Proton NMR Investigation of the [4Fe−4S]$^{1+}$ Cluster Environment of Nitrogenase Iron Protein from *Azotobacter vinelandii*: Defining Nucleotide-Induced Conformational Changes†

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ABSTRACT: This work presents the complete assignment of the isotropically shifted ^{1}H NMR resonances of *Azotobacter vinelandii* nitrogenase iron protein (Fe protein) to β-CH_2 and α-CH protons of the [4Fe−4S]$^{1+}$ cluster cysteinyl ligands. Four resonances were observed for the reduced Fe protein with chemical shifts of 49, 23, 17, and 13 ppm. T_1 measurements and analysis of relative peak areas coupled with one-dimensional nuclear Overhauser effect (NOE) difference spectra were used to assign the two most downfield-shifted resonances (49 and 23 ppm) to cysteinyl ligand β-CH_2 protons and the 17 and 14 ppm resonances to cysteinyl ligand α-CH protons. Temperature dependence studies of the isotropically shifted protons revealed both Curie and anti-Curie behavior. These results, along with previous Mossbauer studies of the Fe protein, allowed the assignment of signal A (49 ppm) to four β-CH_2 protons and signal C (17 ppm) to 2 α-CH protons of two cysteinyl ligands bound to a mixed-valence iron pair (Fe^{3+}−Fe^{2+}) of the [4Fe−4S]$^{1+}$ cluster. Signal B (23 ppm) was assigned to four β-CH_2 protons, and signal C (17 ppm) and D (13 ppm) were assigned to two α-CH protons of two cysteinyl ligands bound to a ferrous pair of irons ($2Fe^{2+}$). The effects of MgATP, MgADP, and Mg-adenosine-β,γ-methylene-5′-triphosphate binding to the Fe protein on the assigned resonances were established and are discussed in the context of nucleotide-induced changes in the protein environment of the [4Fe−4S] cluster. In addition, conditions are described that prevent the long-standing problem of *A. vinelandii* Fe protein self-oxidation.

Nitrogenase catalyzes the biological reduction of dinitrogen according to the overall reaction

$$N_2 + 8H^+ + 16MgATP + 8e \rightarrow 2\,NH_3 + H_2 + 16MgADP + 16P_i \quad (1)$$

Nitrogenase is a complex metalloenzyme consisting of two separable component proteins, the molybdenum−iron protein (MoFe protein)[1] and the iron protein (Fe protein) (Burris, 1991; Smith & Eady, 1992; Dean et al., 1993; Mortenson et al., 1993; Rees et al., 1993). The MoFe protein contains two molybdenum−iron cofactors (FeMoco), which are the site of substrate reduction, and two [8Fe−8S] clusters (P clusters), which appear to mediate electron transfer from the Fe protein to FeMoco. The Fe protein is a 64 kDa homodimer containing a single [4Fe−4S] cluster and two MgATP binding sites (Georgiadis et al., 1992). MgATP binding to the Fe protein appears to serve at least two functions in the nitrogenase mechanism (Seefeldt, 1994). First, MgATP binding to the Fe protein induces a series of conformational changes that are necessary for Fe protein docking to the MoFe protein. Second, MgATP hydrolysis by the Fe protein−MoFe protein complex is required for the transfer of an electron from the [4Fe−4S] cluster of the Fe protein to the MoFe protein and subsequent substrate reduction.

Defining the conformational changes induced in the Fe protein by MgATP or MgADP binding is essential in understanding the mechanism of nitrogenase component docking, MgATP hydrolysis, and intercomponent electron transfer. It is known that nucleotide binding to the Fe protein results in changes in the properties of its [4Fe−4S] cluster (Mortenson et al., 1993). From the recent X-ray structure of the *A. vinelandii* Fe protein, it is known that the [4Fe−4S] cluster is bridged between identical subunits approximately 19 Å away from the two nucleotide binding sites (Georgiadis et al., 1992). Thus, nucleotide binding-induced changes in the [4Fe−4S] cluster must be communicated through protein conformational changes (Mortenson et al., 1993). Significant among the changes in the properties of the [4Fe−4S] cluster upon MgADP or MgATP binding to the Fe protein, is the lowering of the midpoint reduction potential from −290 to −430 mV (Zumft et al., 1974; Morgan et al., 1986). The lower reduction potential of the [4Fe−4S] cluster appears to be necessary, although not sufficient, for intercomponent electron transfer (Lanzilotta et al., 1995). A variety of spectroscopic techniques (e.g., EPR, CD, and Mossbauer spectroscopies) have revealed changes in the electronic properties of the [4Fe−4S] cluster upon MgATP binding to the Fe protein (Zumft et al., 1973;

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[1] Abbreviations: Fe protein (iron protein of nitrogenase); MoFe protein (molybdenum−iron protein of nitrogenase); *Av2*, (Fe protein of *Azotobacter vinelandii*); *Cp2*, (Fe protein of *Clostridium pasteurianum*); ^{1}H NMR (proton nuclear magnetic resonance), EPR (electron paramagnetic resonance), Tris (tris(hydroxymethyl)aminomethane), NOE (nuclear Overhauser effect).

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Stephens et al., 1979; Lindahl et al., 1985). Fe−K edge X-ray absorption studies of Fe proteins have revealed that nucleotide binding does not change the Fe−Fe or Fe−S bond distances in the [4Fe−4S] cluster significantly, suggesting that the changes induced by nucleotide binding to the Fe protein are mediated through changes in the protein environment near the [4Fe−4S] cluster (Lindahl et al., 1987). Very little is currently known about the changes in the protein environment around the [4Fe−4S] cluster upon binding of nucleotides to the Fe protein.

^{1}H NMR spectroscopy has emerged as a valuable technique for the study of paramagnetic [4Fe−4S] cluster-containing proteins (Bertini et al., 1993; La Mar & de Ropp, 1993; Cheng & Markley, 1995). Protons proximate to the cluster are shifted out of the diamagnetic envelope, providing a fingerprint of the cluster environment. These hyperfine shifted resonances are extremely sensitive to the distance and orientation of the protons relative to the electronic spin state of the paramagnetic center. ^{1}H NMR studies of proteins containing [4Fe−4S] clusters have provided a great deal of information about the cluster environment as well as the electronic properties of the cluster (Bertini et al., 1993; La Mar & de Ropp, 1993; Cheng & Markley, 1995). [4Fe−4S] clusters are typically divided into two subgroups according to their redox couple, with the high-potential proteins accessing the 3+/2+ oxidation states and the low-potential or ferredoxin-like proteins accessing the 2+/1+ oxidation[2] states (Johnson, 1994). While proteins containing high-potential $[4Fe-4S]^{3+/2+}$ clusters have been extensively studied by ^{1}H NMR (Bertini et al., 1993; La Mar & de Ropp, 1993; Cheng & Markley, 1995), relatively few studies have been reported on proteins containing $[4Fe-4S]^{1+}$ clusters (Sweeney, 1981; Bertini et al., 1992; Donaire et al., 1994). The single [4Fe−4S] cluster of the nitrogenase Fe protein is a low-potential cluster that is similar to other low-potential [4Fe−4S] clusters in ferredoxins. An earlier ^{1}H NMR study of the $[4Fe-4S]^{1+}$ state of *Clostridium pasteurianum* Fe protein revealed three groups of isotropically shifted protons in the 50−15 ppm chemical shift range (Meyer et al., 1988). MgATP or MgADP binding to the Fe protein was observed to change the chemical shifts of several of these proton signals; however, none of these resonances was assigned.

In order to provide a more detailed understanding of the protein environment around the $[4Fe-4S]^{1+}$ cluster of the nitrogenase Fe protein and the conformational changes induced by MgATP or MgADP binding, we have examined the reduced state of the Fe protein from *Azotobacter vinelandii* by ^{1}H NMR spectroscopy. The long-standing problem of self-oxidation observed for highly concentrated samples of *A. vinelandii* Fe protein was solved, providing stable reduced enzyme for at least 48 h. Analysis of the isotropic shifts, T_1 values, and NOE difference spectra allowed complete assignment of the hyperfine-shifted signals to β-CH_2 and α-CH protons of the $[4Fe-4S]^{1+}$ cluster cysteinyl ligands. The temperature dependence of each isotropically shifted resonance was determined, providing information about the magnetic properties of the $[4Fe-4S]^{1+}$ cluster. In addition, the effects of binding MgATP, MgADP, or the nucleotide analog adenosine-β,γ-methylene 5′-triphosphate to the Fe protein on the isotropically shifted resonances were examined, providing new insights into conformational changes around the cluster upon nucleotide binding.

[2] The redox couples of low-potential [4Fe−4S] clusters can also be defined in terms of the formal charges of the entire cluster [4Fe−4S-(Cys)$_4$] in which the $2^+/1^+$ couple is defined as $2^-/3^-$.

MATERIALS AND METHODS

Nitrogenase Iron Proteins. Nitrogenase Fe protein was purified from *A. vinelandii* cells as previously described (Seefeldt & Mortenson, 1993) and was homogeneous as determined by Coomassie staining of sodium dodecyl sulfate−polyacrylamide gels (Hathaway et al., 1979). Fe protein used in these studies had specific activities of at least 1820 nmol of acetylene reduced min^{-1} (mg of Fe protein)$^{-1}$ and 1890 nmol of H_2 evolved min^{-1} (mg of Fe protein)$^{-1}$ (Seefeldt & Ensign, 1994). Protein concentrations were determined by a modified Biuret method with bovine serum albumin as the standard (Chromy et al., 1974). ATP, ADP, and adenosine-β,γ-methylene 5′-triphosphate were purchased from Sigma Chemical Co. (St. Louis, MO).

NMR Sample Preparation. Fe protein samples for NMR were prepared in an argon-filled glovebox with an oxygen concentration less than 1 ppm. Fe protein samples were exchanged into a Tris-buffered D_2O solution (70 mg of Tris-HCl and 20 mg of Tris-base in 10 mL of D_2O with 5 mM dithionite, pH 7.5) by passage through a Sephadex G-25 column (Lanzilotta et al., 1995). Unless otherwise noted, the Tris-buffered D_2O solution was passed through a Chelex 100 column prior to equilibration of the Sephadex column. Fe protein fractions from the Sephadex column were concentrated to between 65 and 75 mg ml^{-1} by microfiltration with a Microcon-30 (Amicon, Beverly, MA). In all cases, sealed glass (5 mm) NMR tubes (Wilmad, Buena, NJ) were used to maintain an anaerobic environment. Analysis of Fe protein samples after NMR revealed less than a 5% loss in activity when held at 305 K. Nucleotides were prepared as 25 mM stock solutions by dissolving the nucleotide in 1 M Tris-buffered D_2O that had been passed through a Chelex 100 column. $MgCl_2$ was added to a final concentration of 30 mM. As indicated, an aliquot of the nucleotide stock solution was added to the Fe protein to a final concentration of 5 mM.

Physical Methods. Proton NMR spectra were recorded on a Bruker ARX-400 spectrometer at 400.17 MHz. A modified-DEFT multipulse sequence was used to suppress the water signal and the resonances in the diamagnetic region (Hochmann & Kellerhals, 1980). The pulse sequence repetition rate was typically 3 s^{-1} with a spectral window of 83 kHz. Chemical shifts (in ppm) were referenced to the residual water peak at 4.7 ppm. The ^{1}H NMR data were Fourier transformed with an exponential apodization function. A 30 Hz line broadening was also applied. Longitudinal relaxation times (T_1) were measured by the use of an inversion−recovery pulse sequence (180°−τ−90°). Plots of $\ln(I_o - I_\tau)$ vs τ for each signal provided a straight line over all τ values investigated. Peak areas were determined as relative areas based on the 3:1 area ratio of signals C and D for native *Av2*. Non-baseline-subtracted spectra were used to determine these areas by the cut and weigh method. NOE difference spectra were obtained at 305 K by computer manipulation of the free induction decay with the saturation pulse set alternatively on the signal of interest and a reference position for 80 ms. Steady state NOE (η_{ij}) on signal i when signal j is saturated for a period of time t is given by

$$\eta_{ij} = \sigma_{ij}/\rho_i = 0.1\gamma 4h^2 r_{ij}^{-6}\tau_c T_1 \qquad (2)$$

where σ_{ij} is the cross-relaxation between i and j, τ_c is the rotational correlation time of the molecule, ρ_i (T_1) is the spin−lattice relaxation rate of proton i, and r_{ij} is the distance between nuclei i and j. The remaining constants have their usual meaning.

RESULTS

Stabilization of Reduced Av2. A significant limitation to studies of *A. vinelandii* Fe protein (*Av2*) at high concentrations has been stabilization of the reduced state. *Av2* is known to catalyze a time-dependent oxidation of the reductant dithionite, leading to oxidation of the [4Fe−4S] cluster to the 2^+ state. This reaction, termed "self-oxidation", is accelerated by increasing concentrations of *Av2* and the addition of MgATP or MgADP (Stephens et al., 1979, 1982; Braaksma et al., 1982; McKenna et al., 1991). In our initial attempts to record ^{1}H NMR spectra of *Av2* at concentrations above 1 mM, we found that *Av2* would self-oxidize in approximately 3 h, preventing the acquisition of spectra over long periods of time. It seemed possible that trace metals associated with the buffers used might participate in this self-oxidation reaction. Previous studies have found that divalent metal concentrations in protein samples used for NMR and EPR studies could be significantly reduced by pretreatment of the buffer solutions with the ion exchange resin Chelex 100 (Barker et al., 1979; Kochoyan et al., 1990; Ray et al., 1990). Chelex 100 has high affinity for transition metals and is widely used to remove traces of such metals from buffer solutions. To test the possibility that transition metals might participate in the *Av2* self-oxidation reaction, *Av2* samples were prepared in buffers that had been pretreated by passage through Chelex-100 resin. Figure 1 shows the downfield portion of the ^{1}H NMR spectrum of *Av2* samples that were exchanged into buffer that either had, or had not, been pretreated by passage through a Chelex 100 column. Traces 1 and 2 show spectra taken immediately after sample preparation, while traces 3 and 4 show spectra of the same samples after 15 h of incubation at 298 K. The *Av2* sample exchanged into Chelex 100-treated buffer was stable in the reduced state for at least 15 h, while the *Av2* sample in buffer that had not been treated with Chelex was oxidized within 3 h. We have observed stable *Av2* samples exchanged into Chelex 100-treated buffers for up to 2 days with no noticeable changes in the ^{1}H NMR spectrum or acetylene reduction activity.

While the mechanism of *Av2* self-oxidation remains unknown, the observation that this reaction can be greatly reduced by pretreatment of the buffer solutions by passage through a Chelex 100 column suggests that this reaction is mediated by transition metals commonly found in buffer solutions. The stabilization of *Av2* samples in the reduced state provided by the above observation will now allow analysis of highly concentrated *Av2* samples by several spectroscopic techniques.

^{1}H NMR Studies of Av2. The 400 MHz ^{1}H NMR spectrum of reduced *Av2* in Tris buffered D_2O, pH 7.5 at 305 K is shown in Figure 2. Four hyperfine-shifted signals were observed in the 60−10 ppm chemical shift range (Table 1). Hyperfine shifted protons observed for proteins containing [4Fe−4S]$^{1+}$ clusters have previously been assigned to β-CH_2 and α-CH protons of the cluster cysteinyl ligands (Bertini et al., 1993; La Mar & de Ropp, 1993). On the basis of chemical shifts and T_1 ($\propto r^6_{Fe-H}$) values, signal A (49 ppm; <1 ms) and signal B (23 ppm; 5 ms) were tentatively assigned to β-CH_2 protons of cysteinyl ligands, while signal C (17 ppm; 25 ms) and signal D (14 ppm; 17 ms) were tentatively assigned to cysteinyl α-CH protons. The relative areas of these signals indicated that A and B constituted

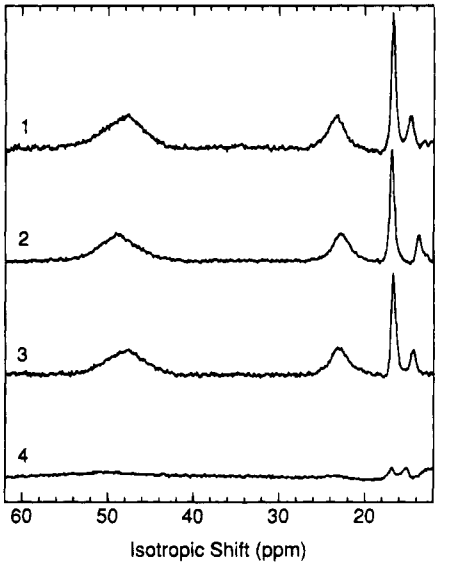


FIGURE 1: Stabilization of *A. vinelandii* nitrogenase Fe protein in the reduced (1+) state monitored by ^{1}H NMR spectroscopy. The downfield portion of the 400 MHz ^{1}H NMR spectrum of the nitrogenase Fe protein samples at 305 K are shown. Fe protein samples (between 70 and 80 mg ml^{-1} or 1.1 and 1.3 mM) were prepared by passage through a G-25 column equilibrated with a Tris-buffered D_2O solution that had been treated by passage through a Chelex 100 column (trace 1) or with a nontreated buffer solution (trace 2). Both samples were allowed to remain at 298 K for 15 h and ^{1}H NMR spectra were rerecorded for the samples (trace 3, Chelex-treated buffer; trace 4, nontreated buffer) under identical conditions.

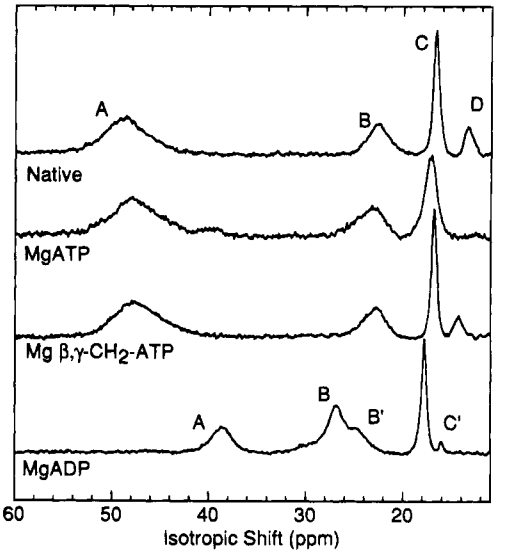


FIGURE 2: Effects of nucleotide binding on the isotropically shifted ^{1}H NMR resonances of Fe protein. The downfield portion of the 400 MHz ^{1}H NMR spectra at 305 K for Fe protein in the absence of nucleotides (Native), in the presence of MgATP (MgATP), in the presence of Mg-adenosine-β,γ-methylene 5′-triphosphate (Mg β,γ-CH_2-ATP), and in the presence of MgADP (MgADP). Fe protein samples (70−80 mg ml^{-1}) were prepared in Chelex-treated Tris-buffered D_2O solution, and spectra were recorded at 305 K. Nucleotides were added to a final concentration of 5 mM from stock solutions.

Table 1: Properties of the Isotropically Shifted β-CH_2 and α-CH Cysteinyl Proton NMR Resonances of the Reduced Nitrogenase FeP from *Azotobacter vinelandii* at 305 K

signal	assgnt	chemical shift (ppm)	width[a] (Hz)	relative area[b]	T_1 (ms)[c]	temp dependence
			no nucleotides			
A	β-CH_2	49	1800	4	<1	Curie
B	β-CH_2	23	1000	4	5	anti-Curie
C	α-CH	17	200	3	25	Curie
D	α-CH	13	500	1	17	anti-Curie
			+MgATP			
A	β-CH_2	48	2000	4		Curie
B	β-CH_2	23	1000	4		anti-Curie
C	α-CH	17	500	4		Curie
			+β,γ-Methylene-ATP			
A	β-CH_2	48	1700	4	1	Curie
B	β-CH_2	23	900	4	5	anti-Curie
C	α-CH_2	17	300	3	17	Curie
D	α-CH_2	14	300	1	8	anti-Curie
			+MgADP			
A	β-CH_2	39	900	4	6	Curie
B	β-CH_2	27	900	5	8	anti-Curie
B′	α-CH	25	800		7	anti-Curie
C	α-CH	18	300	3	16	Curie
C′		16	100	0.1	13	

[a] Full-width at half-maximum. [b] Relative areas are based on the areas of signals C and D. [c] Due to slow hydrolysis of MgATP at 305 K, the longitudinal relaxation times (T_1 values) for the MgATP-bound state could not be determined.

approximately 4 protons each while C and D constituted approximately 3 protons and 1 proton, respectively. The four cysteine ligands bound to the [4Fe−4S] cluster would have a total of 8 β-CH_2 protons and 4 α-CH protons. Our initial assignments are consistent with these stoichiometries.

Proton NMR provides a sensitive way to probe the changes in the cluster ligands induced by nucleotide binding to the Fe protein. Therefore, we recorded ^{1}H NMR spectra of the MgATP- and MgADP-bound states of *Av2*. Reduced *Av2* binds two MgATP molecules with high affinity (K_d = 570 μM) (Ryle et al., 1995). The binding of MgATP to *Av2* was found to result in small shifts for each of the four observed proton signals (Figure 2). Signal D was shifted such that it was no longer observable at 305 K. Signal A (45 ppm) shifted toward the diamagnetic region, while signals B (23 ppm) and C (18 ppm) shifted downfield. The three signals observed for the MgATP bound state (A−C) have relative areas of four protons each, suggesting that signal D had shifted downfield and was concealed beneath signal C.

T_1 values for the three signals in the MgATP-bound state could not be accurately measured since a slow hydrolysis of MgATP at 305 K to MgADP was observed. In order to obtain a stable, nucleotide−triphosphate bound Fe protein for NMR studies, we recorded the ^{1}H NMR spectrum of the Fe protein with the MgATP analog Mg-adenosine-β,γ-methylene triphosphate (Mg-β,γ-CH_2-ATP) bound (Figure 2). Previous studies have revealed that this analog results in identical EPR spectral changes for *Av2* (rhombic to axial) to that seen for MgATP binding (Zumft et al., 1973). In addition, Meyer et al. (1988) indicated that MgATP and the above analog resulted in identical changes in the ^{1}H NMR spectrum for *Cp2*. Since Mg-β,γ-CH_2-ATP was not hydrolyzed by nitrogenase, a stable nucleotide−triphosphate bound state seemed likely. With the analog bound, resonance A exhibited an identical shift to that observed for MgATP binding. However, unlike MgATP binding, the analog resulted in no detectable shifts for signals B or C. Interestingly, resonance D was only slightly shifted upon binding Mg-β,γ-CH_2-ATP, while it was significantly shifted upon binding MgATP. Relative peak areas and T_1 values for signals A and B, and C and D were consistent with their assignment to β-CH_2 and α-CH cysteinyl protons, respectively (Table 1).

Reduced *Av2* also binds two MgADP molecules with high affinity (K_d = 143 μM) (Ryle et al., 1995). MgADP and MgATP binding are competitive, suggesting binding to the same sites on the Fe protein. MgADP binding to the Fe protein is known to result in protein conformational changes; however, these changes appear to be quite different from those induced by MgATP binding. We have examined the effects of MgADP binding on the properties of the hyperfine shifted protons of *Av2*. MgADP binding to *Av2* resulted in the most significant changes in the isotropically shifted resonances for the Fe protein. Five hyperfine-shifted signals were observed in the 40−10 ppm chemical shift range at 305 K (Figure 2). The chemical shift of signal A (38 ppm; 6 ms) was drastically affected by MgADP binding with an 11 ppm shift to higher field compared to the native Fe protein. The two resonances labeled B (27 ppm; 8 ms) and B′ (25 ppm; 7 ms) were observed, in contrast to the single resonance observed for the native state in this chemical shift region. Signal C was shifted downfield by 2 ppm to 19 ppm (T_1 = 16 ms) and a new signal, labeled C′ (16 ppm; 13 ms), was observed. Inspection of the relative areas of the observed resonances of the MgADP-bound Fe protein indicates that signal A corresponds to approximately 4 protons while signal C corresponds to approximately 3 protons. These values are similar to the relative areas observed for the corresponding signals in the native state of the Fe protein. While the relative areas of the overlapping signals B and B′ were difficult to obtain accurately, their approximate areas were consistent with B and B′ integrating to 4 protons and 1 proton, respectively. These data, coupled with the observed T_1 values, suggested that signals A and B resulted from β-CH_2 cysteinyl protons and signals B′ and C resulted from α-CH cysteinyl protons. The remaining signal (C′) had a relative area less than a single proton. Since all of the β-CH_2 and α-CH cysteinyl protons have been

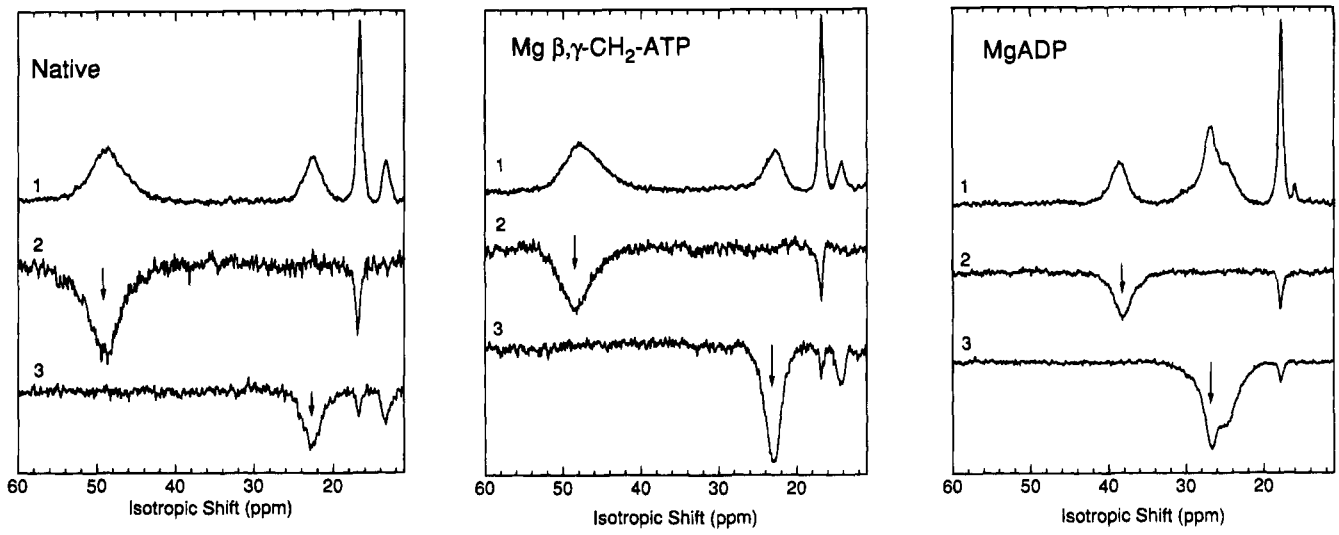


FIGURE 3: One-dimensional saturation transfer (NOE) difference spectra of the Fe protein. Fe protein samples were prepared as described in the legend to Figure 2. NOE difference ^{1}H NMR spectra were recorded at 305 K with the on-resonance decoupler pulse set at the frequency indicated by the arrow. Native panel: ^{1}H NMR spectrum of the Fe protein in the absence of nucleotides (trace 1) and NOE difference spectra with the decoupler pulse centered at 49 ppm (trace 2) and 23 ppm (trace 3). Mg-β,γ-CH_2-ATP panel: ^{1}H NMR spectrum of the Fe protein in the presence of 5 mM Mg-adenosine-β,γ-methylene 5′-triphosphate (trace 1) and NOE difference spectra with the decoupler pulse centered at 47 (trace 2) and 24 ppm (trace 3). MgADP panel: ^{1}H NMR spectrum of the Fe protein in the presence of 5 mM MgADP and NOE difference spectra with the decoupler pulse centered at 38 (trace 2) and 27 ppm (trace 3).

tentatively accounted for, it is possible that signal C′ is a proton in close proximity to the [4Fe−4S] cluster that is not associated with the cysteinyl ligands.

NOE Difference Spectra of the Fe Protein. Definitive assignment of each of the observed isotropically shifted signals for *Av2* was obtained from nuclear Overhauser effect (NOE) saturation transfer experiments. For paramagnetic metalloproteins with favorably short electronic relaxation times, steady state NOE has been shown to be a useful tool for identifying pairs of nuclei in close proximity to each other (Bertini et al., 1993; La Mar & de Ropp, 1993). For native Fe protein, NOE difference spectra were obtained for signals A and B at 305 K (Figure 3, Native). Irradiation of signal A for 80 ms showed clear NOE cross-relaxation to signal C. Likewise, when B was irradiated for 80 ms, clear NOE connection was observed to signals C and D. In order to verify that irradiation of B for 80 ms did not in turn partially irradiate signal C, which is 5 ppm away, the decoupler was calibrated by irradiating a single resonance of ferricytochrome *C* that was less than 2 ppm away from a second resonance. In this experiment, irradiation of either signal showed no irradiation of the second. These data indicate that under our pulsing conditions, decoupler power spillover did not occur for signals greater than or equal to 2 ppm away. In a separate experiment, the decoupler was placed off-resonance of signal B by 2 ppm downfield and clear NOE's were still observed between signals B and C, and B and D, albeit much weaker. These data indicate that protons A and C are in close proximity to one another and therefore, make up a β-CH_2 and α-CH pair from two cysteinyl ligands. Likewise, proton B is in close proximity to protons contributing to signals C and D, consistent with their assignment to β-CH_2 and α-CH protons of two cysteinyl ligands. These data also reveal that signal C is composed of signals from α-CH protons from three of the four cysteinyl ligands.

NOE difference spectra were also recorded for the analog (Mg-β,γ-CH_2-ATP) bound state of the Fe protein (Figure 3, Mg-β,γ-methylene-ATP). Signals A and B were irradiated independently with a decoupler pulse of 80 ms. Saturation of resonance A showed clear NOE cross-relaxation to signal C, similar to that observed for the native Fe protein. Likewise, irradiation of signal B showed clear NOE cross relaxation to signals C and D, consistent with the NOE's observed for the native state. These results confirm that signals A and B represent two nonequivalent groups of β-CH_2 protons while signals C and D represent two nonequivalent groups of α-CH protons.

Irradiation of signal A for the MgADP bound state of *Av2* revealed a clear NOE to signal C (Figure 3, MgADP). Like the native Fe protein, signal A was assigned to cysteine β-CH_2 protons and signal C to α-CH protons of the same pair of cysteines. Since B and B′ were overlapping, NOE studies on each signal individually was difficult, therefore the decoupler pulse was placed in the center of both signals with a saturation time of 80 ms. Clear NOE was observed between B (B′) and C. These data indicate that the protons of either B or B′ are in close proximity to C. Thus, by comparison with the native Fe protein data, signals A and B in all cases arise from β-CH_2 protons and signals C and D arise from α-CH protons (Table 1).

Temperature Studies. The influence of temperature on the isotropic shift of the observed resonances for the native, MgATP-, Mg-β,γ-CH_2-ATP-, and MgADP-bound states of the Fe protein was examined and is presented as Curie plots (Figure 4). Both Curie and anti-Curie temperature dependencies were observed for the isotropically shifted protons. In all cases, signal A followed Curie-like behavior (contact shift decreases with increasing temperature) while signals B and D displayed anti-Curie behavior (contact shift increases with increasing temperature). Signal C in each case was rather invariant to changing temperature but exhibited slight Curie-like behavior. Since D exhibited anti-Curie behavior, and one component of signal D resides under the envelope of signal C, a slight Curie-like behavior for C is not unexpected. At no temperature examined (280−320 K) could the two components of signal C be resolved. For the MgADP bound state, signal B′, like signal B, exhibited anti-Curie behavior indicating these protons reside on residues

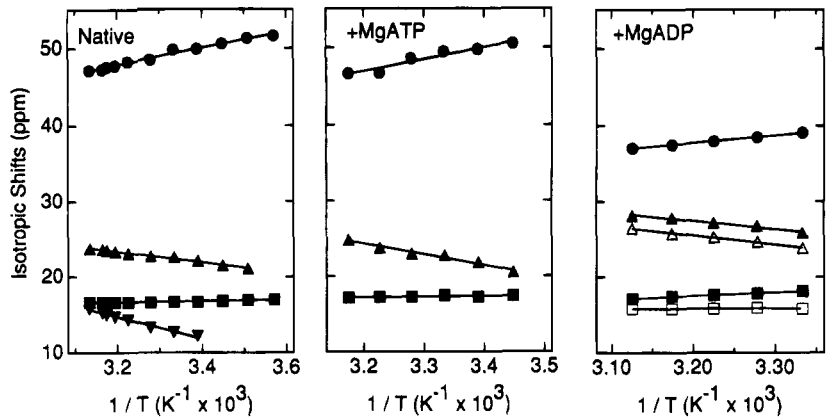


FIGURE 4: Temperature dependence of the isotropically shifted ^{1}H NMR resonances of Fe protein. Curie plots of the isotropically shifted signals observed for *Av2* at acquisition temperatures ranging from 280 to 320 K were recorded. Plots of observed chemical shift versus $1/T$ are shown. Native panel: Temperature dependence of signals A (●), B (▲), C (■), and D (▼) observed for the Fe protein in the absence of nucleotides. +MgATP panel: Temperature dependence of signals A (●), B (▲), and C (■) observed for the Fe protein in the presence of 5 mM MgATP. +MgADP panel: Temperature dependence of signals A (●), B (▲), B′ (△), C (■), and C′ (□) observed for the Fe protein in the presence of 5 mM MgADP.

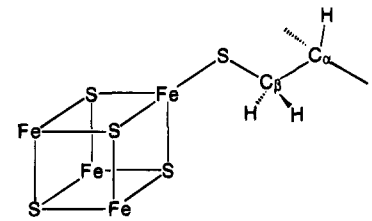
bound to the same iron pair. These data further support the conclusion that signal B is due to β-CH_2 cysteinyl protons and signal B′ is due to α-CH cysteinyl protons.

DISCUSSION

The [4Fe−4S] cluster of the Fe protein functions to reduce, in single-electron steps, the MoFe protein (probably the 8Fe cluster). The current model suggests that two MgATP molecules are hydrolyzed followed by the transfer of an electron to the MoFe protein. The oxidized Fe protein dissociates from the MoFe protein, and a second reduced Fe protein binds to transfer a second electron (Hageman & Burris, 1978; Thorneley & Lowe, 1985). This cycle is repeated until sufficient electrons have been transferred to reduce the substrate bound to the MoFe protein (six for N_2 reduction). The dissociation of the oxidized Fe protein from the MoFe protein is proposed to be the rate-limiting step in the overall nitrogenase reduction reaction (Hageman & Burris, 1978). MgATP serves two essential functions in the nitrogenase mechanism. First, MgATP binding to the Fe protein induces conformational changes around the [4Fe−4S] cluster that are a prerequisite for docking to the MoFe protein. Second, the hydrolysis of MgATP to MgADP + P_i by the Fe protein−MoFe protein complex is absolutely required for the transfer of an electron from the Fe protein to the MoFe protein and subsequent substrate reduction (Lanzilotta et al., 1995). A detailed understanding of the mechanism of conformational changes induced by MgATP binding to Fe protein and the function of its hydrolysis in electron transfer has not been elucidated.

The [4Fe−4S] cluster of nitrogenase Fe protein is bound by four cysteine ligands (Howard et al., 1989; Georgiadis et al., 1992). Unlike most other protein bound [4Fe−4S] clusters, however, the Fe protein is a homodimeric protein with the cluster bound at the interface between the two identical subunits near one end of the protein (Georgiadis et al., 1992). Thus, two Cys 97 residues, one from each subunit, and two Cys 132 residues, one from each subunit, provide the four ligands to the cluster. An approximate 2-fold axis of symmetry through the [4Fe−4S] cluster would suggest nearly identical environments around the two Cys 97 and the two Cys 132 ligands. The protons on the α- and β-carbons (two each on the β-carbons and 1 each on the α-carbons) of the Cys ligands (Scheme 1) of the Fe protein

Scheme 1



[4Fe−4S] cluster should reflect this symmetry due to their close proximity to the paramagnetic cluster. The assignments of two distinct isotropically shifted β-CH_2 ^{1}H NMR signals is consistent with this model for the cluster.

One conclusion from the present NMR studies is that the Fe protein contains two pairs of cysteinyl β-protons that are nonequivalent with respect to each other and yet equivalent within the pair. It appears from the temperature dependence of the observed isotropically shifted signals that one equivalent pair of β-CH_2 protons is either the two Cys 97 or the two Cys 132 ligands. An interesting finding of the current work is that the α-CH protons do not follow exactly the same symmetry as the β-CH_2 protons. While two resonances (C and D) were assigned to α-CH protons, the first resonance (C) was found to arise from three α-CH protons (two from the first Cys set and one from the second set). Resonance D was assigned to a single α-CH proton. These results suggest that for one pair of the cysteinyl ligands, which gives rise to four identical β-protons, their α-proton partners have different chemical shifts. This indicates a slight asymmetry between these two ligands in the absence of nucleotides. This may reflect a small shift in the alignment of the two subunits, creating a slight deviation from an absolute 2-fold axis of symmetry.

An important question is "What is the nature of the changes in the cluster ligands upon Fe protein binding nucleotides?" While nucleotide-induced changes in the properties of the [4Fe−4S] cluster of the Fe protein have been extensively studied, few methods have provided a fingerprint for both MgATP and MgADP conformational changes. For example, while MgATP binding results in differences in the EPR spectrum (Orme-Johnson et al., 1972; Zumft et al., 1973) and chelation reaction rates of the Fe protein (Walker & Mortenson, 1974), MgADP binding does not. Both MgATP and MgADP binding result in the same

changes in the reduction potential of the cluster by −100 mV (Watt et al., 1986; Ryle et al., 1995), suggesting that both induce changes in the environment of the cluster, but again, the two states were not distinguished. Recently, we have found that the visible region circular dichroism spectrum of *Av2* changes markedly upon binding MgADP or MgATP (L. C. Seefeldt, M. J. Ryle, W. J. Lanzilotta, R. C. Scarrow, and G. M. Jensen, unpublished data), providing a unique fingerprint for both bound states.

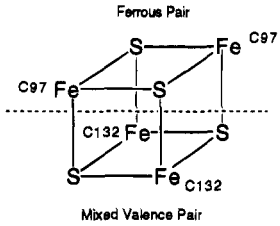
The isotropically shifted ^{1}H NMR resonances of the reduced state of *Av2* presented in the present work provides an opportunity to monitor changes in the cluster ligands upon nucleotide binding. We found that both MgATP and MgADP binding to *Av2* resulted in different types of shifts for the isotropically shifted protons. MgATP binding to *Av2* was found to result in the smallest changes in the proton NMR spectrum. The chemical shifts of the β-CH_2 protons (signals A and B) were only slightly shifted (1 ppm or less), while the chemical shifts of the α-CH protons (C and D) were shifted more significantly. In particular, signal D was shifted by 4 ppm downfield under signal C. The MgATP analog (Mg-β,γ-CH_2-ATP) resulted in similar changes to signals A−C as those observed for MgATP binding. The most shifted signal upon MgATP binding (signal D) was only slightly shifted by 1 ppm downfield by the analog. This suggests that, while the analog induces conformational changes similar to MgATP, the effects on the properties of the α-CH proton contributing to signal D are not identical.

MgADP binding to *Av2* was found to have the greatest effect on the ^{1}H NMR spectrum. The most downfield-shifted signal (A), assigned to β-CH_2 protons, was shifted 10 ppm upfield. Signal B was shifted 4 ppm in the opposite (downfield) direction. The α-CH proton signals (C and D) were also shifted. In particular, it seems likely that signal D (now labeled B′) has shifted downfield by 12 ppm near signal B. Thus, MgADP binding resulted in the greatest change in the cluster ligands, with the largest effects on the β-protons of one Cys set (A) and the α-protons of the other set (D). The present data, in conjunction with recent CD data (L. C. Seefeldt, M. J. Ryle, W. J. Lanzilotta, R. C. Scarrow, and G. M. Jensen, unpublished data), reveal that both MgADP and MgATP binding result in significant and different changes in the ligand environment of the cluster.

One possible model for the function of MgATP hydrolysis in the electron transfer reaction from the Fe protein to the MoFe protein would be that MgATP binding to the Fe protein results in small changes in the protein environment near the cluster. These changes result in a more negative reduction potential, which is necessary to provide a thermodynamically favorable transfer of an electron to the MoFe protein. Changes in the Fe protein that allow docking to the MoFe protein would also occur (Howard & Rees, 1994; Peters et al., 1994; Seefeldt, 1994). While these changes are necessary for docking to the MoFe protein, we know that they are not sufficient to allow the transfer of the electron from the Fe protein to the MoFe protein (Lanzilotta et al., 1995). However, the hydrolysis of MgATP to MgADP is absolutely required. The significantly different ^{1}H NMR spectrum of the MgADP-bound state of the Fe protein, and hence the cluster ligands, when compared to MgATP binding, suggests that during the course of MgATP hydrolysis to MgADP and P_i, a conformational change occurs. The transition from the MgATP-bound state to the MgADP-bound state could be essential in promoting electron transfer from the Fe protein to the MoFe protein. Alternatively, it is possible that this conformational change prevents the back-transfer of an electron from the MoFe protein to the Fe protein, which would result in a futile cycle.

Chart 1

MoFe protein binding face



It is interesting to note that while MgADP or MgATP binding to the Fe protein results in changes in the proton NMR spectrum due to changes in the cluster environment, these changes appear to be subtle. Earlier models suggesting major rearrangements of the subunits upon binding nucleotides are clearly not supported by the current NMR data. Rather, it would appear that minor changes in amino acids near the [4Fe−4S] cluster effects the observed changes in the properties of the cluster. At least one pathway for communication from the nucleotide binding site to the cluster has been proposed. Asp 125 (Wolle et al., 1992) and Asp 129 (Lanzilotta et al., 1995) are located in the phosphate binding sites of *Av2*, only a few amino acid residues away from the two Cys 132 cluster ligands. It has been proposed, and recent analysis of altered *Av2* proteins support, a model in which the binding of the phosphate portion of MgATP or MgADP moves Asp 125 and Asp 129, resulting in changes in the cluster ligands, Cys 132 (Wolle et al., 1992; Lanzilotta et al., 1995). Thus, it seems reasonable to assume that the most significantly shifted signals upon binding MgADP (the proximate pair A and C) could arise from Cys 132, while proton signals B and D might then be assigned to the Cys 97 pair.

Previous Mossbauer studies of the Fe protein revealed two quadrupole doublets, suggesting that in the reduced state, the four irons of the cluster are arranged as two pairs. The first pair would contain a pair of irons in the 2+ state and the second pair would contain a mixed-valence (Fe^{2+} and Fe^{3+}) pair (Lindahl et al., 1985; Lindahl et al., 1987). The observation of both Curie and anti-Curie temperature dependence for β-CH_2 and α-CH pairs is consistent with the earlier Mossbauer studies. Thus, the Cys ligands responsible for signals A and C bind to the mixed-valence Fe pair, while the Cys ligands responsible for signals B and D bind to the ferrous pair of irons. One possible explanation of the above data is presented in Chart 1. In this model, the two Cys 97 ligands would bind to the all ferrous iron pair at the top of the Fe protein. This would result in anti-Curie temperature dependence for the protons of the Cys 97 ligands, corresponding to signals B and D. Having the diferrous pair at the top of the Fe protein nearest the MoFe protein binding site is consistent with the single-electron oxidation of this pair followed by transfer of this electron to the MoFe protein. This places the Cys 132 ligands at the bottom of the cluster bound to the mixed-valence iron atoms. This model would thus predict the largest changes in the Cys 132 ligands upon binding of nucleotides, consistent with our ^{1}H NMR data and the proposed roles for Asp 125 and Asp 129. Further evidence for this model will require sequence specific assignments for the isotropically shifted resonances.

Comparison of the NMR spectrum observed for *Av2* to that previously reported for *Cp2* (Meyer et al., 1988) is of interest to define differences between these two Fe proteins. Since the isotropically shifted ^{1}H NMR resonances for *Cp2* were not assigned, only general comparisons are possible. In the absence of nucleotides, *Cp2* revealed three groups of resonances (centered at 45, 25, and 16 ppm). This compares with the four resonances observed for *Av2* centered at 49, 23, 17, and 13 ppm. These data suggest that while the cluster environments of the two Fe proteins are similar, they are not identical. The most significant differences between the two proteins are observed upon binding nucleotides. Upon binding MgATP, *Cp2* shows two resonances in the 25 ppm chemical shift range, while *Av2* showed only a single resonance (B). MgADP binding resulted in significant upfield shifts for signal A of *Av2* and for the downfield resonance of *Cp2*. No changes in the two signals centered at 25 ppm were observed upon binding either MgATP or MgADP to *Cp2*, while significant differences in the chemical shifts of signal B and B′ were observed for the MgATP- and MgADP-bound states of *Av2*. These results suggest that, although *Cp2* and *Av2* have many similarities, there are significant differences in the cluster ligand environments in both the presence and absence of bound nucleotides. That *Cp2* and *Av2* are fundamentally different is apparent from the observation that when *Cp2* is combined with the MoFe protein from *A. vinelandii* (*Av1*), a tight complex is observed, which is not observed for the homologous complexes (Smith et al., 1976; Emerich & Burris, 1978).

In summary, the present work has provided insights into the conformational changes in the [4Fe−4S] cluster ligands of the nitrogenase Fe protein upon binding of nucleotides. The assignment of the isotropically shifted resonances to β-CH_2 and α-CH protons of the cysteinyl cluster ligands, in conjunction with the preparation of site-specifically altered Fe proteins, should provide a way to better define the specific protein environmental changes near the [4Fe−4S] cluster affected by nucleotide binding.

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