Mn²⁺-adenosine nucleotide complexes in the presence of the nitrogenase iron-protein: detection of conformational rearrangements directly at the nucleotide binding site by EPR and 2D-ESEEM (two-dimensional electron spin-echo envelope modulation spectroscopy)

Jan PETERSEN^{*1}, Christof GESSNER[†], Karl FISHER^{*2}, Claire J. MITCHELL^{*}, David J. LOWE^{*} and Wolfgang LUBITZ^{†‡}

^{*}Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Colney Lane, Norwich NR4 7UH, U.K., [†]Max-Volmer-Institut für Biophysikalische Chemie, Technische Universität Berlin, Str. des 17 Juni 135, 10623 Berlin, Germany, and [‡]Max-Planck-Institut für Bioorganische Chemie, Stiftstrasse 34-36, 45470 Mülheim/Ruhr, Germany

Both ATP and a bivalent nucleotide-bound metal activator, normally Mg²⁺, are required for nitrogenase activity. EPR and ESEEM (electron spin-echo envelope modulation) measurements have been carried out on adenosine nucleotides in which the Mg²⁺ ion that is usually bound is replaced by Mn²⁺ in the presence of Kp2 (nitrogenase Fe-protein from *Klebsiella pneumoniae*). The Mn²⁺ zero-field splitting parameters have been determined from the EPR-spectrum to be $|D| = 0.0125 \text{ cm}^{-1}$ with a rhombicity $\lambda = E/D = 0.31$ by direct diagonalization of the complete spin Hamiltonian. ESEEM spectra of the Fe-protein with MnADP and MnATP both show an ESEEM line pair with one signal component at about 3.6 MHz and a relatively broad resonance at 8 MHz originating from a superhyperfine coupling to a ³¹P nuclear spin from one or more directly co-ordinated phospho group(s) of the nucleotide. A pronounced resonance overlapping the low-frequency component of the ³¹P-signal at about 3.5 MHz is attributed to an interaction of Mn²⁺ with univalent ²³Na nuclei. ESEEM lines at frequencies < 3.5 MHz have been ascribed to interactions with ¹⁴N nuclei. Differences in the ¹⁴N features that depend on the type of nucleotide are consistent with substantial

conformational rearrangements at the nucleotide-binding site upon hydrolysis. In addition, four-pulse HYSCORE (hyperfine sublevel correlation spectroscopy) experiments not only confirm the three-pulse ESEEM results, but also achieve significantly better spectral deconvolution, especially of the ³¹P-couplings, and demonstrate that the nucleotide is at least a unidentate ligand of Mn²⁺. Moreover it was also possible to identify peaks from an ¹⁴N interaction more clearly; these most probably arise from outer-sphere interactions with nitrogen atom(s) of non-co-ordinated residues which are affected by conformational rearrangements upon nucleotide hydrolysis. In addition, different redox states of the [4Fe-4S] cluster of the Fe-protein show disparate conformations of the metal–nucleotide co-ordination environment, demonstrating that also the cluster site communicates with the nucleotide binding site.

Key words: electron paramagnetic resonance (EPR), electron spin-echo envelope modulation (ESEEM), Fe-protein, hydrolysis, manganese, nitrogenase.

INTRODUCTION

Nitrogenases are the key enzymes by which bacteria convert the relatively inert gas dinitrogen, in an ATP-dependent process, into a biologically usable form, i.e. ammonia (see, e.g., [1,2]). Molybdenum nitrogenase comprises two proteins, the homodimeric iron protein (encoded by *nifH*) and the $\alpha_2\beta_2$ tetrameric molybdenum-iron protein (encoded by *nifD* and *nifK*), which form the catalytically active heteroprotein complex. Neither protein has catalytic activity alone. The MoFe-protein, $M_r \approx 220 \text{ kDa}$, hosts two types of prosthetic metal centres: the P-cluster and the FeMo-cofactor. The latter is a [MoFe₇S₉] multinuclear cluster that is believed to be the site of substrate binding and reduction. The smaller Fe-protein, $M_r \approx 62 \text{ kDa}$, encloses a [4Fe-4S] cluster at the interface between the two subunits. Both ATP and a bivalent metal ion, usually Mg²⁺, are required for nitrogenase activity, with one molecule of ATP binding to each of the Fe-protein subunits.

The Fe-protein is believed to undergo a substantial conformational rearrangement upon binding of nucleotides, and the ATP hydrolysis necessary for protein complex formation/dissociation

and electron transfer to the MoFe-protein. However, the evidence for this has always been indirect and based on, for example, a shift of the redox potential of the [4Fe-4S] cluster by about + 100 mV in the presence of nucleotides [3], and a number of other indicators, including a change in lineshape of the FeS cluster $S = 1/2$ EPR signal [4,5].

The nucleotide-binding site of the Fe-protein shares a remarkable amino acid sequence homology with other nucleotide-binding proteins. These ‘classical’ nucleotide-binding proteins or regulatory ‘switch’ proteins, such as the GTP-binding proto-oncogene product p21Ras, possess a highly homologous P-loop motif with a consensus amino acid sequence of the form Gly-Xaa-Xaa-Xaa-Gly-Lys-Ser/Thr (with Xaa being any amino acid residue) known as the ‘Walker A motif’ [6], which is highly conserved throughout a vast family of ATPases and GTPases. X-ray crystal structures revealed that, for Av2 (the Fe-protein from *Azotobacter vinelandii* [7,8]), a number of the amino acid residues of this motif either co-ordinate the metal centre directly or interact with the nucleotide. In addition, two inhibitory nitrogenase heteroprotein complexes have been analysed by X-ray

Abbreviations used: cw-EPR, continuous-wave EPR; 2D, two-dimensional; ENDOR, electron nuclear double resonance; ESEEM, electron spin-echo envelope modulation; FFT, fast Fourier transform; hf(c), hyperfine (coupling); HYSCORE, hyperfine sublevel correlation spectroscopy; Kp2 (Av2), nitrogenase Fe-protein from *Klebsiella pneumoniae* (or *Azotobacter vinelandii*); Kp2^{ox}, oxidized Kp2; Kp2^{red}, reduced Kp2; NQR(I), nuclear quadrupole resonance (interaction); shf, superhyperfine; zfs, zero-field splitting.

¹ To whom correspondence should be addressed (email jan.petersen@bbsrc.ac.uk).

² Present address: Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA, U.S.A.

Figure 1 Schematic representation of the immediate nucleotide-bound octahedral metal-centre environment of 'classical' nucleotide binding proteins, to which the nitrogenase Fe-protein also belongs, in the ADP-bound (left) and ATP-bound form (right)

The question mark indicates a ligand that is a threonine in the homologous family of G-proteins that co-ordinates via its hydroxy side chain to the metal centre; a corresponding metal-co-ordinating amino acid has not yet been identified in the native 'on'-state of the Fe-protein.

crystallography in the presence of MgATP [9,10]. These studies suggested that conformational rearrangements occur almost exclusively in two regions that are called, in accordance with those of other 'classical' nucleotide binding proteins, 'switch I' and 'switch II' regions. These domains can relay structural rearrangements at the metal–nucleotide binding site to other regions of the protein, such as the [4Fe–4S] cluster or the Fe-protein/MoFe-protein interface. The inner-sphere metal co-ordination configuration, generally highly homologous throughout this superfamily of nucleotide-binding proteins, is essential for the structural rearrangements, as depicted schematically in Figure 1. However, the electronic structure at the metal–nucleotide binding site before and after hydrolysis, which is the trigger for the structural rearrangements, and the influence of the metal ion, remains largely uncharacterized.

We have previously investigated the Fe-protein, substituting the nucleotide-bound Mg^{2+} with paramagnetic VO^{2+} [11]. It is of interest that substrate reduction by nitrogenase is inhibited by the VO^{2+} substitution [11], whereas enzymatic activity is retained with Mn^{2+} [12–14]. It has been suggested that this is due to alterations in, or the absence of, some of the critical rearrangements of the nucleotide-binding site that modulate various protein functions and ultimately lead to nitrogen fixation. It is therefore especially important to investigate the fully functional enzyme complex to understand what alterations in the protein might be responsible for rendering the protein active or inactive. Mn^{2+} is a versatile substitute for Mg^{2+} , since, in many cases, it retains enzymatic activity [15], as is the case for nitrogenase, although with diminished turnover rate [12–14]. Yet it demonstrates that Mn^{2+} binds at the active site.

In the present work we use cw-EPR (continuous-wave EPR) and pulsed EPR techniques to investigate the local metal-centre co-ordination environment of the Fe-protein nucleotide-binding site using the active nucleotide metal analogue, with paramagnetic Mn^{2+} bound to the nucleotide in place of diamagnetic Mg^{2+} . It also allows the application of ESEEM (electron spin-echo envelope modulation spectroscopy) techniques for the spectroscopic investigation of the local nucleotide-binding-site environment; this has previously been used successfully for the study of some other ATPases and GTPases [17–24]. ESEEM spectroscopy is a pulsed EPR method that can be used to study shf (superhyperfine) interactions that arise from the (usually) weak couplings of an electron-spin to nuclear-spin centres in its vicinity. These give rise to modulations of the echo decay traces that can provide very

detailed information about the nuclear environment of the electron spin [25–27]. In the present study we further demonstrate that 2D (two-dimensional) four-pulse ESEEM [HYSCORE (hyperfine sublevel correlation spectroscopy)] spectroscopy can significantly enhance the spectral resolution, especially of the phosphorus interactions and deconvolute these from contributions of other nuclear species such as ^{14}N , thus considerably aiding their interpretation.

MATERIALS AND METHODS

Sample preparation

The Fe-protein was isolated and purified from *Klebsiella pneumoniae* N.C.I.B 12204 as described previously [13]. The MgCl_2 normally used in the Fe-protein buffer solutions was removed by anaerobic gel filtration (Bio-Gel P-6DG; Bio-Rad), followed by exchange into Mg-free 25 mM Hepes buffer solution containing 250 mM NaCl. The pH was adjusted to pH 7.4 with HCl. Preparations of the reduced proteins 'as isolated' contained 2 mM sodium dithionite. Fe-protein concentrations after reconcentration using Minicon macrosolute concentrators (Amicon) were about 110 mg/ml.

MnCl_2 and ADP or ATP were, except where otherwise stated, added as the ADP or ATP sodium salt with a 9-fold excess of nucleotide over metal. Samples were prepared inside an anaerobic glove box in 4.9-mm-outside-diameter SUPRASIL® quartz tubes and frozen anaerobically in a liquid- N_2 -cooled cold block containing methanol inside the glove box before being stored at liquid- N_2 temperatures for subsequent EPR and ESEEM measurements.

The [4Fe–4S] cluster of the Fe-protein was oxidized using the dye Indigo Carmine (AnalaR grade; BDH Chemicals) as described previously [28]. The change of the oxidation state was checked by EPR.

The sample with KCl substituting for NaCl was similarly prepared by equilibration of the column with 25 mM Hepes, 250 mM KCl and 0.5 mM $\text{Na}_2\text{S}_2\text{O}_4$.

Activity assay

The specific activities of Kp2 (nitrogenase Fe-protein from *Klebsiella pneumoniae*) in the presence of MgATP were assayed as described by Eady et al. [13] and were in the range 1600–2000 nmol of ethylene produced/min per mg of protein. Samples in the presence of nucleotides, in which the Mg^{2+} was replaced by Mn^{2+} , were assayed for ethylene activity at pH 7.4 and with 0.5 mg of total protein at an Fe-protein/MoFe-protein ratio of approx. 20:1 (w/w) to which MnCl_2 and ATP in a 1:9 stoichiometry were added. These activities were compared with the MgATP activity at two different MnCl_2 concentrations, namely 1 and 10 mM, and for reactions stopped at 1 and 2 min after mixing. This gave specific activities ranging between 50% (long assay, low concentration) and 34% (short assay, high concentration) of the corresponding MgATP activity. The results are in good agreement with previous activity assays of Kp- [13] and Av-nitrogenase with Mn-substituted ATP chelate complexes [14].

EPR and ESEEM spectroscopy

cw-EPR spectra were recorded using a X-band Bruker ESP300 spectrometer equipped with an Oxford Instruments ER900 gas flow-cryostat. EPR simulations were conducted according to the method described in [29], using the MAGRES program [30], not accounting for any kind of D, E or g-strain, even though these may

contribute to the overall line width. In particular D- and E-strain may obscure features from higher-spin multiplicity transitions.

EPR simulations of Mn²⁺ systems have generally been carried out using perturbation theory, with corrections up to third order [16]. Here we have used a different approach in order directly to simulate the EPR spectrum recorded at X-band frequencies, namely to diagonalize the full spin Hamiltonian, which is essentially similar to the method used by Coffino and Peisach [31]. The complete ⁵⁵Mn²⁺ spin Hamiltonian used for the simulations has the form:

$$H = g\beta B_0 \hat{S} + D \left\{ \hat{S}_z^2 - (1/3)S(S+1) \right\} + E \left\{ \hat{S}_x^2 - \hat{S}_y^2 \right\} + \hat{S}A\hat{I} \quad (1)$$

where the first term is the Zeeman energy, the following two the zfs (zero-field splitting) interaction with *D* and *E*, the axial and rhombic zfs tensor parameters respectively, the last term is the manganese hyperfine interaction and the co-ordinates *x*, *y*, *z* refer to the principal axis system of the zfs tensor. The rhombicity of a metal centre is often defined as $\lambda = E/D$. λ can range between 0 for total axial symmetry and 1/3 in the limit for a maximum rhombic distortion. We have tested such an approach previously for other spin $S > 1/2$ systems with large zfs values as observed for iron-sulphur 'super'-clusters, and demonstrated that the simulation routine yields good results [29]. With this most rigorous approach, EPR spectra can be simulated at any microwave frequency and any magnitude of the zfs; this enables us to simulate Mn²⁺ spectra also at X-band frequencies.

Electron-spin-echo measurements were carried out as described previously [32] with a Bruker ESP380E pulsed EPR spectrometer equipped with a dielectric ring cavity (ESP380-1052 DLQ-H) and fitted with an Oxford Instruments CF 935 continuous-flow cryostat. Three- and four-pulse ESEEM spectra were measured for pulsewidths of typically 16 ns. The spectrometer dead time was ≈ 100 ns. A four-step phase cycle routine analogous to the one described by Fauth et al. [33] was employed to suppress unwanted echoes. For the three-pulse ESEEM spectra, the echo intensity was recorded as a function of the continuously varied time, *T*, between the second and third pulse versus incrementation of the time τ between the first and second pulse (cf. the pulse sequence in Figure 3 below). 2D data sets were recorded for all preparations in order to avoid 'blind spots' which occur for specific τ values at which the amplitude of certain harmonics in the stimulated echo experiment is at a minimum. In the Fourier-transform spectra, the echo decay envelope was subtracted using a multi-exponential decay function in both directions and a Hamming apodization. The skyline projections shown were obtained after zero-filling, Fourier transformation and magnitude calculation. Four-pulse HSCORE spectra were recorded for several different values of otherwise fixed time τ for optimization of τ before 2D data sets were recorded.

For the reduced Fe-protein the excitation field was generally set to the lowest-field Mn²⁺ hyperfine line ($g \approx 2.15$). This setting was chosen in order to avoid spectral overlap with the $S = 1/2$ EPR-signal of the [4Fe-4S]⁺ cluster of the reduced Fe-protein with *g*-tensor principal axes components all found at *g*-values ≤ 2.04 . This is not necessary for the oxidized protein with an $S = 0$ FeS-cluster state; nonetheless this field setting was also used for Kp2^{ox}. (oxidized Kp2) for better comparability. In order to test whether, at the field setting used, the nuclear couplings can be regarded as originating from an effective $S = 1/2$ spin system, the field dependence was monitored as described by Singel and co-workers [34]. This is necessary, because manganese has an electron spin, *S*, of 5/2, so that effects from higher multiplicity transitions have to be considered.

Figure 2 EPR spectra of Mn²⁺ATP in the presence of dithionite-reduced Kp2

The frozen solution X-band cw-EPR spectrum of the Fe-protein preparation in 25 mM Hepes buffer, pH 7.4, is shown, together with simulations of the manganese spin $S = 5/2$, $I = 5/2$ system. Inflections originating from the intrinsic [4Fe-4S]-cluster $S = 1/2$ signal are marked by arrows. The asterisk indicates the field position generally used for recording the ESEEM and HSCORE spectra. The magnetic-field regions indicated by the thick horizontal bars represent regions which are likely to be broadened by *D*- and *E*-strain. The insert shows spectral contributions on the low field side originating from the FeS-cluster $S = 3/2$ signal of the Fe-protein. The parameters for the simulation were a 'real' *g*-value of 2.002, a linewidth parameter of 2.2 mT, an isotropic *hfc* of $A = -8.2$ mT and zfs parameters of $|D| = 0.0125$ cm⁻¹ and a rhombicity ($\lambda = E/D$) of 0.307. Experimental conditions: temperature, 8.0 K; microwave power, 2 mW; modulation amplitude, 0.5 mT (1.0 mT for the insert); modulation frequency, 100 kHz; microwave frequency, 9.43 GHz.

RESULTS

EPR

It has been previously shown that the bivalent metal ion associated with nucleotide in the nitrogenase Fe-protein can be replaced by paramagnetic Mn²⁺ [13,14]. However, zfs parameters have not yet been reported for the Mn²⁺-substituted Fe-protein. In the case where the zfs parameters of Mn²⁺ are not too large, they usually give rise to a six-line spectrum. The zfs is characterized by two parameters, *D* and *E*, which provide insight into the local symmetry of the Mn²⁺ ligation sphere. The EPR spectrum of Mn²⁺-ATP in the presence of Kp2^{red}. (reduced Kp2) is shown in Figure 2. Six clearly resolved EPR lines are observed in the spectrum centered at $g = 2.0$. This indicates that the zfs of the Mn²⁺-complex is relatively small. The spectrum is typical of a system for the limiting condition where $|D| \ll g\beta B_0$ (where the resonant microwave quantum energy, $h\nu_{MW}$, at X-band is ≈ 0.33 cm⁻¹). This sextet signal primarily arises from the six allowed $\Delta M_S = 1$ hf (hyperfine) transitions in the electron spin $M_S = | + 1/2 \rangle \leftrightarrow | - 1/2 \rangle$ manifold. A slight distortion is noticeable at the high-field side of the spectrum; this is due to the

underlying $S = 1/2$ signal of the Fe-protein [4Fe-4S] cluster with inflections at about $g = 2.04$ and 1.94 .

For the EPR simulations, the Mn^{2+} hf coupling is taken as isotropic, as expected for an S -ground state ion and was assumed, in accord with the literature [35–38], to be negative for Mn^{2+} , even though the sign of the hf interaction has only a minor influence on the spectral characteristics of the central sextet spectrum (result not shown). The undulations seen in the wings of the central sextet originate from transitions in the higher-spin manifolds, especially of the fully allowed $| -1/2, M_1 \rangle \leftrightarrow | -3/2, M_1 \rangle$ and $| +1/2, M_1 \rangle \leftrightarrow | +3/2, M_1 \rangle$ transitions. These are not resolved experimentally, or are only seen as very broad features. They are also not reproduced in simulations using the perturbation approach [16]. Nonetheless, the broadness of the high- and low-field flanks is indicative of contributions from transitions in the higher-spin manifolds broadened by substantial D - and/or E -strain [39–41]. D - and/or E -strain, as often encountered in biological systems, should be most pronounced for transitions in the higher-spin manifolds, so that these are broadened beyond recognition and only the $M_S = | -1/2, M_1 \rangle \leftrightarrow | +1/2, M_1 \rangle$ transition is observed. The differences between experiment and simulation at the high-field side of the central sextet spectrum are due to the underlying FeS-cluster $S = 1/2$ signal.

The best correspondence between experiment and simulation is achieved for zfs parameters, $|D| = 0.0125 \pm 0.0005 \text{ cm}^{-1}$ and $|E| = 0.0038 \pm 0.0005 \text{ cm}^{-1}$. The Mn^{2+} EPR spectra are almost identical for Kp2 with ATP or ADP bound, corresponding to local metal-centre symmetries that are indistinguishable using solely the zfs parameters. The comparison of the zfs and hfc (hyperfine coupling) parameters determined here with those of other nucleotide-binding proteins is listed in Table 1 and demonstrates that they correlate well with the ‘classical’ nucleotide-binding proteins that contain the consensus P-loop motif. For example, they all show relatively large E/D -values, indicating that they all exhibit

Table 1 Axial (D) and rhombic (E) zero-field splitting parameters of Mn^{2+} -nucleotide enzyme complexes for some GTPases and ATPases

Abbreviation: n.r., not reported.

Complex	Zero-field splitting (cm^{-1})	Reference
$\text{Mn}^{2+} \cdot \text{GTP} \cdot \text{EF-Tu}^*$	n.r.	Wilson and Cohn [67]
$\text{Mn}^{2+} \cdot \text{ADP} \cdot \text{SF}_1^\dagger$	$D = 0.0224$ $E = 0.0075$	Webb et al. [68]
$\text{Mn}^{2+} \cdot \text{GDP} \cdot \text{p21Ras}$	$D = 0.0105$ $E = 0.0029$	Latwesen et al. [69]
$\text{Mn}^{2+} \cdot (\text{ADP?}) \cdot \text{CF}_1^\ddagger$	$D \approx 0.0200$ $E \approx 0.0040$	Buy et al. [22]
$\text{Mn}^{2+} \cdot \text{ADP} \cdot \text{TF}_1^\S$	$D = 0.0180$ $E = 0.0050$	Schneider et al. [23]
$\text{Mn}^{2+} \cdot \text{ATP} \cdot \text{Kp2} $	$D = 0.0125\P$ $E = 0.0038\P$	The present study

* Elongation factor Tu from *Escherichia coli*.

† Myosin chymotryptic subfragment 1 from rabbit skeletal muscle.

‡ H^+ -ATPase from spinach (*Spinacia oleracea*) chloroplasts (some tightly bound ADP was probably present).

§ H^+ -ATPase from the thermophilic *Bacillus* PS3.

|| Dithionite-reduced nitrogenase Fe-protein from *K. pneumoniae*.

¶ Estimated errors are $\pm 0.0005 \text{ cm}^{-1}$.

a considerable deviation from tetragonal symmetry of the Mn^{2+} -centre.

Three-pulse ESEEM

The 2D ESEEM time domain data set for the Mn^{2+} -ADP complex in the presence of reduced Kp2 after Fourier transformation is shown in Figure 3. The most intense ESEEM line in the

Figure 3 2D-ESEEM spectra recorded for frozen solutions of Mn^{2+} ADP in the presence of dithionite-reduced Kp2

ESEEM spectra recorded at X-band frequencies are shown after magnitude calculation and Fourier transformation in both directions, together with the skyline projection on to the ν_T axis. Traces were recorded for a field setting positioned at the lowest field hf line of the manganese EPR sextet ($B_0 = 324.2 \text{ mT}$) for various τ values. The inset shows the three-pulse sequence used for the experiment. Experimental conditions: temperature, 8 K; microwave frequency, 9.79 GHz; time incrementation in τ direction, 8 ns; pulse widths, 16 ns. Shot repetition time was 1 ms with 100 shots per loop.

Figure 4 Comparison of the stimulated echo ESEEM spectra of Mn²⁺-nucleotide-Kp2 ternary complexes with ADP and ATP respectively

Spectra represent skyline projection of the full 2D-ESEEM data sets after Fourier transformation in both directions and magnitude calculation. Spectra are for reduced Kp2 in the presence of MnADP (A) and in the presence of MnATP (B) recorded with the field centred at $B_0 = 324.1$ mT. (C) is the MnATP-Kp2^{red} spectrum for preparations in which Na⁺ was exchanged for K⁺. All spectra are at a metal/protein ratio of 1:1 with a 9-fold excess of nucleotide over metal. The line pair attributed to an interaction with ³¹P and the nuclear Zeeman frequencies of ¹H, ³¹P, and ¹⁴N nuclei are indicated. Spectra were recorded at a sample temperature of 8 K, a microwave frequency of 9.7895 GHz and pulse widths of 16 ns.

frequency domain is found at about 13.8 MHz and is assigned to interactions of Mn²⁺ with weakly coupled protons, corresponding to the high-frequency component in the time domain, resonating at their respective nuclear Zeeman frequency ($\nu_n = 13.804$ MHz at the B_0 -field used with $\nu_n = g_n \beta_n B_0 / h$ for a given nucleus).

A comparison of the FFT (fast Fourier transform) ESEEM frequency spectrum (Figure 4) for the ternary complexes of Kp2^{red} with Mn²⁺ADP (trace A) and Mn²⁺ATP (trace B) shows that, apart from the dominant signal at 13.8 MHz, an intense line is detected close to 3.8 MHz. This signal is asymmetric, as most clearly seen for the MnADP-Kp2 complex (cf. Figure 4A), with a sharp peak at 3.6 MHz and a broad shoulder on the high-frequency side. The 3.6 MHz resonance is approximately at the nuclear Zeeman frequency of ²³Na ($\nu_n = 3.65$ MHz at $B_0 = 324.1$ mT, with a natural abundance of 100 %), which was generally present in high concentrations in the preparations.

In order to confirm the assignment of this resonance to Na⁺, we also performed ESEEM measurements with preparations in which the high NaCl concentration was replaced by using the corresponding K⁺-containing salt (cf. Figure 4C). The pronounced, sharp spike at the nuclear Zeeman frequency of Na⁺, seen in Figures 4(A) and 4(B) at 3.6 MHz, is clearly absent in the spectrum of the K⁺-containing preparation. The remaining broad

ESEEM line at about 3.9 MHz is much less intense and is shifted to higher frequency by about 0.3 MHz. This resonance is attributed to an electron nuclear coupling to a different nuclear species underlying the ²³Na signal, as described below. Signals from natural-abundance K⁺ in the spectrum of the preparation with potassium were not observed, owing to the low nuclear Zeeman frequencies of the two major K⁺ isotopes, ³⁹K and ⁴¹K, with $\nu_n = 0.696$ and 0.382 MHz at 350 mT respectively. Nonetheless, K⁺ may substitute for Na⁺ as the univalent metal centre at the nucleotide-binding site. Despite the fact that ²³Na with $I = 3/2$ has a quadrupole moment, Q , only a single line without any additional structure is observed, suggesting that the quadrupole coupling of the ²³Na ion is small. This observation is consistent with results for Nd³⁺-nucleotides [42], which showed a similar relatively sharp signal, likewise attributed to an interaction with ²³Na. This resonance is present with MnADP or MnATP and therefore the interaction with the Na⁺ ion is essentially unaffected by the hydrolysis state of the nucleotide.

A broad ESEEM line is observed at 7.7 MHz for all our preparations. The resonance has been assigned to one component of a line pair arising from an electron-nuclear shf interaction of the Mn²⁺ centre electron spin with a phosphorus ³¹P nuclear spin ($I = 1/2$). To first order, nuclear transitions for an interaction with an $I = 1/2$ nuclear spin are found for shf-couplings $|A| < 2 \nu_n$, at the two ENDOR (electron nuclear double resonance) frequencies [43], $\nu_{\text{ENDOR}}^{\pm} = \nu_n \pm |A|/2$. With the nuclear Zeeman frequency of natural-abundance phosphorus, $\nu_n(^{31}\text{P}) = 5.59$ MHz (at a field setting of $B_0 = 324.1$ mT) and one ³¹P resonance of the ESEEM line pair at 7.7 MHz the corresponding low frequency component should be found symmetrically disposed about the nuclear Zeeman frequency at about 3.8 MHz, where the Na⁺ resonance is also observed. We therefore assume that the feature in the 3.8 MHz region is a superposition of two signals from different nuclear species, namely from ³¹P and ²³Na. The assignment that ESEEM resonances at about 3.8 and 7.7 MHz arise from an electron-nuclear interaction with a ³¹P nucleus is supported by the frequency shift as a function of the external field in measurements conducted at different field settings and by the HSCORE experiments described below. The ³¹P shf coupling for MnATP in the presence of Kp2^{red} was determined to be $|A| = 3.9$ MHz. This is in good agreement with the ³¹P couplings measured for other adenosine- and guanosine-nucleotide-binding proteins.

Another relatively broad ESEEM feature is detected for the Mn²⁺ATP-Kp2^{red} ternary complex at about 1.0 MHz (cf. Figure 4A). This is close to the ¹⁴N nuclear Zeeman frequency at this field setting of $\nu_n = 0.996$ MHz which, together with the frequency dependence of the field setting, suggest that this ESEEM line is due to a ¹⁴N interaction. There is another, yet unassigned, resonance observed for the Mn²⁺ADP-Kp2^{red} ternary complex at a frequency of about 2.2 MHz which cannot be attributed to an interaction with either ³¹P or ²³Na, and hence it seems reasonable, likewise, to ascribe this resonance to interactions with ¹⁴N nuclei. From a comparison of the ESEEM spectra for Kp2 with MnADP (Figure 4A) and MnATP (Figure 4B) it is apparent that the feature at 2.2 MHz is not detected for the ternary complex with ATP, yet the signal at about the nuclear Zeeman frequency of ¹⁴N-nuclei is observed, suggesting that the nitrogen environment differs quite substantially in the two states.

Another difference is that the ³¹P resonances are significantly broader in spectra with MnATP, so that the asymmetric ESEEM line with MnADP at about 3.7 MHz alters the lineshape and the resonance at 7.8 MHz, attributed to the high-frequency component of the ³¹P line pair, is almost unrecognizable. Despite this, the four-pulse HSCORE experiment clearly demonstrates that a coupling to a ³¹P nucleus still exists (see below).

Figure 5 Comparison of the skyline projection of the stimulated echo FFT-ESEEM spectra with those of Figure 3 for the dithionite-reduced and Indigo-Carmine-oxidized Fe-protein

(A) is the spectrum for MnATP-Kp2^{red}. and (B) is the spectrum for MnATP-Kp2^{ox}. The metal/protein molar ratio was 2:1, with a 9-fold excess of nucleotide over metal. Experimental conditions were the same as for Figure 3, except that the field setting for the reduced protein was $B_0 = 324.6$ mT.

In order to test whether the electronic spin of the metal bound to one nucleotide can interact with the second nucleotide-binding site of the Fe-protein, we also recorded ESEEM spectra for a more natural metal-nucleotide/protein stoichiometry. The 1:1 (Figure 4B) and the 2:1 (Figure 5A) complexes are essentially identical, showing no evidence for variations between the two stoichiometries. Note that, at the higher metal-nucleotide/protein ratio, the ESEEM line at 2.2 MHz, which is most sensitive to the type of nucleotide, is again absent. Corresponding ESEEM spectra were also recorded for the MnADP-Kp2 complexes for the 2:1 molar ratio (results not shown); these again gave spectra essentially identical with those using a 1:1 ratio. However, the slightly more pronounced low-frequency ESEEM features for MnADP-Kp2 confirm even more clearly the absence of spectral variations between the two stoichiometries. Thus the binding of a second nucleotide neither changes the existing interactions nor induces additional interactions, such as ^{14}N couplings from the base nitrogen atoms bound within interacting distance at the second site.

We have also investigated the effect of oxidation of the [4Fe-4S] cluster of the Fe-protein on the ESEEM spectrum of the Mn^{2+} centre at the nucleotide-binding site. This is depicted in Figure 5 for a full supplement of Mn^{2+} , namely a 2:1 metal-nucleotide/protein stoichiometry. Here the FFT-ESEEM skyline projection of the MnATP complex in the presence of dithionite-reduced Kp2 (Figure 5A) is compared with that of the Indigo Carmine-oxidized protein (Figure 5B). The most intense resonance, observed for the reduced protein in the low-frequency region at a frequency

of 3.6 MHz, is again observed for the oxidized MnATP-Kp2 complex. However, the lineshape of the signal for the latter complex resembles much more closely that of MnADP with reduced Kp2 (Figure 4A) as compared with the one with MnATP (Figure 5A). The reappearance of the resonance at about 2.2 MHz is also notable, since it is not detected for the ATP complex with reduced protein. Thus the lineshape of the ESEEM signal at 3.6 MHz and the signal at 2.2 MHz of the oxidized protein are more akin to those from MnADP-Kp2^{red}. than to those from MnATP-Kp2^{red}.

HYSCORE

Four-pulse HYSCORE experiments were performed in order further to substantiate the observation that the broad three-pulse ESEEM features at 3.8 and 7.7 MHz originate from interactions with nucleotide phospho group(s) and possibly to disentangle better contributions from different nuclear species. The HYSCORE method generates correlations between the nuclear coherences belonging to different electron spin (M_S) manifolds and has proven to be particularly useful for substantiating metal phosphate couplings [23]. Figure 6 shows the 2D HYSCORE frequency domain data set for MnATP in the presence of reduced Kp2 after Fourier transformation and magnitude calculation. In the three-dimensional representation of HYSCORE spectra, the time interval, t_1 (see the pulse sequence in Figure 6), is varied as a function of the time t_2 for a fixed τ . Two clearly discernible features can be seen at off-diagonal positions in the upper quadrant of Figure 6, indicating that they arise from an interaction with a shf coupling $A < 2\nu_n$. The peaks at $(\nu^\alpha, \nu^\beta) \cong (3.1 \text{ MHz}, 8.5 \text{ MHz})$ are equally disposed around the ^{31}P nuclear Zeeman frequency and are thus assigned, in accordance with the three-pulse ESEEM results, to interactions with ^{31}P nuclei with a shf coupling of $|A| \approx 5.4 \text{ MHz}$. Except for the ^{31}P peaks, no other pronounced HYSCORE features are detected in the frequency region below 10 MHz for Kp2^{red}. with MnATP bound.

In contrast, HYSCORE spectra of MnADP with Kp2 display a number of features originating from interactions with other nuclear species than ^{31}P interactions. This is illustrated in Figure 7, which shows essentially similar off-diagonal peaks from the shf interaction with the nucleotide phosphate group(s) in both spectra. The other features below 4.0 MHz, observed predominantly on the diagonal of the $(++)$ quadrant, are assigned to interactions with either ^{14}N or ^{23}Na . However, after replacement of the Na^+ ion with K^+ , both Kp2^{red}-MnADP and also Kp2^{red}-MnATP exhibit noticeable features in the frequency range below 4.0 MHz. The features for Kp2 at frequencies $\leq 3.0 \text{ MHz}$, primarily on the diagonal of the positive quadrant, are provisionally assigned to the interaction of Mn^{2+} with ^{14}N nuclei. The peak on the diagonal at $(\nu^\alpha, \nu^\beta) \cong [+3.0, +3.0] \text{ MHz}$ has been attributed to the $(\nu_{\text{dq}}^+, \nu_{\text{dq}}^+)$ ^{14}N double quantum transition. This peak is hidden from detection in the three-pulse ESEEM, since it underlies the more prominent ^{31}P feature at this frequency. Cross-peaks are observed at $(\nu^\alpha, \nu^\beta) \cong [+3.0, +0.6] \text{ MHz}$ and $[+0.6, +3.0] \text{ MHz}$, which clearly correlate $\nu_{\text{dq}}^+, \nu_{\text{dq}}^+$ with at least one other peak on the diagonal at $(\nu^\alpha, \nu^\beta) \cong [+0.6, +0.6] \text{ MHz}$, which is hence attributed to a $(\nu_{\text{sq}}^+, \nu_{\text{sq}}^+)$ single quantum transition of the same spin manifold. This allows the clear separation of resonances from ^{31}P and ^{14}N , which have both frequency components at about 3 MHz and hence only generate a single broad peak from ^{31}P in the ESEEM spectrum. There are two other transitions on the diagonal at 1.2 and 2.0 MHz; the latter probably corresponds to the 2.2 MHz signal in the MnADP-Kp2 ESEEM spectrum. However, no obvious correlations are observable, so that it is presently unclear

Figure 6 2D amplitude spectra (top) and respective contour plot (bottom) of the four-pulse 2D-HYSCORE spectrum of MnATP in the presence of Kp2^{red}.

HYSCORE spectra (with the pulse sequence indicated) are recorded for a fixed τ of 160 ns after 2D Fourier transformation and magnitude calculation. The magnetic field was centred at the lowest field Mn²⁺ hf line ($B_0 = 323.1$ mT). Two quadrants are shown out of the complete 2D data set, labelled '(++)' and '(+-)'. The line connecting the ³¹P cross-peaks (indicated by the dotted line) intersects the diagonal at the phosphorus nuclear Zeemann frequency, ν_n , as indicated by the broken line. The microwave frequency was 9.653 GHz and the temperature 8 K. The sharp features detected along the diagonal are caused by unwanted echoes, occurring at frequencies related to the pulse programmer step frequency of 8 ns. These are often more persistent in the four- than in the three-pulse echo experiment, owing to insufficient suppression by the phase cycling routine.

whether these resonances represent the missing single quantum transitions or belong to transitions from a different ¹⁴N nucleus.

The frequency of the double quantum transitions is given by eqn (2):

$$\nu_{dq} \approx 2[(\nu_n \pm |A|/2)^2 + K^2(3 + \eta^2)]^{1/2} \quad (2)$$

showing that ν_{dq} depends on the effective shf-coupling $|A|$ and the NQI (nuclear quadrupole interaction) parameters that are given for this $I > 1/2$ system by $K = e^2qQ/4h$ and η [25]. Assuming that the NQI is negligible, one can estimate $|A|$ to be in the order of 1.0 MHz, which is far from the 'exact cancellation' condition (where $|A| = 2\nu_n$ so that the local field is zero in one M_S -state).

However, eqn (1) strictly only applies for systems close to the 'exact cancellation' condition, so that the coupling determined can only be regarded as a rough estimate. Even though the nuclear quadrupole coupling can probably not be ignored for this kind of nitrogen interaction, for the purpose of demonstrating the presence of a fundamental structural rearrangement upon hydrolysis, it is justifiable to do so. No other correlation peaks can be identified unequivocally and no contributions from transitions in the higher-spin manifolds of Mn²⁺ appear to be present.

2D HYSCORE experiments conducted with Kp2^{red}-MnATP after Na⁺ replacement by K⁺ (cf. Figure 7B) have shown that there is a noticeable difference in the region on the diagonal of the (++) quadrant where the Na⁺ peak would be expected. The

Figure 7 Frozen solution 2D-HYSCORE spectra of MnADP in the presence of reduced Kp2

Comparison of spectra for the standard preparation conditions (**A**) and of MnADP in the presence of Kp2^{red} after exchange of NaCl by KCl (**B**) shown as contour plots together with its HYSCORE skyline projections. Peaks labelled by the asterisk in the skyline projections represent experimental timing artifacts and the ²³Na peak is indicated in (**A**). The experimental conditions are the same as for Figure 6, except that the magnetic field setting was $B_0 = 324.6$ mT and microwave field $\nu_{MW} = 9.726$ GHz for (**A**) and $B_0 = 325.6$ mT and $\nu_{MW} = 9.671$ GHz for (**B**).

spectrum demonstrates that the transitions close to the ²³Na nuclear Zeeman frequency in the sample containing high Na⁺ concentrations (Figure 7A) are clearly absent for the sample exchanged into KCl (Figure 7B).

Apart from the absence of Na⁺ features, the contour plot apparently also lacks the $(\nu^\alpha, \nu^\beta) \cong [+3.0, +3.0]$ MHz double quantum peak observed with MnADP and instead displays a pronounced peak on the diagonal at $(\nu^\alpha, \nu^\beta) \cong [+2.2, +2.2]$ MHz with cross-peaks at $(\nu^\alpha, \nu^\beta) \cong [+2.2, +0.6]$ MHz and $[+0.6, +2.2]$ MHz respectively. These, again, presumably correlate the double quantum peak with one of the respective single quantum transitions at $(\nu^\alpha, \nu^\beta) \cong [+0.6, +0.6]$ MHz. An additional feature that has also been detected by three-pulse ESEEM is observed at $[+1.0, +1.0]$ MHz, but this shows no obviously assignable cross-correlations. Using the same strategy to determine the effective shf coupling constant as with MnADP, a value of $|A|$ of about 0.2 MHz is estimated. This value is fundamentally different from that with MnADP, which confirms the presence of substantial rearrangements in the nitrogen environment of the metal centre.

DISCUSSION

It is now well established that Mn²⁺ can readily substitute for Mg²⁺ in biological environments. Apart from their similar effective ionic radii, this is partially due to the preference of these two metal ions for similar types of ligands, especially oxygen atoms [50], although Mn²⁺ has a slightly higher tendency to bind nitrogen ligands, at least when it is in an octahedral co-ordination environment. The co-ordination environments of 'classical' nucleotide-binding proteins, in general, are all oxygen ligands where Mn²⁺, like Mg²⁺, presumably adopts an octahedral co-ordination geometry. Even though Mn²⁺ can replace Mg²⁺ in nitrogenase functioning, the all-oxygen ligand environment provides a plausible reason for the preference of 'classical' nucleotide-binding proteins for Mg²⁺.

In the present work we have studied the interactions of Mn²⁺ in ternary complexes with nucleotides and the nitrogenase Fe-protein. Such studies are of particular interest, since the catalytically functional enzyme can be investigated in the presence of ADP and ATP, presumably reproducing all the natural conformational rearrangements that regulate the nitrogenase substrate reduction reaction.

A perturbation approach, even for complexes with low zfs values, already fails at X-band, because the condition that $|D| \ll h\nu_{MW}$ is no longer strictly fulfilled. Therefore simulations using a perturbation approach for systems even with relatively small zfs values mandated the use of higher frequencies, so that spectra have generally been recorded at Q-band, where this approach remains applicable [16]. Here a method has been applied to simulate Mn²⁺ EPR spectra that works at any microwave frequency and can therefore serve as a versatile tool to determine the metal-centre geometry under any experimental conditions. Interestingly the Mn²⁺ zfs parameters for the nitrogenase Fe-protein are almost identical with those for Mn²⁺-nucleotides bound to other Walker-A-motif-containing proteins such as the GTP-binding proteins elongation factor Tu and p21Ras, and also the ATP-binding chloroplast H⁺-ATPase (cf. Table 1). Although the zfs parameters were not explicitly stated for elongation factor Tu, visual inspection of the spectrum indicates that they must be very similar to those observed in the present study, suggesting that the local metal-centre symmetries of all of these proteins are highly homologous. The axial zfs parameter determined for the nucleotide-bound Mn²⁺-centre in the presence of the Fe-protein is not very different from that found in the Mn²⁺(H₂O)₆ complex, and hence the Mn²⁺-binding symmetry can be regarded as typical for systems of low zfs.

The zfs parameters are indistinguishable for Mn²⁺ ADP or ATP complexes in the presence of the Fe-protein. Thus we conclude that conformational rearrangements of the Fe-protein derived from the local metal-centre symmetry are not identifiable purely on the basis of an EPR analysis, and higher-resolution techniques are required.

Table 2 Comparison of ³¹P shf couplings observed by ESEEM for some Mn²⁺-substituted ATPases and GTPases and an inorganic Mn²⁺-doped single crystal

Measurements were recorded using different magnetic-field settings throughout the Mn²⁺ sextet signal.

	$\nu_{\text{obs.}}$ (MHz)	$ A(^{31}\text{P}) $ (MHz)	Reference
Creatine kinase–Mn ²⁺ ADP–NO ₃ [–] – creatine	4.0		LoBrutto et al. [17]
	8.0	4.0	
Pyruvate kinase–Mn ²⁺ –MgATP–oxalate	3.9		Tipton et al. [18]
	8.0	4.1	
Staphylococcal nuclease–Mn ²⁺ –pdTp*	3.8		Serpensu et al. [19]
	7.7	3.9	
N-Ras ^{p21} –Mn ²⁺ GDP	4		Larsen et al. [21]
	8	4.0	
N-/H-Ras ^{p21} –Mn ²⁺ guanosine 5'- [β,γ -imido]triphosphate	3.52		Halkides et al. [20]
	7.29	3.77	
TF1–Mn ²⁺ ATP	3.7		Buy et al. [22]
	8.2	4.5	
GDPMH†–Mn ²⁺ GDP	3.7		Legler et al. [24]
	8.0	4.3	
Kp2–Mn ²⁺ ATP	3.8		The present study
	7.7	3.9	
Mn ²⁺ GDP	3.7		Legler et al. [24]
	8.0	4.3	
Na ₄ P ₂ O ₇ · 10H ₂ O single crystal	3.5		LoBrutto et al. [17]
	7.5	4.0	

*pdTp, thymidine 3',5'-diphosphate.

†GDPMH, GDP-mannose mannosylhydrolase.

³¹P interactions

The ³¹P shf coupling observed by ESEEM is assigned to an inner-sphere interaction of the Mn²⁺ centre with a phosphorus nucleus from the nucleotide phosphate moiety co-ordinated to the metal via at least one of the phospho oxygen atoms. The presence of this coupling clearly demonstrates that Mn²⁺, in the presence of the Fe-protein, forms a stable Mn²⁺–nucleotide complex. The comparison of the shf coupling measured here for the nitrogenase Fe-protein with those from other proteins are listed in Table 2. Although ³¹P resonances were observed in the stimulated echo ESEEM experiments, the HYSCORE spectrum best demonstrates the Mn²⁺–phosphate couplings.

The shf coupling of the directly co-ordinated ³¹P group is dominated by the Fermi contact coupling, indicating partial delocalization of the unpaired electron spin on to the phosphorus 3s orbital. We also obtained evidence from the HYSCORE data that a non-negligible electron-nuclear dipole interaction exists for the ³¹P coupling of the directly co-ordinating phosphate group(s). The shape of the off-diagonal ν^{α} , ν^{β} and $\nu^{\beta}, \nu^{\alpha}$ peaks in the HYSCORE contour plot is associated with the relative values of nuclear Zeeman as well as isotropic and anisotropic portions of the shf interactions [51,52], although a full interpretation of HYSCORE spectra for $S > 1/2$ systems is only just emerging [45,46,53,54]. Nonetheless the lineshape of the non-diagonal ³¹P resonances can be used to estimate the anisotropic contribution to the ³¹P shf coupling. The extension along the long axis of the lens-shaped ³¹P peaks is linked to the extent of the dipolar contribution to the shf tensor. Such an analysis might hold at least for bound MnADP, where Mn²⁺ presumably forms only monodentate nucleotide complexes. For the ATP-bound case, however, the

oblong shape of the off-diagonal peaks could be caused by a second ³¹P shf coupling from an additional co-ordinated phosphate group with a slightly inequivalent ³¹P shf coupling. Assuming that the short distance in the oval equals the intrinsic line width, measured to be ≈ 1.2 MHz, then the dipolar shf coupling can be determined from the difference between these two values. An estimate for the dipolar contribution to the ³¹P shf coupling yields 0.9 ± 0.2 MHz for the MnADP–Kp2^{red} ternary complex. This value is in good agreement with that for A^{dip} (the dipolar contribution to the hyperfine coupling) of 0.86 MHz [21] determined for the ³¹P coupling of a co-ordinating nucleotide phosphate group observed by multifrequency ESEEM for the Mn²⁺–GDP–p21Ras ternary complex. On the basis of a simple point dipole approximation, the distance between the Mn²⁺ centre and the phosphorus nucleus is estimated to be ≈ 0.33 nm (3.3 Å). This is in good agreement with the distance between Mg²⁺ and co-ordinating phosphate groups measured in the X-ray crystal structure of MgGTP bound to the Ras oncogene product H-p21 of 0.325 nm (3.25 Å) [55] and an average distance of 0.357 nm (3.57 Å) for the two subunits for the MgADP complex with Av2 [8].

The similarity of the observed ³¹P shf couplings for the metal chelate complexes with ADP and ATP implies that the electron-nuclear interactions of the metal ion with the directly co-ordinated nucleotide phosphate group(s) are relatively unaffected by structural rearrangements. However, the similarity does not necessarily also apply to the number of co-ordinated phosphate groups. In fact, no indication for couplings due to inequivalent phosphate groups was obtained for Kp2 in the presence of ATP by either ESEEM or HYSCORE. This is in marked contrast with the spectroscopic investigation of the F₁-ATPase [22,23], where separate shf couplings to P _{β} - and P _{γ} -phosphate groups were resolved with ATP and its analogue adenosine 5'-[β,γ -imido]triphosphate. There are two potential reasons for this: (i) Mn²⁺ is only a monodentate ligand of ATP in the Fe-protein, or (ii) Mn²⁺ is a multidentate ligand, but the couplings to the disparate phosphate groups are almost identical. Unfortunately the number of co-ordinated phosphate groups can not be determined by ESEEM or HYSCORE if they possess equivalent ³¹P shf couplings and they are too small to be resolved by EPR.

Even though there are a few occasions where a monodentate nucleotide coordination of Mg²⁺ is adopted in nitrogenase [9,10], it is probable that ATP is a bidentate metal ligand in the native Fe-protein in which the ³¹P couplings have to be nearly equivalent; this contrasts with the behaviour in F₁-ATPase, despite the considerable homology between the nucleotide-binding folds of the proteins. On the other hand it is similar to the MnATP complex in the absence of protein where only a single resonance is observed even though Mn²⁺ forms a bidentate complex with the nucleotide [23]. Thus we conclude, that the two ³¹P couplings to the directly co-ordinated phosphate groups in nitrogenase change much less upon binding to the protein than they do for F₁-ATPase.

The ³¹P shf coupling constants determined for the Fe-protein are very similar to those from Mn²⁺-nucleotide complexes found in various other ATPases and GTPases, such as the GTP binding switch proteins (G-proteins) (cf. Table 2). This includes both the 'classical' guanosine-nucleotide binding proteins, such as p21Ras, which possess the typical Walker A motif, and the 'non-classical' nucleotide-binding proteins with no such conserved amino acid sequences. The latter are, for example, the phosphogroup transferases such as pyruvate kinase [57,58]. This suggests that at least the immediate metal centre co-ordination, especially that of the co-ordinated phosphate groups, is similar for all these diverse proteins. The ³¹P shf-couplings derived by HYSCORE for the only two biological manganese nucleotide binding systems investigated to date are listed in Table 3, together

Table 3 Mn^{2+} -phosphorus shf couplings observed by HYSCORE for nucleotide-enzyme complexes with the *Bacillus* PS3 (TF1) ^1H -ATPase and Kp2^{red} , and comparison with the three-pulse ESEEM results for Mn^{2+} -nucleotides in the absence of protein

	$\nu^{\text{obs.}}$ (MHz)		Assignment	$ A(^{31}\text{P}) $ (MHz)	Reference
TF1- Mn^{2+} -ADP	3.50*	8.55	P_β	5.20	Schneider et al. [23]
TF1- Mn^{2+} -ATP	3.65*	8.25	P_β	4.60	Schneider et al. [23]
	3.05	8.90	P_γ	5.90	
Kp2- Mn^{2+} -ADP	3.25†	7.60	$(\text{P}_\beta)?$	4.40	The present study
Kp2- Mn^{2+} -ATP	3.00‡	8.40	$(\text{P}_\beta \text{ and } \text{P}_\gamma)?$	5.40	The present study
Mn^{2+} -ADP	3.70*	8.20	P_β (P_α)	4.50	Schneider et al. [23]
Mn^{2+} -ATP	3.70*	8.20	P_β and P_γ	4.50	Schneider et al. [23]

* Measured at 345.4 mT.
† Measured at 323.1 mT.
‡ Measured at 324.1 mT.

with the results for MnADP and MnATP complexes in the absence of protein.

^{14}N interaction

In the present study on the Fe-protein, interactions with nuclear species other than ^{31}P -couplings are identified, as opposed to the ESEEM or HYSCORE investigations on F_1 -ATPase in the presence of ADP or ATP [22,23]. For the Fe-protein, a number of potential nucleotide and protein nitrogen atoms exist in the vicinity of the nucleotide-bound metal centre. It is unlikely that these arise from the adenine base nitrogen atoms of the nucleotide, because the distances between the metal centre and any of these nitrogen atoms are much too large. Moreover, no ^{14}N resonances, which would provide evidence for metal base-nitrogen interactions, are reported for metal-nucleotide complexes studied either by ESEEM [26,42] or ENDOR [61]. Thus we assume that ^{14}N resonances must arise from interactions with protein nitrogen atom(s).

Site-directed mutagenesis experiments with the Fe-protein suggested that lysine (Lys¹⁵ in the Av2 sequence) is involved in ATP hydrolysis [64] and might be essential for rendering the terminal phosphate more susceptible to nucleophilic attack by water. ESEEM experiments for VO^{2+} -substituted nucleotides indicated that the amino side chain of such a lysine residue co-ordinates the metal directly in the related F_1 -ATPase in the absence of nucleotides [65]. Resonances from the $^{14}\text{N}_\epsilon$ nitrogen atom of the histidyl imidazole [17,62] or of amine lysine nitrogen atoms [18,22,65] directly co-ordinated to Mn^{2+} have usually shown ^{14}N NQR lines at substantially higher frequencies than observed here, thus excluding the likelihood that these residues are inner-sphere ligands of Mn^{2+} bound to the Fe-protein. Nonetheless, it is possible that the nitrogen atom of the lysine side-chain could come sufficiently close to the metal centre to represent a non-co-ordinated residue giving rise to a weak through-space interaction. Since Lys¹⁵ is in the same relatively rigid P-loop region as Ser¹⁶, we consider it unlikely that it causes the noticeable spectral changes observed, on the same grounds as stated below for Ser¹⁶. Therefore the resonances have been attributed to arise either from other backbone nitrogen atoms of the polypeptide or more remote, non-co-ordinated amino acid side groups. Polypeptide backbone nitrogen interactions with Rieske-type FeS cluster have been analysed by HYSCORE [63].

The closest outer-sphere protein nitrogen atom is the amide main-chain nitrogen atom of the co-ordinated serine residue

Figure 8 X-ray crystal structure of the Av2-MgADP ternary complex highlighting the nitrogen environment of the Mg^{2+} centre

Mg^{2+} (black sphere in the centre) possesses an all-oxygen inner-sphere co-ordination composition consisting of one P_β oxygen atom, one serine-side-chain oxygen atom and four water oxygen atoms complete the octahedral metal co-ordination environment. ADP is shown in a ball-and-stick representation. Surrounding protein residues within a 0.7 nm (7 Å) radius are depicted as sticks with the nitrogen atoms highlighted as dark grey balls.

(Ser¹⁶) at about a distance of 0.41 nm (4.1 Å) from the bivalent-metal-binding site. This residue is in the conserved P-loop region and remains relatively invariant in the ADP and ATP structures, and hence it may not be responsible for the noticeable nucleotide-dependent alterations in the ^{14}N -ESEEM spectra. However, for the serine residue differences are also observable when the MgADP and $\text{MgADP} \cdot \text{AlF}_4^-$ structures with Av2 are compared, so that a contribution of this residue to the ^{14}N -ESEEM spectra cannot be ruled out completely.

Six other residues lie within a radius of 0.5–0.6 nm (5.0–6.0 Å) of Mn^{2+} ; these are the side-chain amine nitrogen atoms of Lys⁴¹ and the main-chain nitrogen atoms of Asp⁴³, Ser⁴⁴, Val¹²⁶, Leu¹²⁷ and Gly¹²⁸ (Figure 8). All these nitrogen atoms are closely associated with the so-called ‘switch regions’ of the protein. Lys⁴¹, Asp⁴³ and Ser⁴⁴ belong to the switch I region and all undergo a considerable change in distance from Mg^{2+} when the Av2 structures with MgADP and the transition state are compared, with greater Mg^{2+} -to-nitrogen-atom distances for the latter. Conversely, backbone nitrogen atoms of Val¹²⁶, Leu¹²⁷ and Gly¹²⁸, which originate from the switch II region, move significantly closer to Mg^{2+} in the structure with $\text{MgADP} \cdot \text{AlF}_4^-$. The most dramatic change in distance [by nearly 0.39 nm (3.9 Å)] is observed for the Gly¹²⁸. Thus the ESEEM nitrogen environment of the metal centre is likely to be a superposition of contrasting effects; we favour an assignment of the ^{14}N resonances to peptide amino groups of the switch II domain or the Lys⁴¹ $^{14}\text{N}_\epsilon$ atom of the NH_3 side chain of the switch I domain.

Despite the inability to assign the ^{14}N resonances definitively to a specific amino acid residue, owing to the sheer numbers of surrounding nitrogen atoms, the data provide direct spectroscopic evidence for conformational rearrangements of the Fe-protein observed directly at the nucleotide-binding site. Earlier ESEEM measurements on the $S = 1/2$ EPR signal of [4Fe-4S] cluster of the Fe-protein did not detect any changes at the site of the cluster upon addition of MgATP [66], despite the obvious changes in the shape of the EPR signal.

Interestingly, when the Fe-protein was oxidized, the characteristic 2.2 MHz ESEEM resonance reappeared for bound MnATP, indicating that a second conformational change occurs at the Fe-protein nucleotide-binding site upon electron abstraction from the [4Fe-4S] cluster. The overall similarity of the ESEEM spectra from MnADP-Kp2^{red} and MnATP-Kp2^{ox} implies that, surprisingly, a remarkably homologous conformation is adopted for ATP bound after electron transfer, converting the distinctly

Scheme 1 Simplified and slightly modified Thorneley-Lowe model for the reductant-dependent nitrogenase cycle of the Fe-protein

The six stages of the cycle are: protein-protein association, ATP hydrolysis, Fe-protein oxidation and electron transfer to the MoFe-protein, protein-protein dissociation and inorganic phosphate release, Fe-protein re-reduction and, finally, re-activation of the Fe-protein through nucleotide exchange. The model assumes that bound Mn²⁺ cycles through the same stages as natural Mg²⁺ so that $\text{Mn}^{2+} = \text{Mg}^{2+}$ or Mn^{2+} . The model displays where in the cycle the conformational rearrangements, detected in the present work, are observed.

different MnATP–Kp2^{red} configuration back into a conformation comparable with that for MnADP–Kp2^{red}. We speculate that an equally dramatic conformational rearrangement occurs upon electron transfer as that upon hydrolysis.

Conformational rearrangements upon ATP hydrolysis are well established for nitrogenase; this is not the case for the electron transfer intermediate from the [4Fe-4S] cluster to the MoFe-protein. Here we show that the information about the redox state of the cluster is relayed back to the nucleotide-binding site, which demonstrates for the first time that conformational rearrangements are not unidirectional but, instead, that information is mediated in both directions, from the nucleotide binding site to the cluster and vice versa. We also note that this rearrangement requires only a single MnATP molecule to be bound, since spectra of the 1:1 MnATP–protein complex already show this rearrangement, with little change for the 2:1 complex. Subtle differences in the spectra are detected that depend critically on which nucleotide is bound and on the oxidation state of the Fe-protein.

These structurally distinct nucleotide co-ordination environments most directly indicate that the Fe-protein indeed undergoes a conformational rearrangement upon ATP hydrolysis and electron transfer. The stages of the conformational rearrangements within the hydrolysis-coupled redox cycle, identified in the present work, are depicted in Scheme 1.

²³Na interactions

Interestingly, an intense ESEEM line is observed that is attributed to an electron-nuclear interaction with Na⁺. Whether ADP or ATP was bound, this single, sharp ESEEM resonance was not significantly affected. The presence of an alkali cation, closely associated with the metal-nucleotide binding site, is consistent with observations made earlier by ESEEM for Nd³⁺ATP [42], Mn²⁺ATP complexes [26] and Mn²⁺ in the presence of the hammerhead ribozyme [59]. The Na⁺ resonance of these complexes exhibited no resolvable quadrupole splitting, the absence of which is probably the result of an almost cubic crystal field environment of the closed-shell ²³Na ion. A univalent ion was also found in a phospho-group transfer protein, pyruvate kinase [15], where it is presumably involved in a process which activates the protein which gives rise to similarly sharp Na⁺ ESEEM resonance for the Mn²⁺–pyruvate kinase–MgATP–oxalate complex [18] akin to that observed in the present study. Whether the alkali metal ion has a real structural or functional role

in nitrogenase, such as being a further source for compensation of the negative charge on the nucleotide, is not clear at present.

The difficulties in observing ²³Na correlation features in the HYSORE is probably due to the relatively complex pattern expected for an interaction with $I = 3/2$ ²³Na nuclei. In fact very little information exists concerning $S = 5/2$, $I = 3/2$ systems; only the simpler systems, such as $S = 5/2$ interacting with $I = 1/2$ or $I = 1$ nuclei or $S = 1/2$, $I = 3/2$ systems are somewhat better understood [44–47]. ²³Na interactions have also been observed in mixed alkali silicate glasses, and the HYSORE spectrum for such an Na⁺ interaction has been simulated [48]. The only other example, to our knowledge, for an Na⁺ ion interacting with Mn²⁺ studied by HYSORE in a biological matrix is the hammerhead ribozyme [49], where again no tangible correlation features could be assigned.

Finally, it has been noted that it is important to have a means for monitoring univalent-metal binding sites, such as K⁺ and Na⁺ in Mg²⁺ binding proteins [60]. Those proteins include ribozymes, which depend on uni- and bi-valent metals as cofactors. ESEEM might be a versatile tool to track two of the most common metal ions in Nature at nucleotide binding sites.

Supporting information

The following supporting information is available at <http://www.BiochemJ.org/bj/391/bj3910527add.htm>: (i) comparison of EPR simulations for opposite signs of the Mn²⁺ hfc; (ii) 2D-ESEEM time domain traces of MnADP–Kp2^{red}; (iii) comparison of MnADP–Kp2 preparations for disparate Mn²⁺ stoichiometries; and (iv) plot of the Larmor frequency shifts as a function of the excitation field setting.

This work was supported by a DTI LINK grant with BBSRC (Biotechnology and Biological Sciences Research Council), Zeneca Agrochemicals and Bruker Spectrospin, by a travel grant of the British Council (to J.P.), by Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie (to W.L.). D.J.L. thanks the BBSRC for financial support through the Core Strategic Grant to the John Innes Centre. We are also grateful to Dr H. Käss (Technische Universität, Berlin, Germany) for his expert experimental advice concerning the HYSORE measurements.

REFERENCES

- 1 Mortenson, L. E., Seefeldt, L. C., Morgan, T. V. and Bolin, J. T. (1993) The role of metal clusters and MgATP in nitrogenase catalysis. *Adv. Enzymol.* **67**, 299–374

- 2 Burgess, B. K. and Lowe, D. J. (1996) Mechanism of molybdenum nitrogenase. *Chem. Rev.* **96**, 2983–3011
- 3 Zumft, W. G., Mortenson, L. E. and Palmer, G. (1974) Electron-paramagnetic-resonance studies on nitrogenase; Investigation of the oxidation–reduction behaviour of azoferrodoxin and molybdoferrodoxin with potentiometric and rapid-freeze techniques. *Eur. J. Biochem.* **46**, 525–535
- 4 Orme-Johnson, W. H., Hamilton, W. D., Ljones, T., Tso, M.-Y. W., Burris, R. H., Shak, V. K. and Brill, W. J. (1972) Electron paramagnetic resonance of nitrogenase and nitrogenase components from *Clostridium pasteurianum* W5 and *Azotobacter vinelandii* OP. *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3142–3145
- 5 Smith, B. E., Lowe, D. J. and Bray, R. C. (1973) Studies by electron paramagnetic resonance on the catalytic mechanism of nitrogenase of *Klebsiella pneumoniae*. *Biochem. J.* **135**, 331–341
- 6 Walker, J. E., Saraste, M., Runswick, M. J. and Gay, N. J. (1982) Distantly related sequences in the α - and β -subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**, 945–981
- 7 Georgiadis, M. M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J. and Rees, D. C. (1992) Crystallographic structure of the nitrogenase iron protein from *Azotobacter vinelandii*. *Science* **257**, 1653–1659
- 8 Jang, S. B., Seefeldt, L. C. and Peters, J. W. (2000) Insights into signal transduction in nitrogenase: Structure of an iron protein with MgADP bound. *Biochemistry* **39**, 14745–14752
- 9 Schindelin, H., Kisker, C., Schlessman, J. L., Howard, J. B. and Rees, D. C. (1997) Structure of ADP · AlF_4^- -stabilized nitrogenase complex and its implications for signal transduction. *Nature (London)* **387**, 370–376
- 10 Chiu, H.-J., Peters, J. W., Lanzilotta, W. N., Ryle, M. J., Seefeldt, L. C., Howard, J. B. and Rees, D. C. (2001) MgATP-bound and nucleotide-free structures of a nitrogenase protein complex between the Leu 127 Δ -Fe-protein and the MoFe-protein. *Biochemistry* **40**, 641–650
- 11 Petersen, J., Fisher, K., Mitchell, C. J. and Lowe, D. J. (2002) Multiple inequivalent metal-nucleotide coordination environments in the presence of the VO^{2+} -inhibited iron protein: pH-dependent structural rearrangements at the nucleotide binding site. *Biochemistry* **41**, 13253–13263
- 12 Burns, R. C. (1969) The nitrogenase system from *Azotobacter*; activation energy and divalent cation requirement. *Biochim. Biophys. Acta* **171**, 253–259
- 13 Eady, R. R., Smith, B. E., Cook, K. A. and Postgate, J. R. (1972) Nitrogenase of *Klebsiella pneumoniae*: purification and properties of the component proteins. *Biochem. J.* **128**, 655–675
- 14 Seefeldt, L. C. and Mortenson, L. E. (1993) Increasing nitrogenase catalytic efficiency for MgATP by changing serine 16 of its Fe-protein to threonine: use of Mn^{2+} to show interaction of serine 16 with Mg^{2+} . *Protein Sci.* **2**, 93–102
- 15 Mildvan, A. S. (1974) Mechanism of enzyme action. *Ann. Rev. Biochem.* **43**, 357–399
- 16 Reed, G. H. and Markham, G. D. (1984) EPR of Mn(II) complexes with enzymes and other proteins. In *Biological Magnetic Resonance*, vol. 6 (Berliner, L. J. and Reuben, J., eds.), pp. 73–142, Plenum Press, New York
- 17 LoBrutto, R., Smithers, G. W., Reed, G. H., Orme-Johnson, W. H., Tan, S. L. and Leigh, J. S. (1986) Observation of manganese(II)-ligand superhyperfine couplings in complexes with proteins by electron spin-echo spectroscopy. *Biochemistry* **25**, 5654–5660
- 18 Tipton, P. A., McCracken, J., Cornelius, J. B. and Peisach, J. (1989) Electron spin echo envelope modulation studies of pyruvate kinase active-site complexes. *Biochemistry* **28**, 5720–5728
- 19 Serspers, E. H., McCracken, J., Peisach, J. and Mildvan, A. S. (1988) Electron spin echo modulation and nuclear relaxation studies of staphylococcal nuclease and its metal-coordinating mutants. *Biochemistry* **27**, 8034–8044
- 20 Halkides, C. J., Farrar, C. T., Larsen, R. G., Redfield, A. G. and Singel, D. J. (1994) Characterization of the active site of p21 ras by electron spin-echo envelope modulation spectroscopy with selective labeling: Comparisons between GDP and GTP forms. *Biochemistry* **33**, 4019–4035
- 21 Larsen, R. G., Halkides, C. J., Redfield, A. G. and Singel, D. J. (1992) Electron spin-echo envelope modulation spectroscopy of Mn^{2+} · GDP complexes in N-ras p21 with selective ^{15}N labeling. *J. Am. Chem. Soc.* **114**, 9608–9611
- 22 Buy, C., Girault, G. and Zimmermann, J.-L. (1996) Metal binding sites in H^+ -ATPase from chloroplast and *Bacillus* PS3 studied by EPR and pulsed EPR spectroscopy of bound manganese(II). *Biochemistry* **35**, 9880–9891
- 23 Schneider, B., Sigalat, C., Amano, T. and Zimmermann, J.-L. (2000) Evidence for changes in the nucleotide conformation in the active site of H^+ -ATPase as determined by pulsed EPR spectroscopy. *Biochemistry* **39**, 15500–15512
- 24 Legler, P. M., Lee, H. C., Peisach, J. and Mildvan, A. S. (2002) Kinetic and magnetic resonance studies of the role of metal ions in the mechanism of *Escherichia coli* GDP-mannose mannosyl hydrolase, an unusual nudix enzyme. *Biochemistry* **41**, 4655–4668
- 25 Dikanov, S. A. and Astashkin, A. V. (1989) ESEEM of disordered systems: theory and application. In *Advanced EPR, Applications in Biology and Biochemistry* (Hoff, A. J., ed.), pp. 59–117, Elsevier, Amsterdam
- 26 Mims, W. B. and Peisach, J. (1989) ESEEM and LEFE of metalloproteins and model compounds. In *Advanced EPR, Applications in Biology and Biochemistry* (Hoff, A. J., ed.), pp. 1–55, Elsevier, Amsterdam
- 27 Dikanov, S. A. and Tsvetkov, Y. D. (1992) *Electron Spin Echo Envelope Modulation (ESEEM) Spectroscopy*, CRC Press, Boca Raton, FL
- 28 Ashby, G. A. and Thorneley, R. N. F. (1987) Nitrogenase of *Klebsiella pneumoniae*. Kinetic studies on the Fe-protein involving reduction by sodium dithionite, the binding of MgADP and a conformation change that alters the reactivity of the 4Fe-4S centre. *Biochem. J.* **246**, 455–465
- 29 Petersen, J. and Lowe, D. J. (2002) Probing the electronic structure of polynuclear metal clusters with total electron spin $S > 1/2$ and significant zero-field splitting: Application to the clusters of the nitrogenase MoFe-protein. *Phys. Chem. Chem. Phys.* **4**, 2356–2364
- 30 Keijzers, C. P., Reijerse, E. J., Stam, P., Dumont, M. F. and Gribnau, M. C. M. (1987) MAGRES: A general program for electron spin resonance, ENDOR and ESEEM. *J. Chem. Soc., Faraday Trans.* **83**, 3493–3503
- 31 Coffino, A. R. and Peisach, J. (1996) Nuclear modulation effects in high-spin electron systems with small zero-field splittings. *J. Magn. Reson.* **111 B**, 127–134
- 32 Käss, H., Fromme, P. and Lubitz, W. (1996) Quadrupole parameters of nitrogen nuclei in the cation radical P^{+}_{700} determined by ESEEM of single crystals of photosystem I. *Chem. Phys. Lett.* **257**, 197–206
- 33 Fauth, J.-M., Schweiger, A., Braunschweiler, L., Forrer, J. and Ernst, R. R. (1986) Elimination of unwanted echoes and reduction of dead time in three-pulse electron spin-echo spectroscopy. *J. Magn. Reson.* **66**, 74–85
- 34 Larsen, R. G., Halkides, C. J. and Singel, D. J. (1993) A geometric representation of nuclear modulation effects: the effects of high electron spin multiplicity on the electron spin echo envelope modulation spectra of Mn^{2+} complexes of N-ras p21. *J. Chem. Phys.* **98**, 6704–6721
- 35 Goodman, B. A. and Raynor, J. B. (1970) Electron spin resonance of transition metal complexes. *Adv. Inorg. Chem. Radiochem.* **13**, 135–362
- 36 Korkmaz, M., Dupont, M. and Aktas, B. (1984) EPR of Mn^{2+} in $\text{Cd}(\text{COO})_2 \cdot 3\text{H}_2\text{O}$ single crystals. *J. Phys. Chem. Solids* **45**, 465–470
- 37 Anthonisamy, V. S. X., Padiyan, D. P. and Murugesan, R. (1998) Single-crystal EPR studies on Ni(II)- and Mn(II)-doped hexakis(pyrazole) complexes of Zn(II) and Cd(II): A trigonally distorted cubic environment. *Mol. Phys.* **94**, 275–281
- 38 Padiyan, D. P., Muthukrishnan, C. and Murugesan, R. (2002) Single crystal EPR studies on Mn(II)-doped sarcosine cadmium chloride and sarcosine cadmium bromide: Study of zero-field splitting tensor in iso-structural complexes. *Spectrochim. Acta* **A58**, 509–517
- 39 Brill, A. S., Fiamingo, F. G., Hampton, D. A., Levin, P. D. and Thorkildsen, R. (1985) Density of low-energy vibrational states in a protein solution. *Phys. Rev. Lett.* **54**, 1864–1867
- 40 Hendrich, M. P. and Debrunner, P. G. (1988) EPR spectra of quintet ferrous myoglobin and a model heme compound. *J. Magn. Reson.* **78**, 133–141
- 41 Misra, S. K. (1996) Interpretation of Mn^{2+} spectra in disordered materials. *Appl. Magn. Reson.* **10**, 193–216
- 42 Shimizu, T., Mims, W. B., Peisach, J. and Davis, J. L. (1979) Analysis of the electron spin echo decay envelope for Nd^{3+} :ATP complexes. *J. Chem. Phys.* **70**, 2249–2254
- 43 Scholes, C. P. (1979) In *Multiple Electron Resonance Spectroscopy* (Dorio, M. M. and Freed, J. H., eds.), pp. 297–330, Plenum, New York
- 44 Gutjahr, M., Böttcher, R. and Pöppel, A. (2002) Analysis of correlation patterns in hyperfine sublevel correlation spectroscopy of $S = 1/2$, $I = 3/2$ systems. *Appl. Magn. Reson.* **22**, 401–414
- 45 Benetis, N. P., Dave, P. C. and Goldfarb, D. (2002) Characteristics of ESEEM and HYSCORE spectra of $S > 1/2$ centers in orientationally disordered systems. *J. Magn. Reson.* **158**, 126–142
- 46 Madi, Z. L., Van Doorslaer, S. and Schweiger, A. (2002) Numerical simulation of one- and two-dimensional ESEEM experiments. *J. Magn. Reson.* **154**, 181–191
- 47 Finazzo, C., Harmer, J., Bauer, C., Jaun, B., Duin, E. C., Mählert, F., Goenrich, M., Thauer, R. K., Van Doorslaer, S. and Schweiger, A. (2003) Coenzyme B induced coordination of coenzyme M via its thiol group to Ni(I) of F_{430} in active methyl-coenzyme M reductase. *J. Am. Chem. Soc.* **125**, 4988–4989
- 48 Astrakas, L. and Kordas, G. (1999) Electron spin echo envelope modulation spectroscopy in mixed alkali silicate glasses. *J. Chem. Phys.* **110**, 6871–6875
- 49 Schiemann, O., Fritscher, J., Kisseleva, N., Sigurdsson, S. T. and Prisner, T. F. (2003) Structural investigation of a high-affinity Mn^{II} binding site in the hammerhead ribozyme by EPR spectroscopy and DFT calculations. Effects of neomycin B on metal-ion binding. *Chem. Biochem.* **4**, 1057–1065
- 50 Bock, C. W., Katz, A. K., Markham, G. D. and Glusker, J. P. (1999) Manganese as a replacement for magnesium and zinc: Functional comparison of the divalent ions. *J. Am. Chem. Soc.* **121**, 7360–7372

- 51 Reijerse, E. J. and Dikanov, S. A. (1992) Exploiting lineshape singularities in ESEEM of orientationally disordered systems. *Pure Appl. Chem.* **64**, 789–797
- 52 Dikanov, S. A. and Bowman, M. K. (1995) Cross-peak lineshape of two-dimensional ESEEM spectra in disordered $S = 1/2$, $I = 1/2$ spin systems. *J. Magn. Reson.* **A116**, 125–128
- 53 Coffino, A. R. and Peisach, J. (1992) Nuclear modulation effects in high-spin electron systems with small zero-field splittings. *J. Chem. Phys.* **97**, 3072–3091
- 54 Astashkin, A. V. and Raitsimring, A. M. (2002) Electron spin echo envelope modulation theory for high electron spin systems in weak crystal field. *J. Chem. Phys.* **117**, 6121–6132
- 55 Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W. and Wittinghofer, A. (1990) Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 Å resolution: Implications for the mechanism of GTP hydrolysis. *EMBO J.* **9**, 2351–2359
- 56 Reference deleted
- 57 Buchbinder, J. L. and Reed, G. H. (1990) Electron paramagnetic resonance studies of the coordination schemes and site selectivities for divalent metal ions in complexes with pyruvate kinase. *Biochemistry* **29**, 1799–1806
- 58 Tan, X., Poyner, R., Reed, G. H. and Scholes, C. P. (1993) Electron nuclear double resonance study of the Mn²⁺ environs in the oxalate–ATP complex of pyruvate kinase. *Biochemistry* **32**, 7799–7810
- 59 Hoogstraten, C. G., Grant, C. V., Horton, T. E., DeRose, V. J. and Britt, R. D. (2002) Structural analysis of metal ion ligation to nucleotides and nucleic acids using pulsed EPR spectroscopy. *J. Am. Chem. Soc.* **124**, 834–842
- 60 Pyle, A. M. (2002) Metal ions in the structure and function of RNA. *J. Biol. Inorg. Chem.* **7**, 679–690
- 61 Mustafi, D. and Makinen, M. W. (1988) ENDOR-determined solvation structure of VO²⁺ in frozen solutions. *Inorg. Chem.* **27**, 3360–3368
- 62 McCracken, J., Peisach, J., Bhattacharyya, L. and Brewer, F. (1991) Electron spin echo envelope modulation studies of lectins: Evidence for a conserved Mn²⁺-binding site. *Biochemistry* **30**, 4486–4491
- 63 Dikanov, S. A., Xun, L., Karpel, A. B., Tyryshkin, A. M. and Bowman, M. K. (1996) Electron spin echo envelope modulation studies of lectins: evidence for a conserved Mn²⁺-binding site. *J. Am. Chem. Soc.* **118**, 8408–8416
- 64 Seefeldt, L. C., Morgan, T. V., Dean, D. R. and Mortenson, L. E. (1992) Electron spin echo envelope modulation studies of lectins: evidence for a conserved Mn²⁺-binding site. *J. Biol. Chem.* **267**, 6680–6688
- 65 Houseman, A. L. P., LoBrutto, R. and Frasch, W. D. (1995) Effects of nucleotides on the protein ligands to metals at the M2 and M3 metal-binding sites of the spinach chloroplast F₁-ATPase. *Biochemistry* **34**, 3277–3285
- 66 Morgan, T. V., McCracken, J., Orme-Johnson, W. H., Mims, W. B., Mortenson, L. E. and Peisach, J. (1990) Pulsed electron paramagnetic resonance studies of the interaction of MgATP and D₂O with the iron protein of nitrogenase. *Biochemistry* **29**, 3077–3082
- 67 Wilson, G. E. and Cohn, M. (1977) Magnetic resonance studies of the manganese guanosine di- and triphosphate complexes with elongation factor Tu. *J. Biol. Chem.* **252**, 2004–2009
- 68 Webb, M. R., Ash, D. E., Leyh, T. S., Trentham, D. R. and Reed, G. H. (1982) Electron paramagnetic resonance studies of Mn(II) complexes with myosin subfragment 1 and oxygen 17-labeled ligands. *J. Biol. Chem.* **257**, 3068–3072
- 69 Latwesen, D. G., Poe, M., Leigh, J. S. and Reed, G. H. (1992) Electron paramagnetic resonance studies of a ras p21–Mn^{II}GDP complex in solution. *Biochemistry* **31**, 4946–4950

Received 4 February 2005/6 June 2005; accepted 21 June 2005

Published as BJ Immediate Publication 21 June 2005, doi:10.1042/BJ20050226