process of dinitrogen reduction.

INTRODUCTION

Molybdenum nitrogenases consist of two metalloproteins, the iron protein (Fe protein) and the molybdenum-iron protein (MoFe protein), which together catalyse the reduction of dinitrogen to ammonia in an ATP-dependent reaction (reviewed in [1–3]). During catalytic turnover, the reduced Fe protein of *Klebsiella pneumoniae* nitrogenase (Kp2) binds MgATP^{2-} then forms a complex with the MoFe protein (Kp1) and transfers a single electron. ATP is hydrolysed to ADP in this reaction but the mechanism of energy transduction, whereby ATP hydrolysis facilitates substrate reduction, requires clarification. Dissociation of Kp2 with bound MgADP^- from the MoFe protein is the rate-limiting step of the reaction cycle, which is repeated eight times before N_2 is fully reduced to ammonia [4]. Pre-steady-state studies have shown that the release of P_i formed by ATP hydrolysis occurs before dissociation of the electron transfer complex [5]. The overall eight-electron process is coupled to the hydrolysis of a minimum of 16 ATP *in vitro* and is accompanied by the formation of 1 mol of H_2 per mol of N_2 converted to ammonia:

The consensus view of nitrogenase function is that the ATP bound to the Fe protein is hydrolysed to provide the energy necessary for completion of the catalytic cycle. A recently published 3 Å resolution structure of an AlF_4^- -stabilized puta-

tive transition-state complex of the *Azotobacter vinelandii* nitrogenase proteins contains an Av2 molecule bound to each half of the tetrameric MoFe protein [6]. The two Av2 nucleotide-binding sites at the interface of the subunits of the Av2 homodimer are fully occupied by MgADP^- and AlF_4^- in the crystallized complex model. The Walker-type binding sites of the Fe protein subunits consist of P loop motifs comparable to those of other nucleotide binding proteins [7,8]. However, the catalytic group(s) responsible for ATP hydrolysis in the nitrogenase complex remain to be ascertained with certainty and it should be noted that, in isolation, Fe proteins do not catalyse the rapid hydrolysis of ATP.

Chemical shifts of the ^{31}P resonances of bound nucleotides reflect bonding constraints that alter the shielding of the P nucleus, environmental factors and chemical exchange rates [9]. Chemical shifts of bound nucleotide phosphate groups in slow exchange can reflect the extent of distortion of R-O-P and P-O-P bonds from the tetrahedral configuration of free phosphates [10]. Thus ^{31}P NMR presents the additional possibility of directly studying subtle, time-dependent or redox-state-dependent changes in protein conformation that alter chemical-shift and line-width parameters. These effects can potentially provide information on nitrogenase-nucleotide interactions that are not accessible by other spectroscopic methods. ^{31}P NMR spectroscopy has been used to acquire evidence for an AMP adduct of Kp1 that was formed in a process involving the hydrolysis of reversibly bound ADP [11]; the tight binding of MgADP^- and MgATP^{2-} to the *Clostridium pasteurianum* Fe protein (Cp2) has also been observed by this method [12]. There is strong evidence from a range of techniques that conformational

Abbreviations used: K_d , average dissociation constant for a protein ligand/binding system; S-1, truncated motor domain of myosin. The nitrogenase metalloproteins are abbreviated according to the genus and species of the diazotrophs from which they were obtained, e.g. the Fe protein from *Klebsiella pneumoniae* is Kp2, the *Azotobacter vinelandii* Fe protein is Av2 and the *Clostridium pasteurianum* Fe protein is Cp2; the MoFe protein from *A. vinelandii* is Av1, etc.

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changes occur in the isolated Fe protein on binding nucleotides [3,6]. The major effects of these changes that have been identified are a decrease in redox potential of the Fe_4S_4 cluster and an associated increase in accessibility of the cluster to oxygen and Fe chelators. It is also clear from the three-dimensional structure of the nitrogenase complex that the Fe protein containing bound ADP-AlF_4^- has undergone major conformational changes [6]. Confirmation of the importance of the primary and secondary structures of the Fe protein to catalytic activity of the nitrogenase complex can be deduced from a Lys-127 deletion mutant of Av2, which assumes the nucleotide-bound conformation in the absence of nucleotide. The mutant Av2 also binds tightly to the MoFe protein and transfers a single electron to it but cannot complete the catalytic cycle and consequently has no nitrogenase activity [13,14].

Here we report a ^{31}P NMR study of the interaction of MgADP^- and MgATP^{2-} with catalytically important oxidized and reduced forms of Kp2. The reduced iron-sulphur cluster of the free Fe protein is located approx. 20 Å from the putative nucleotide-binding sites [2] and is probably too remote to cause paramagnetic broadening of bound nucleotide phosphorus NMR resonances. We also present results indicating that the intact cluster is required for maintenance of the nucleotide-binding site structure. In the course of these studies we observed slow changes in the NMR spectra of bound nucleotides. Further insight into these processes was obtained from flow dialysis experiments with MgATP^{2-} [15,16].

Equilibria between multiple conformational states have been postulated for the truncated heavy chain (S-1) of myosin on binding nucleotides [17]. Here we compare the mode of binding of MgADP^- by the nitrogenase Fe protein and the motor domain of myosin and show that the changes in the NMR spectra of the nucleotide are very similar. In the Fe protein, oxidation-state-dependent changes in the NMR signals arising from bound ADP indicate the existence of previously undetected conformational states of the nitrogenase Fe protein that are potentially important in the catalytic cycle of this system [18,19].

MATERIALS AND METHODS

Fe protein purification

Kp2 was purified from extracts of *Klebsiella pneumoniae* (oxytoca) N.C.I.B. 12204 in the presence of 2 mM dithionite and assayed with added Kp1 as described previously [20]. All subsequent operations were performed in an anaerobic chamber at or below 1 p.p.m. O_2 . Fractions with a minimum specific activity for acetylene reduction of 1500 nmol of ethylene formed/min per mg of protein were pooled and concentrated in single-use mini-concentrators (Amicon). Background nitrogenase activity due to residual Kp1 in purified Kp2 was less than 1 nmol of ethylene/min per mg. Adenylate kinase (EC 2.7.4.3) activity was not detected in purified Kp2 under aerobic conditions [11]. Adenine nucleotides were hydrolysed by Kp2 at low rates (not more than 0.05 nmol/min per mg) detectable at protein concentrations greater than 10 mg/ml. This process could be followed by observing time-dependent changes in NMR spectra. This activity was present in all Kp2 preparations and was not removed by further purification. Calculations based on the low residual nitrogenase activity of Kp2 due to contaminating Kp1 suggest that this could account for the accumulation of some ADP from ATP. This would be consistent with a reported requirement for both nitrogenase proteins for the formation of AMP from ADP when the Fe protein is in excess [21]. Oxidized Kp2 was more effective than the reduced protein in hydrolysing adenine nucleo-

tides and we attribute this to an inherent reactivity of Kp2 detectable at the high protein concentrations used.

Dithionite-reduced Kp2 was freed of dithionite by passage through 1.2 cm $\times$ 18 cm columns of Bio-Gel P-6 DG gel filtration medium (Bio-Rad) equilibrated with buffer containing 50 mM Tris/HCl, pH 7.4, 50 mM NaCl and 10 mM MgCl_2 . Indigocarmine (Sigma Chem. Co.) bound to Dowex AG1-X8 ion-exchange resin was used to oxidize Kp2 reversibly [22]. A 1.2 cm $\times$ 2.5 cm column of the dye-saturated resin was layered on the top of the gel-filtration medium, and aliquots of the concentrated protein were allowed to percolate into the Dowex layer. After remaining in contact with the resin for 15 min, the protein was washed through the layered column with the same buffer and collected in a minimum volume. This procedure removed traces of the dye. The oxidation state of Kp2 samples was confirmed by EPR or by recording the visible spectra of small aliquots after dilution to 1 mg/ml with anaerobic buffer. Indigocarmine oxidation of the 66 kDa Kp2 protein resulted in the loss of 90% of both the $S = 1/2$ and the $S = 3/2$ EPR signals of the reduced Kp2 iron-sulphur centres and caused an increase in absorbance at 430 nm equivalent to an absorbance change of 4.4 mM^{-1} .

Determination of nucleotide binding

^{31}P NMR

^{31}P NMR spectra of the free nucleotides and nucleotides bound to Kp2 were recorded at 22 °C with a JEOL GSX270 spectrometer with a 10 mm multifrequency probe (^{31}P freq. = 109.25 MHz) or with a JEOL λ -400 spectrometer with a 5 mm multifrequency probe (^{31}P freq. = 161.70 MHz). A bilevel, proton-decoupling pulse sequence was repeated 1000–4000 times with minimal temperature rise (less than 1 °C) measured with a thermocouple placed in the samples after data acquisition. Integration of free and bound nucleotide resonances was used to calculate the concentrations of nucleotide species and free P_i in the absence of paramagnetic or extreme exchange broadening.

Samples (1–3 ml) of protein were dialysed against nucleotide solutions for 16 h at 16 °C inside an anaerobic chamber before the addition of 10% (v/v) $^2\text{H}_2\text{O}$ and transfer to the NMR spectrometer. In these experiments the protein was in dynamic equilibrium with the ligand before placement in the spectrometer. In an alternative procedure, MgADP^- or MgATP^{2-} was added anaerobically to Kp2 in 5 or 10 mm NMR sample tubes and data acquisition commenced immediately. Satisfactory signal-to-noise ratios were obtained after 10–30 min, depending on the protein and nucleotide concentrations. Spectra were acquired at subsequent time intervals to determine the effect of slow hydrolysis on nucleotide concentrations.

Flow dialysis

Estimates of ATP bound per mol of Kp2 at equilibrium were made under a continuous flow of oxygen-free nitrogen at 22 °C in a magnetically stirred flow dialysis cell. The spiral dialysate chamber had a volume of 50 μl [15]. The flow dialysis cell was thermally isolated from the magnetic stirrer with a 6 mm polystyrene foam sheet, limiting the temperature rise in the cell to less than 0.3 °C/h. $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ was added to 3.5 mg of dithionite-reduced protein (110 μM) in 0.5 ml of 50 mM Tris/HCl buffer, pH 7.4, containing 10 mM MgCl_2 and 50 mM NaCl. The rate of dialysis was measured as the amount of ATP passing through the membrane per unit time at a constant flow rate of 0.5 ml/min. Fractions (0.5 ml) of the continuously flowing dialysate were collected and the radioactivity was determined by

liquid-scintillation counting of 50 μ l aliquots. After equilibration of Kp2 with a predetermined concentration of radionucleotide (range 20–200 μ M) for 5 min, a pulse of unlabelled nucleotide (1.2 mM) was added to the cell in a small volume (5 μ l) while dialysate collection was continued. A non-binding control containing 17 mg of highly purified catalase (Sigma Chemical Co.) was treated in the same way as the Kp2 samples.

Equilibration of MgATP^{2-} across the dialysis membrane was more rapid than equilibration between nucleotides and Kp2-binding sites at the flow rates employed. The initial appearance of the ligand in the dialysate reflected the equilibration of MgATP^{2-} across the dialysis membrane in both the control and Kp2. Subsequent changes in radioactivity in the Kp2 dialysate indicated the approach to equilibrium binding of MgATP^{2-} by the protein at non-saturating levels of ligand. The catalase control also allowed an assessment of the effects of possible artifacts such as depletion of ATP from the dialysis chamber or non-specific changes in the dialysis rate.

The rate of exchange of nucleotide bound to Kp2 in the presence of saturating ligand (more than 1.2 mM) can be observed by flow dialysis provided that the release of radiolabelled nucleotide from the protein is slower than equilibration of the ligand across the dialysis membrane. Bound and free nucleotide concentrations in the dialysis chamber at equilibrium were calculated for each initial concentration of ATP and the results were analysed with Scatchard plots.

RESULTS AND DISCUSSION

NMR spectra of MgADP^- bound to reduced Kp2

In preliminary experiments designed to investigate the binding of nucleotides to Kp2 we observed time-dependent changes in NMR spectra. To minimize this complicating factor we used bulk dialysis to generate samples in which the protein was initially at equilibrium with a specified nucleotide concentration. Spectrum A in Figure 1 was obtained after reduced Kp2 had been dialysed for 18 h against a 3-fold excess of MgADP^- .

The rate of dissociation of the reduced Kp2– MgADP^- complex that was formed on dialysis of reduced Kp2 against MgADP^- was sufficiently slow to allow the separation of the Kp2– MgADP^- complex from free nucleotide by anaerobic gel filtration (Figure 1, spectrum C). On the basis of nucleotide standards (see Figure 2, spectrum E), we assign resonances 1 and 2 of spectrum C in Figure 1 to free AMP (+4.5 p.p.m.) and P_i (+3.1 p.p.m.) formed by further slow hydrolysis of the bound MgADP^- . Resonances 3 and 4 correspond to resonances 1 and 3 of spectrum A in Figure 1 and this allows the unequivocal assignment of these resonances at –1.6 p.p.m. and –9.1 p.p.m. to the β and α phosphates respectively of MgADP^- bound to reduced Kp2. Thus the β resonance of ADP was shifted to a higher frequency from –5.2 to –1.6 p.p.m. on binding to the protein, whereas the α resonance did not shift by more than 0.2 p.p.m. on binding. Similar results have been reported for ADP bound to the S-1 chain of mammalian myosin [17]. In both cases the bound nucleotide exchanges slowly with free nucleotide, allowing the resolution of the free and bound β phosphate resonances.

Integration of the ADP β phosphate resonances of spectrum A in Figure 1 indicated that 0.76 mM MgADP^- was bound by 0.82 mM reduced Kp2, with 1.7 mM MgADP^- remaining free in solution. In a similar experiment where 2.9 mM ADP was added directly to reduced Kp2, 0.80 mM MgADP^- was bound to 0.75 mM Kp2 in equilibrium with 2.1 mM free MgADP^- . On the basis of the assumption of two ADP binding sites on Kp2 [7,22], approximately one-half of the total binding sites remained

Figure 1 ^{31}P NMR spectra of Kp2– MgADP^- complexes recorded at 22 °C

Kp2 was reacted with, or dialysed against, MgADP^- as described in the Materials and methods section. Spectrum A: dithionite-reduced Kp2 (0.82 mM) dialysed against MgADP^- (2.46 mM). The NMR spectrum was recorded in a 10 mm sample tube within 20 min of termination of dialysis. Resonances of free and protein-bound ADP are indicated as: resonance 1, β phosphate group of MgADP^- bound to Kp2 (β_b^{red}); resonance 2, β phosphate group of MgADP^- free in solution (β_f); resonance 3, overlapping resonances of α phosphate groups of bound and free ADP ($\alpha_f + \alpha_b^{\text{red}}$). Spectrum B: sample of spectrum A treated with excess $\text{K}_3\text{Fe}(\text{CN})_6$ (6 mM) for 20 min. Resonance 1, β resonance of MgADP^- remaining bound to inactivated Kp2; resonance 2, β_f ; resonance 3, α_f superimposed on α resonance of MgADP^- remaining bound. Spectrum C: reduced Kp2 with bound MgADP^- isolated after dialysis by rapid gel-filtration chromatography. Kp2, 0.5 mM. Resonances 1 and 2, AMP and P_i formed by hydrolysis of ADP; resonance 3, β_b^{red} ; resonance 4, β_b^{red} . Spectrum D: reduced Kp2 after dialysis and incubation for 2 h in a 5 mm NMR sample tube under nitrogen. Approximately half of the total Kp2 present initially was auto-oxidized in the absence of dithionite. Resonances 1 and 2, free AMP and P_i ; resonance 3, β_b^{ox} ; resonance 4, β_b^{red} ; resonance 5, β_f ; resonance 6, $\alpha_b^{\text{red}} + \alpha_f + \alpha_b^{\text{ox}}$. Spectrum E: indigocarmine-oxidized Kp2 (1 mM) dialysed against MgADP^- (3 mM). The spectrum was recorded 20 min after the cessation of dialysis. Resonance 1, β_b^{ox} ; resonance 2, β_f ; resonance 3, $\alpha_b^{\text{ox}} + \alpha_f$. Spectrum F: indigocarmine-oxidized Kp2 (same sample as in spectrum E) incubated at 22 °C for 2.5 h. All free nucleotide was hydrolysed to AMP and P_i . Resonance 1, AMP and P_i ; resonance 2, β_b^{ox} ; resonance 3, α_b^{ox} .

unoccupied in these spectra and bound-to-free ratios were in the range 0.28–0.45.

Spectrum D in Figure 1 was obtained with the sample of spectrum C after 2 h of incubation at 22 °C. Some free MgADP^- was observed (resonances 5 and 6) owing to slow dissociation of the isolated Kp2– MgADP^- complex. The intensity of resonances 1 and 2 increased during this time, indicating the slow hydrolysis of ADP. The notably slow rate of dissociation of MgADP^- from isolated Kp2 observed under the conditions of the NMR experiments prevented the determination of the dissociation constant owing to difficulty in achieving equilibrium mixtures of the reactants. This effect could in turn account for the great variability in the reported dissociation constants of Fe protein– MgADP^- complexes [3].

Table 1 ^{31}P NMR chemical shifts (δ) of adenine nucleotide phosphate groups in solution and bound to the nitrogenase proteins of *K. pneumoniae*

Sample buffer contained 50 mM Tris/HCl, pH 7.4, 10 mM MgCl_2 and 50 mM NaCl. NMR spectra were recorded as specified in the Materials and methods section.

Phosphate group	Nucleotide status	Protein oxidation state	δ (p.p.m.)
ATP γ	Free	—	—4.4, —4.5
	Kp2 bound	Reduced	+3.0
	Kp2 bound	Oxidized	+3.2
ATP α	Free	—	—9.5, —9.6
	Kp2 bound	Reduced	—8.9
	Kp2 bound	Oxidized	—8.9
ATP β	Free	—	—18.3 (triplet)
	Kp2 bound	Reduced	—4.9
	Kp2 bound	Oxidized	—4.2
ADP β	Free	—	—5.2 (doublet)
	Kp2 bound	Reduced	—1.6
	Kp2 bound	Oxidized	—0.35 (and —1.6; see the text)
ADP α	Free	—	—9.3 (doublet)
	Kp2 bound	Reduced	—9.1
	Kp2 bound	Oxidized	—9.1
ADP β	Kp1 bound	Oxidized	—5.2 (see [11])
ADP α	Kp1 bound	Oxidized	—9.1 (see [11])
AMP	Free	—	+4.5
	Kp1 bound	Oxidized	—1.0 (see [11])
P_i	Free	—	+3.04

An additional resonance (designated 3) appeared in spectrum 1D in Figure 1 at -0.35 p.p.m., which was typical of MgADP^- bound to indigocarmine-oxidized Kp2 (see below). Because the gel-filtration step also removed dithionite from the sample, we assume that this arose from the partial oxidation of Kp2 in the NMR tube, giving a mixture of oxidized and reduced Kp2. EPR spectroscopy confirmed that complete oxidation had not occurred, however, and we conclude that in this case a mixture of two conformers corresponding to oxidized and reduced Kp2 with bound MgADP^- had been formed.

A summary of chemical-shift data for free adenine nucleotides and nucleotides reversibly bound to Kp2 is given in Table 1. The chemical shifts of the phosphate groups of MgADP^- in the presence of Kp1 and the chemical shift of AMP covalently bound to Kp1 are included in Table 1 for comparison. The ^{31}P resonances of free MgAMP , MgADP^- and MgATP^{2-} in the absence of protein are shown in spectrum E in Figure 2 (see below).

NMR spectra of MgADP^- bound to oxidized Kp2

Dialysis of indigocarmine-oxidized Kp2 against excess MgADP^- gave spectrum E in Figure 1. Only a single resonance at -0.35 p.p.m. was detected from the ADP β phosphate of the indigocarmine-oxidized Kp2 complex. Hydrolysis of ADP with the formation of P_i and AMP was a significant factor in acquiring NMR spectra with oxidized Kp2. Spectrum F in Figure 1 shows that, after 2.5 h, little free detectable MgADP^- remained in the NMR sample mixture even though Kp2 retained slowly dissociating bound MgADP^- . The increased hydrolysis of MgADP^- to AMP and P_i observed on the oxidation of Kp2 confirms that this property is associated with nitrogenase. At this stage we cannot exclude the involvement in this process of the very low level of Kp1 remaining in our preparations of Kp2.

Oxidative inactivation of Kp2 with a 9-fold excess of ferricyanide ions resulted in the irreversible inactivation of the iron-sulphur cluster. After this treatment, some MgADP^- remained bound to Kp2 (Figure 1, spectrum B); interestingly,

the chemical shift of the β phosphate resonance is the same as that observed with reduced Kp2, presumably because the inactivated Kp2 could not assume the reversibly oxidized conformation. Some ADP was released by treatment with excess ferricyanide and the line widths of the α and β resonances of the free nucleotide were diminished to the point that the doublet structure of free MgADP^- was resolved. This effect was due to the elimination of slow exchange between free and bound MgADP^- after the oxidative inactivation of Kp2. Taken together, these results show that resonance 1 of spectrum E arises from MgADP^- bound to catalytically active oxidized Kp2.

It is evident that the bonding constraint and/or the chemical environment of the β phosphate group of the nucleotide bound to oxidized, active Kp2 is altered significantly compared with that of the reduced Kp2 complex. The results illustrated in Figure 1 are consistent with a reversible change in conformation of the Kp2- MgADP^- -binding site on controlled oxidation of the iron-sulphur cluster. A 1.3 p.p.m. chemical shift to higher frequency of the β phosphate group suggests that increased deshielding of the phosphorus atom and tighter binding to Kp2 in the oxidized conformation results from the removal of a single electron from the Fe_4S_4 cluster. The consensus view based on redox potentials and lability of the Fe_4S_4 cluster is that the ATP-bound and ADP-bound conformations of nitrogenase Fe proteins are different (see [3] for a summary). The results presented here show that the interaction of ADP with Fe protein is more complex than is generally supposed because the ADP-binding sites of the oxidized and reduced Kp2 conformers also differ.

NMR spectra of MgATP^{2-} bound to reduced Kp2

Incubation of reduced Kp2 with MgATP^{2-} resulted in complex NMR spectra with overlapping resonances (Figure 2). At the shortest time required for data acquisition (20 min) resonances assignable to protein-bound ADP were also present but not resolved (results not shown). At reaction times longer than 2 h

Figure 2 ^{31}P NMR spectra of ATP complexes with Kp2

The concentrated protein was incubated with, or dialysed against, MgATP^{2-} . Spectra of nucleotides bound to Kp2 were recorded at 22 °C. Spectrum A: dithionite-reduced Kp2 (1.5 mM) was incubated for 1 h with MgATP^{2-} (2.6 mM). Overlapping resonances of free and bound nucleotides are identified as: resonance 1, γ phosphate group of ATP bound to Kp2 ($\gamma_{\text{b}}^{\text{red}}$) plus some P_i ; resonance 2, β phosphate group of ADP bound to Kp2 (see Figure 1); resonance 3, β phosphate group of ATP bound to Kp2 ($\beta_{\text{b}}^{\text{red}}$) plus γ phosphate of free ATP (γ_{f}) and a small amount of β phosphate of free ADP; resonance 4, α phosphate of bound ATP ($\alpha_{\text{b}}^{\text{red}}$) and small amounts of α phosphates of bound and free ADP; resonance 5, α phosphate of free ATP (α_{f}); resonance 6, β phosphate of free ATP (β_{f}). Spectrum B: indigocarmine-oxidized Kp2 (0.55 mM) was dialysed for 16 h against MgATP^{2-} (2.0 mM). The NMR spectrum was recorded 30 min after the termination of dialysis. Resonance 1, free AMP; resonance 2, γ phosphate of ATP bound to Kp2 ($\gamma_{\text{b}}^{\text{ox}}$) plus some P_i ; resonance 3, β phosphate of bound ADP; resonance 4, β phosphate of bound ATP ($\beta_{\text{b}}^{\text{ox}}$) and small amounts of γ phosphate of free ATP (γ_{f}) and β phosphates of free ADP; resonance 5, α phosphate of bound ATP ($\alpha_{\text{b}}^{\text{ox}}$) plus small amount of α phosphates of free ADP; resonance 6, α_1 (ATP); resonance 7, β_1 (ATP). Spectrum C: NMR spectrum recorded with the same protein as in spectrum B after a further 40 min. Resonance 3 (β phosphate of bound ADP) of the previous spectrum has split into two transient resonances, 3 and 4, representing two conformers of Kp2 containing bound ADP. Resonances 5–8 correspond to resonances 4–7 of spectrum B. Spectrum D: reduced Kp2 (1.5 mM) was dialysed against MgATP^{2-} (2.6 mM). The NMR spectrum was recorded 2 h after the termination of dialysis. Resonance 1, free AMP; resonance 2, $\gamma_{\text{b}}^{\text{red}}$ plus P_i ; resonance 3, β phosphate of ADP bound to reduced Kp2; resonance 4, $\beta_{\text{b}}^{\text{red}}$ plus a small amount of γ_{f} ; resonance 5, resolved resonance of β phosphate of free ADP; resonance 6, $\alpha_{\text{b}}^{\text{red}}$ plus a small amount of α phosphates of ADP; resonance 7, α_1 (ATP); resonance 8, β_1 (ATP). Spectrum E: ^{31}P NMR resonances of free adenine nucleotides in dialysis buffer containing 10 mM MgCl_2 at pH 7.4. Resonance 1, AMP (1 mM); resonance 2, γ phosphate (γ_{f}) of MgATP^{2-} (3 mM); resonance 3, β phosphate of MgATP^{2-} (1 mM); resonance 4, α phosphate of MgADP^{-} ; resonance 5, α phosphate of MgATP^{2-} (α_{f}); resonance 6, β phosphate (β_{f}) of MgATP^{2-} .

the slow, partial hydrolysis of ATP in these highly concentrated solutions of protein further complicated the NMR spectra of the reaction mixtures. However, as we had already assigned the resonances to free and bound MgADP^{-} as well as those of P_i and AMP (Figure 2, spectrum E), it was possible to assign resonances to the three phosphate groups of ATP bound to Kp2.

Spectrum A in Figure 2 was obtained 60 min after the addition of MgATP^{2-} to Kp2. The β peak characteristic of ADP bound to reduced Kp2 was detected at -1.6 p.p.m. owing to the slow hydrolysis of ATP. We can thus assign resonance 2 and a small

amount of resonance 4 to the β and α phosphates respectively of MgADP^{-} bound to Kp2, and resonances 5, 6 and some of 3 to the α , β and γ phosphate groups respectively of free MgATP^{2-} (see standards, Figure 2, spectrum E). Therefore resonances 4, the remainder of 3, and resonance 1 can be assigned to the α , β and γ phosphate groups of MgATP^{2-} bound to reduced Kp2. These assignments indicate that the α , β and γ resonances of bound ATP are at -8.9 , -4.9 and $+3.0$ p.p.m. respectively. Resonance 3 of spectrum A in Figure 2 is partly resolved in spectrum D, which was acquired 2 h after the cessation of dialysis of reduced Kp2 against MgATP^{2-} . This spectrum also shows a resonance at $+4.6$ p.p.m. owing to free AMP. When compared with the spectra of free nucleotide, all resonances of bound MgATP^{2-} moved to a higher frequency. The change in chemical shift of the β peak of bound ATP was greatest (13.4 p.p.m.) followed by the γ peak, which was shifted by 7.5 p.p.m. and superimposed on the resonance of P_i . There is also a change (1 p.p.m.) in the α resonance of bound ATP. This is greater than the change in the chemical shift of the α resonance of MgADP^{-} on binding to Kp2 (approx. 0.2 p.p.m.; Figure 1, spectrum A).

These assignments differ from those made in a preliminary investigation of the interaction of MgATP^{2-} with the homologous Fe protein Cp2 [12]. These authors suggested that the α resonance of MgATP^{2-} shifted to higher frequencies by 8.7 p.p.m. on binding to the protein. This would imply a highly significant change in the torsion angle and/or the R–O–P bond angle of the phosphate ribose ester [10]. We assign the maximally shifted resonance to the β phosphate of bound ATP. This resonance is superimposed on the β resonance of free ADP formed by ATP hydrolysis. The possibility that significant ATP hydrolysis occurred during lengthy NMR experiments was not considered in the earlier work, leading to a misinterpretation of the resulting spectrum.

NMR spectra of MgATP^{2-} bound to oxidized Kp2

Dialysis of oxidized Kp2 against MgATP^{2-} yielded spectrum B in Figure 2, which was recorded 30 min after the cessation of dialysis. The γ peak of bound MgATP^{2-} and a small peak of P_i remained superimposed, whereas other resonances of free and bound ATP remained essentially the same as seen with reduced Kp2 (Figure 2, spectra A and D). Small changes in chemical shifts of ATP phosphates to higher frequencies ($\gamma = +3.2$ p.p.m., $\beta = -4.2$ p.p.m.) are consistent with somewhat tighter binding of MgATP^{2-} to oxidized Kp2 compared with the protein with a reduced iron–sulphur cluster. However, there does not seem to be a major difference in the bonding constraint or chemical environment of the γ and β resonances of ATP bound to oxidized or reduced Kp2, in contrast with the β phosphate of bound ADP.

These results are in agreement with several reports in the literature and are consistent with the bound ADP and ATP conformations of the Fe protein being different (see [3]). It can now be seen for the first time that the interaction of the β phosphate of MgADP^{-} with Kp2 might be crucial in mediating conformational changes associated with the oxidation of the iron–sulphur cluster of active Kp2.

Despite the large chemical shift of the γ phosphate of ATP on binding to Kp2, bonding constraints apparently do not permit exchange of this group with P_i . The latter reaction was observed previously with the MoFe protein Kp1 [23].

Further changes in the spectrum of bound MgADP^{-} formed by the hydrolysis of ATP were observed after extended incubation

Figure 3 [α - ^{32}P]ATP binding to Kp2 as determined by flow dialysis (solid line), and interaction of ATP with non-binding control (catalase) (broken line)

Inset: Scatchard plot of equilibrium binding data; dotted lines, 95% confidence levels of linear plot. Abbreviation: B/F, ratio of bound ligand to free ligand in an equilibrium mixture with protein-binding sites.

of active oxidized Kp2 for a total of 2 h (Figure 2, spectrum C). Although Kp2 remained oxidized, the appearance of a resonance at -1.6 p.p.m. indicated that the binding-site conformation of reduced Kp2 with bound MgADP $^{-}$ can arise transiently in the oxidized protein (compare resonances 3 and 4 of spectrum C in Figure 2). The conformation responsible for this signal was observed during several similar experiments with oxidized Kp2 and MgATP $^{2-}$ but was not stable because only the -0.35 p.p.m. resonance was detected in subsequent spectra. This conformer is apparently not stable unless the iron-sulphur cluster is reduced.

MgATP $^{2-}$ binding to Kp2 determined by flow dialysis

Owing to the complex and time-dependent NMR spectra observed when MgATP $^{2-}$ was bound to Kp2, it was necessary to measure initial nucleotide binding by using an independent, relatively rapid method to minimize the products of ATP hydrolysis. Flow dialysis with a micro-scale dialysate chamber (50 μl) made it possible to estimate bound and free nucleotide concentrations and allowed the assessment of initial time-dependent changes in ligand binding affinity on a scale of minutes [16].

Typical results of a flow dialysis experiment with 110 μM Kp2 and 45 μM [α - ^{32}P]ATP are shown in Figure 3. The initial rate of dialysis, as indicated by the radioactivity in samples collected at fixed intervals, increased to a maximum in 2–3 min then slowly declined. This decrease was not due to the depletion of ATP from the dialysis chamber because it was not observed in control experiments with catalase (Figure 3, broken line). Equilibrium binding of MgATP $^{2-}$ at Kp2 sites was achieved after approx.

10 min, as evidenced by a constant rate of dialysis of free radiolabelled MgATP $^{2-}$. The subsequent addition of a large pulse of unlabelled ATP resulted in the establishment of a new equilibrium dialysis rate. This final dialysis rate of free nucleotide was stable and did not decrease significantly from the initial plateau rate as was the case at lower nucleotide concentrations. The initial decrease in dialysis rate that was observed at all sub-stoichiometric ATP concentrations could have resulted from a slow change in protein conformation, resulting in a tighter binding of nucleotide, after occupation of some of the nucleotide-binding sites.

The flow dialysis procedure was repeated over a 15-fold range of initial MgATP $^{2-}$ concentrations and bound-to-free ratios were calculated for the equilibrium dialysis rates. The results are represented by a linear Scatchard plot (Figure 3, inset). An average K_d for the binding sites ($N = 1.9 \pm 0.2$) was estimated to be 145 μM . The Scatchard plot shows no evidence of co-operative binding of MgATP $^{2-}$ to Kp2. Both co-operative and non-co-operative binding of nucleotides to the nitrogenase Fe proteins have been reported previously but the variation observed might be due to species or experimental differences [3].

Conclusions

1. We have demonstrated here that ^{31}P NMR can be used to provide fresh insight to the interaction of nucleotides and the Fe protein of nitrogenase and it is clear from the results presented that such interactions are more complex than has previously been supposed.

2. Comparison of the NMR data for Kp2 and the S-1 fragment of myosin indicate that MgADP $^{-}$ binds in a similar mode to both proteins and the bonding constraints of the β phosphate sites are similar in both reduced Kp2 and S-1. Both proteins hydrolyse ADP slowly.

3. For the first time, our NMR studies have shown that there are two redox-state-dependent conformers of Kp2, and that the oxidized conformer binds MgADP $^{-}$ with greater constraint of the β phosphate.

4. Our results show that, consistently with the earlier pre-steady-state kinetic data [22], the rate of dissociation of MgADP $^{-}$ from isolated oxidized Kp2 is too slow to be of importance in catalytic turnover. The α resonance of MgADP $^{-}$ bound to Kp2 or S-1 was not shifted significantly and was not affected by the oxidation state of Kp2, showing that in both cases the binding sites interact preferentially with the β phosphate group of MgADP $^{-}$.

5. Interpretation of published data on the homologous Fe protein Av2 suggests that the protonated primary amino group of Lys-15 is likely to interact directly with the γ phosphate in the MgATP $^{2-}$ complex (reviewed in [3]). Our NMR and flow dialysis results suggest that the γ and the β phosphate groups interact strongly at two equivalent binding sites; a slow conformational change was observed during the first 10 min of reaction.

6. Because some ADP was formed in all of the incubation mixtures containing MgATP $^{2-}$, and remained bound to the protein, some binding sites of Kp2 were occupied by ADP even in the presence of excess ATP. This condition might resemble the situation *in vivo*, where the nitrogenase proteins encounter a mixture of nucleotides, in contrast with studies *in vitro*, where an ATP-regenerating system is usually added.

7. During the nitrogenase catalytic cycle the reduced Fe protein, with bound MgATP $^{2-}$, associates with the MoFe protein to form a protein complex. The formation of a putative ADP-AlF $_4$ -stabilized transition state complex that mimics subsequent steps in this process is accompanied by con-

formational changes in both *K. pneumoniae* nitrogenase proteins, as determined by small-angle X-ray scattering [24]. Similarly, the crystal structure of the isolated Av1–Av2 transition-state complex shows, in detail, alterations in the conformation of the Fe protein [6]. Our ^{31}P NMR results indicate that, because the bonding constraints of the Fe protein MgADP^- -binding sites are magnified in the oxidized Fe protein, the hydrolysis of ATP within the nitrogenase complex leads to stabilization of the oxidized conformation after electron transfer to the MoFe protein. This conformation might then trigger the dissociation of the Kp2-MgADP^- complex from the MoFe protein, which is the rate-limiting step in nitrogenase turnover at 23 °C.

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