# Elucidating the Mechanism of Nucleotide-Dependent Changes in the Redox Potential of the [4Fe-4S] Cluster in Nitrogenase Iron Protein: The Role of Phenylalanine 135[†]

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*Received April 10, 1996; Revised Manuscript Received May 22, 1996*[⊗]

ABSTRACT: Nucleotide binding to the nitrogenase iron (Fe) protein results in a lowering of the redox potential of its [4Fe-4S] cluster by over 100 mV, and this is thought to be essential for electron transfer to the molybdenum−iron (MoFe) protein for substrate reduction. This work presents evidence for an important role of the strictly conserved phenylalanine at position 135, located near the [4Fe-4S] cluster of nitrogenase Fe protein, in defining both the redox potential and the nucleotide-induced changes in the redox potential of the [4Fe-4S] cluster. Phe 135 was changed by means of site-directed mutagenesis to the amino acids Tyr (F135Y), Ile (F135I), Trp (F135W), and His (F135H), and the altered proteins were purified to homogeneity. Minor changes in the UV/visible and EPR spectra arising from the [4Fe-4S] cluster were detected in the altered proteins, while dramatic changes were observed in the visible region circular dichroism (CD) spectrum, suggesting that Phe 135 contributes significantly to the chiroptical properties of the [4Fe-4S] cluster. Likewise, significant changes in the redox potentials of the Phe altered Fe proteins were observed, with shifts of +50 to +120 mV compared to the redox potential of the wild-type Fe protein (−300 mV). The shifts in redox potential for the altered Fe proteins appeared to correlate with changes in isotropically shifted proton NMR resonances assigned to cluster ligands. All of the Phe 135 altered Fe proteins were found to bind either MgADP or MgATP, while the reduced and oxidized states of the F135W and F135H altered Fe proteins had significantly higher affinities for binding MgATP when compared to the wild-type Fe protein. While MgATP binding to the wild-type and Phe 135 altered Fe proteins resulted in approximately −100 mV shifts in the redox potentials for all proteins, MgADP binding resulted in only −30 to −50 mV shifts for the altered proteins compared to a −160 mV shift for the wild-type Fe protein. The current results suggest that Phe 135 is important in defining the redox potential of the [4Fe-4S] cluster in the Fe protein and influences the MgADP (but not MgATP) induced modulation of the redox potential.

Nitrogenase is the two component metalloenzyme that catalyzes the biological reduction of dinitrogen to ammonia according to the overall reaction:

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16\ P_i$$

During substrate reduction, MgATP hydrolysis is coupled to the transfer of an electron from the single [4Fe-4S] cluster of the nitrogenase iron (Fe) protein to the molybdenum-iron (MoFe)[1] protein. The MoFe protein contains two unique molybdenum−iron−sulfur−homocitrate cofactors (FeMoco) (Kim & Rees, 1992), which are the sites of substrate binding and reduction (Shah & Brill, 1977; Hawkes et al., 1984), and two [8Fe-(7−8)S] clusters (P or 8Fe) (Kim & Rees, 1992; Bolin et al., 1993), which are thought to initially accept the electron from the Fe protein before transferring it to FeMoco (Lowe et al., 1993; Peters et al., 1995). The Fe protein component is a homodimeric protein containing a single [4Fe-4S] cluster bound between its subunits and two nucleotide binding sites, one per subunit (Georgiadis et al., 1992). The current model for the catalytic mechanism of nitrogenase suggests that the reduced Fe protein, with two bound MgATP molecules, binds to the MoFe protein and transfers a single electron to the MoFe protein with the concomitant hydrolysis of two MgATP molecules (Thorneley & Lowe, 1983; Howard & Rees, 1994). Following electron transfer, the oxidized Fe protein, with two bound MgADP molecules, dissociates from the MoFe protein, and another reduced Fe protein, with two bound MgATP molecules, associates with the MoFe protein for a second round of MgATP hydrolysis and electron transfer (Hageman & Burris, 1978). This cycle is repeated until a sufficient number of electrons have been transferred to FeMoco to catalyze substrate reduction.

How MgATP hydrolysis is coupled to electron transfer from the Fe protein to the MoFe protein and to substrate

[†] This work was supported by National Science Foundation Grant MCB-9315835. The Bruker ESP300E EPR spectrometer was purchased with funds provided by the National Science Foundation (BIR-9413530) and Utah State University.

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[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations: Fe protein, iron protein of nitrogenase; MoFe protein, molybdenum−iron protein of nitrogenase; EPR, electron paramagnetic resonance; CD, circular dichroism; $^1$H NMR, proton nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; BPS, bathophenanthrolinedisulfonic acid; IDS, indigo disulfonate; Tricine, *N*-(tris(hydroxymethyl)methyl)glycine; $E_m$, midpoint potential.

S0006-2960(96)00857-4 CCC: $12.00 

reduction remains largely unknown. The nitrogenase Fe protein is known to bind two molecules of either MgATP or MgADP with high affinity and the binding of these nucleotides induces protein conformational changes which appear to be essential for the electron transfer mechanism. Nucleotide-induced protein conformational changes are known to result in changes in the electronic properties of the Fe protein [4Fe-4S] cluster (Mortenson et al., 1993). For example, the binding of MgATP or MgADP to the Fe protein changes the line shape of the EPR spectrum (Zumft et al., 1973; Lindahl et al., 1987b), the visible region circular dichroism spectrum (Stephens et al., 1979; Ryle et al., 1996), and the downfield resonances in the proton NMR spectrum (Meyer et al., 1988; Lanzilotta et al., 1995a). Significantly, MgATP or MgADP binding to the Fe protein have been shown to induce a −120 to −160 mV change in the redox potential of the [4Fe-4S] cluster (Zumft et al., 1974; Watt et al., 1986). The recent X-ray crystal structure of Fe protein from *Azotobacter vinelandii* (Georgiadis et al., 1992), coupled with the analysis of several site specifically altered proteins (Seefeldt et al., 1992; Wolle et al., 1992; Seefeldt & Mortenson, 1993; Ryle et al., 1995; Lanzilotta et al., 1995b), has revealed that the Fe protein contains two nucleotide binding sites, one on each of the two subunits, and that these nucleotide binding sites are located 19 Å away from the [4Fe-4S] cluster.

How then does MgATP or MgADP binding to the Fe protein induce changes in the spectroscopic properties and the redox potential of the [4Fe-4S] cluster over 19 Å away? A possible signal transduction pathway within the Fe protein has been suggested (Howard & Rees, 1994), and recent work defines a role for the protein chain from Asp 125, in the MgATP binding site, to Cys 132, a ligand to the [4Fe-4S] cluster, in this transduction pathway (Ryle & Seefeldt, 1996). Defining the nature of the nucleotide-induced changes in the protein environment around the [4Fe-4S] cluster of the Fe protein would provide insights into both how the protein controls the redox potential of the [4Fe-4S] cluster and the way protein conformational changes might control changes in this potential as part of the electron transfer mechanism. X-ray absorption spectroscopic results show that nucleotide binding to the Fe protein does not result in changes in the Fe−Fe or Fe−S bond distances of the [4Fe-4S] cluster (Lindahl et al., 1987a; Ryle et al., 1996), clearly suggesting that nucleotide induced changes are mediated by changes in the protein environment around the cluster. Several models have been proposed to explain how the properties of [Fe-S] clusters and hemes are defined by the protein environments (Adman et al., 1975; Kassner & Yang, 1977; Churg & Warshel, 1986; Ortiz de Montellano, 1987; Backes et al., 1991; Langen et al., 1992; Huang et al., 1994; Jensen et al., 1994; Vidakovic et al., 1995; Yu et al., 1995). Examination of the X-ray crystal structures of proteins containing [4Fe-4S] clusters has suggested that the polarity around the [4Fe-4S] cluster controls the redox potential of the cluster (Adman et al., 1975; Jensen et al., 1994), with contributions from amino acid side chains (including aromatic residues), accessibility of water to the cluster, and dipole interactions from the amide groups of the peptide bond. In support of this polarity model, work with model [4Fe-4S(SR)$_4$] clusters has shown that the polarity of the solvent can change the redox potential of these clusters by as much as 850 mV, with water-solvated clusters having more positive redox potentials (Mayerle et al., 1973; Kassner & Yang, 1977).

Therefore, it seems likely that the protein environment around the [4Fe-4S] cluster of nitrogenase Fe protein defines both the redox potential and the nucleotide-induced changes in the redox potential. An analysis of the X-ray structure of the *A. vinelandii* nitrogenase Fe protein (Georgiadis et al., 1992) reveals six potential peptide amide to sulfur interactions to the [4Fe-4S] cluster, three from each subunit, arising from amino acids Ala 98, Gly 99, and Gly 134. In addition, the side chains of six amino acids are predicted to be within van der Waals contact of the [4Fe-4S] cluster, namely, Ala 98, Val 130, and Phe 135, one from each subunit (Georgiadis et al., 1992). These six amino acids appear to create a hydrophobic pocket under the [4Fe-4S] cluster of the Fe protein. In order to gain an understanding of the protein factors defining the properties of the nitrogenase Fe protein [4Fe-4S] cluster, we have examined the role of the phenylalanine at position 135. This residue was changed by means of site-directed mutagenesis to the amino acids Tyr (F135Y), Ile (F135I), Trp (F135W), and His (F135H). Each of the altered proteins was purified to homogeneity, and characterization of the proteins revealed an important role for Phe 135 in defining both the spectroscopic properties and the nucleotide-induced changes in the redox potential of the [4Fe-4S] cluster.

## EXPERIMENTAL PROCEDURES

*Site-Directed Mutagenesis, Expression, and Purification of Fe Proteins.* Site-directed mutagenesis of the gene that encodes the Fe protein subunits of *A. vinelandii* nitrogenase, *nif H*, was carried out as previously described (Jacobson et al., 1989; Seefeldt & Mortenson, 1993). Expression and purification of wild-type and altered Fe proteins were performed as previously described (Seefeldt & Mortenson, 1993). All Fe proteins were purified in 50 mM Tris buffer, pH 8.0, in the presence of 2 mM sodium dithionite and included 20% glycerol as a stabilizing agent (Ryle & Seefeldt, 1996). Prior to freezing, the glycerol concentration was reduced to less than 5% by dilution with buffer without glycerol. Protein concentrations were determined by a modified biuret method (Chromy et al., 1974) using bovine serum albumin as the standard. All proteins were homogeneous as determined by SDS gels stained with Coomassie blue (Hathaway et al., 1979). Proteins were protected from oxygen by manipulation in sealed serum vials with 2 mM dithionite or in an argon atmosphere glove box (Vacuum Atmospheres, Hawthorne, CA) with less than 0.5 ppm oxygen.

*Activity Assays and MgATP Hydrolysis.* Acetylene and proton reduction rates, along with MgATP hydrolysis rates, were determined as previously described (Seefeldt & Ensign, 1994) except that the buffer was 100 mM MOPS, pH 7.0.

*Nucleotide Binding to Wild-Type and Phe 135 Altered Fe Proteins*. The affinities for binding nucleotides of both the one electron oxidized ([4Fe-4S]$^{2+}$) and reduced ([4Fe-4S]$^{1+}$) states of Fe protein samples were determined essentially as described (Ryle et al., 1995). Fe protein was maintained in the reduced state by the presence of 2 mM dithionite. One electron oxidized Fe protein samples were prepared by the addition of 20 mM indigo disulfonate (IDS) until a green color remained. The stoichiometries of bound nucleotides

to either reduced or oxidized Fe proteins were determined using an equilibrium column binding technique (Ryle et al., 1995) in which Fe proteins were passed through a Sephadex G-25 column (0.7 × 15 cm) equilibrated with 50 mM Tris buffer, pH 8.0, and known concentrations of nucleotides ranging from 0 to 1500 $\mu$M for MgATP or from 0 to 350 $\mu$M for MgADP. The protein containing fraction collected from the column was split into two aliquots. One aliquot was analyzed for protein concentration using the modified biuret method, while the other was analyzed for nucleotide concentration by the HPLC method previously described (Seefeldt & Mortenson, 1993). Apparent dissociation constants ($K_d$) were determined by fitting the data to the Hill equation as previously described (Ryle et al., 1995).

*MgATP-Dependent Chelation of $Fe^{2+}$ from Wild-Type and Phe 135 Altered Fe Proteins.* The MgATP-dependent rates of $Fe^{2+}$ chelation from both wild-type and Phe 135 altered Fe proteins were followed continuously by the formation of the $Fe^{2+}$-($\alpha$,$\alpha'$-dipyridyl) complex or by the formation of the $Fe^{2+}$-(4,7-diphenyl-1,10-bathophenanthrolinedisulfonic acid) (BPS) complex, which were monitored spectrophotometrically at 520 nm (8400 $M^{-1}$ $cm^{-1}$) or at 534 nm (22 140 $M^{-1}$ $cm^{-1}$), respectively (Walker & Mortenson, 1973; Ljones & Burris, 1978). Spectrophotometric measurements were performed on a Hewlett Packard 8452A diode array spectrophotometer.

*EPR.* EPR spectra of the dithionite reduced Fe protein were recorded as previously described (Lanzilotta et al., 1995b). Where indicated, MgATP was added to a concentration 10 times the protein concentration prior to freezing. All spectra were recorded on a Bruker ER300E spectrometer with an Oxford ESR 900 liquid helium cryostat.

*Circular Dichroism Spectra of Wild-Type and Phe 135 Altered Fe Proteins.* The circular dichroism spectra of IDS oxidized wild-type and Phe 135 altered Fe proteins were recorded as previously described (Ryle et al., 1996). Samples of Fe protein (20 mg) were desalted by passage down a Sephadex G-25 column (0.5 × 10 cm) equilibrated with 100 mM Tris buffer, pH 8.0. The Fe protein samples were then oxidized by the addition of 25 $\mu$L of 20 mM IDS, and the IDS was removed using a Dowex-1 column. The oxidized Fe protein was diluted to 4 mL and split into two equal samples. Each sample was then transferred into a sealed 1-cm path length quartz cuvette under a nitrogen atmosphere. Nucleotides were added to a final concentration of 1 mM from an anaerobic stock solution. CD spectra were recorded on an Aviv 62DS spectropolarimeter and were baseline subtracted. The concentration of oxidized Fe protein was determined from the absorption at 400 nm using 13 $mM^{-1}$ $cm^{-1}$ as the absorption coefficient (Lanzilotta et al., 1995b).

*UV/Visible Absorption Spectra of Oxidized Wild-Type and Phe 135 Altered Fe Proteins.* Oxidized Fe proteins were prepared by desalting 6 mg of Fe protein on a Sephadex G-25 column (0.5 × 10 cm) equilibrated with 100 mM Tris buffer, pH 8.0 followed by the addition of 5 $\mu$L aliquots of 20 mM IDS until a green color remained. The dye was separated from the Fe protein by passage through a Dowex-1 column (1.0 × 5 cm) equilibrated with 100 mM Tris buffer, pH 8.0. The concentrations of oxidized Fe protein samples were determined from the absorbance at 400 nm. Fe protein samples were diluted to 4 mg $mL^{-1}$ in sealed 1-cm path length quartz cuvettes for spectrophotometric analysis.

*$^1$H NMR Spectra of the Isotropically Shifted Protons for Wild-Type and Phe 135 Altered Fe Proteins.* $^1$H NMR spectra of reduced Fe proteins were recorded as previously described (Lanzilotta et al., 1995a). All spectra were recorded at 320 K in anaerobic glass NMR tubes fitted with degassed Teflon stoppers (Wilmad, Buena, NJ). Fe protein samples were passed through a G-25 column (1.0 × 10 cm) equilibrated with buffer (50 mM Tris buffer, pH 7.5, made in $D_2O$) treated with Chelex-100 to remove trace metals. Samples were concentrated to between 1.0 and 1.5 mM using a Centricon-30 concentrator (Amicon Division, Beverly, MA).

*Potentiometric Titrations.* Potentiometric titrations were performed essentially as previously described (Dutton, 1978; Morgan et al., 1986). Iron protein samples (40 mg) were desalted to remove excess dithionite by passage through a Sephadex G-25 column (1.0 × 15 cm) equilibrated with 100 mM Tricine buffer, pH 8.0. The dithionite-free Fe protein was oxidized by the addition of a minimum volume of 20 mM IDS solution. IDS was removed from the Fe protein sample by passage through a 3-mL Dowex-1 column equilibrated with 100 mM Tricine buffer, pH 8.0. The oxidized Fe protein was diluted to a final concentration between 10 and 15 mg $mL^{-1}$ in a total volume of 2−3 mL with a final buffer composition of 50 mM Tricine buffer (pH 8.0) containing 250 mM NaCl, 50 $\mu$M methyl viologen, 50 $\mu$M benzyl viologen, and 50 $\mu$M flavin mononucleotide. When nucleotides were present, the solution also contained either 2 mM MgADP or 2 mM MgATP. Tricine buffer was used to minimize pH changes upon freezing of the samples for EPR (Williams-Smith et al., 1977). Redox potentiometry was carried out in a stirred cell in an argon filled glovebox essentially as previously described (Morgan et al., 1986) except that the indicator electrode was a gold wire and the reference electrode was Ag/AgCl. The reference electrode was calibrated against a saturated calomel electrode. Sodium dithionite was used as the reductant and IDS as the oxidant. Aliquots (250 $\mu$L) were removed at defined potentials and frozen in calibrated EPR tubes. The fraction of reduced Fe protein was determined by measuring the peak to peak height between the $g = 2.03$ and $g = 1.88$ signals for Fe proteins with rhombic EPR line shapes. The fraction of reduced Fe protein was determined by measuring the peak to peak height between the $g = 2.05$ and the $g = 1.91$ EPR signals for Fe proteins with axial EPR line shapes. EPR spectra were recorded at 15 K with 6.36 mW microwave power. All potentials are reported with respect to the normal hydrogen electrode (NHE). Midpoint potentials ($E_m$) were determined from least-squares fits of the data to the Nernst equation with one faraday per mole using the program Igor Pro (Wavemetrics, Lake Oswego, OR). Midpoint potentials have an estimated error of ±10 mV.

## RESULTS

*Substitution of Phe 135 of A. vinelandii Fe Protein by Site-Directed Mutagenesis.* Analysis of the *A. vinelandii* Fe protein X-ray crystal structure (Georgiadis et al., 1992) suggests that Ala 98, Val 130, and Phe 135, all of which are strictly conserved amino acids among all known Fe protein sequences, are within van der Waals contact with the [4Fe-4S] cluster (Figure 1). It seemed likely that Phe 135 in the Fe protein might, in part, define the properties of the [4Fe-4S] cluster, including the redox potential and nucleotide-

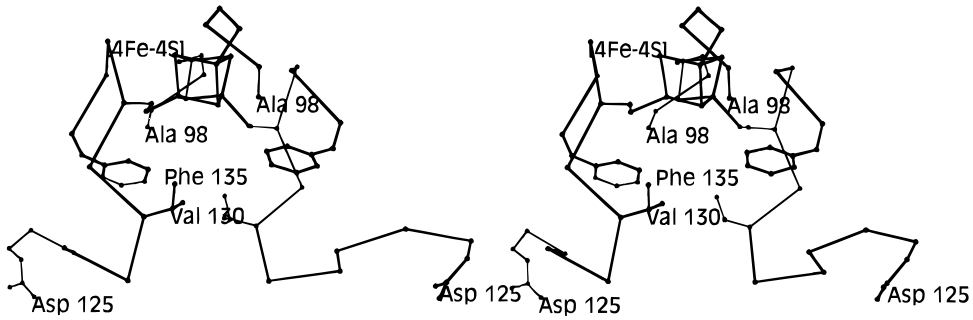


FIGURE 1: Stereoview of the [4Fe-4S] cluster protein environment and a MgATP signal transduction pathway in *A. vinelandii* nitrogenase Fe protein. The model shows the [4Fe-4S] cluster (cubane structure) ligated by two Cys 97 and two Cys 132 residues, one from each subunit. The model also depicts the side chains of the six amino acids (Ala 98, Val 130, and Phe 135) proposed to be within van der Waals contact of the [4Fe-4S] cluster. Also presented are α-carbon backbone traces for each subunit from Asp 125, located in the nucleotide binding site, to Phe 135. The molecular model was generated using the program Molecular Images (U. S. Science, San Diego, CA) from the X-ray coordinates of the *A. vinelandii* Fe protein (Georgiadis et al., 1992).

induced changes in the redox potential. This possibility was supported by recent work defining the protein chain from Asp 125 to Cys 132 as a MgATP signal transduction pathway from the nucleotide binding site to the [4Fe-4S] cluster (Ryle & Seefeldt, 1996). Phe 135 is two amino acids away from the Cys 132 cluster ligand and thus might be expected to be influenced by changes in the signal transduction domain. Thus, it seemed possible that Phe 135 might also function in the nucleotide-dependent changes in the properties of the [4Fe-4S] cluster. In order to probe the role of Phe 135 in defining the properties of the [4Fe-4S] cluster in the Fe protein, Phe 135 was changed by site-directed mutagenesis to the amino acids tyrosine (Y), tryptophan (W), isoleucine (I), histidine (H), and arginine (R). These amino acid substitutions were chosen to probe the function of side chain polarity, size, charge, hydrogen bonding, and aromatic interactions with the [4Fe-4S] cluster. The altered Fe proteins were expressed in *A. vinelandii* cells in place of the wild-type Fe protein by means of a chromosomal gene replacement strategy (Jacobson et al., 1989). The *A. vinelandii* cells expressing each of the altered Fe proteins were tested for their ability to grow under nitrogen fixing conditions, providing an opportunity to analyze the functional consequences of the amino acid changes. None of the mutated *A. vinelandii* strains could grow under nitrogen fixing conditions, revealing that changing Phe 135 to each of these residues rendered the Fe protein inactive. It was of interest to further investigate the properties of the purified altered Fe proteins to define the functional consequences of the amino acid changes, and thus the function of Phe 135. Each of the altered Fe proteins was overexpressed in *A. vinelandii* cells using a derepression protocol previously described (Seefeldt et al., 1992). The F135R altered Fe protein could not be purified, probably reflecting instability in the altered protein. In contrast, the F135Y, F135I, F135W, and F135H Fe proteins could be purified to homogeneity with yields of 200%, 250%, 135%, and 35% of typical wild-type Fe protein yields.

*Activities of Wild-Type and Phe 135 Altered Fe Proteins.* The wild-type Fe protein−MoFe protein complex was found to reduce acetylene at a rate of 1958 nmol of $C_2H_2$ reduced (min·mg of Fe protein)$^{-1}$ and protons at a rate of 2038 nmol of $H_2$ formed (min·mg of Fe protein)$^{-1}$. In contrast, none of the Phe 135 substituted Fe proteins, when combined with wild-type MoFe protein, showed detectable acetylene or proton reduction activities. The wild-type Fe protein−MoFe protein complex was found to hydrolyze MgATP at a rate of 7350 nmol of MgADP formed (min·mg of Fe protein)$^{-1}$, with a stoichiometry of 2.3 ± 0.4 MgATP hydrolyzed per electron transferred. No MgATP hydrolysis could be detected for any of the Phe 135 altered Fe proteins when mixed with wild-type MoFe protein.

In order to determine if the Phe 135 altered Fe proteins could compete with wild-type Fe protein for binding to the MoFe protein, a competitive binding activity assay was employed (Lanzilotta et al., 1995b). Such assays have previously been used as a way to monitor the binding of altered Fe proteins to the MoFe protein. None of the Phe 135 altered Fe proteins were found to influence the rate of substrate reduction by the wild-type Fe protein−MoFe protein complex.

*Absorption Spectra and Total Iron Content of Phe 135 Altered Fe Proteins.* To examine the possible impact of changing Phe 135 on the [4Fe-4S] cluster, spectroscopic properties of the altered proteins were examined. Comparison of the visible region absorption spectra of the reduced or oxidized states of each of the Phe 135 altered Fe proteins with that of the wild-type Fe protein revealed no significant changes in the wavelength maxima for the altered proteins. The oxidized state of F135W and F135H Fe proteins did show slightly reduced absorbance intensity between 350 and 550 nm when the protein concentration was determined by the biuret assay. The lower molar absorbance of F135W and F135H Fe proteins suggested that these proteins might not contain a full complement of [4Fe-4S] cluster. The total Fe content of the wild-type and Phe 135 altered Fe proteins was determined using a procedure previously described (Seefeldt et al., 1992). The wild-type Fe protein was found to contain 3.2 nmol of Fe (nmol of Fe protein)$^{-1}$, while the F135Y Fe protein contained 3.1 nmol of Fe (nmol of Fe protein)$^{-1}$ and the F135I Fe protein contained 3.1 nmol of Fe (nmol of Fe protein)$^{-1}$. In contrast, the F135W Fe protein was found to contain 2.6 nmol of Fe (nmol of Fe protein)$^{-1}$ and the F135H Fe protein 2.4 nmol of Fe (nmol of Fe protein)$^{-1}$. When the concentrations of F135W and F135H Fe proteins were normalized for total iron content, the visible region absorption spectra were nearly identical to that of the wild-type Fe protein. These data suggest that changing Phe 135 has not resulted in dramatic changes in the electronic charge transfer transitions for the [4Fe-4S] cluster.

*Nucleotide Affinities of Wild-Type and Phe 135 Altered Fe Proteins.* It was important to establish that changing Phe

Table 1: Nucleotide Binding to Wild-Type and Phe 135 Altered Fe Proteins

| Fe protein | $K_d$ ($\mu$M)[a] | | | | | |
|---|---|---|---|---|---|---|
| | MgATP | | | | MgADP | |
| | reduced | fold change[b] | oxidized | fold change | reduced | oxidized |
| wild type | 590 | | 440 | | 100 | 60 |
| F135Y | 570 | 1.0 | 340 | 1.3 | 110 | 70 |
| F135I | 570 | 1.0 | 330 | 1.3 | 110 | 80 |
| F135W | 390 | 1.5 | 380 | 1.2 | 100 | 60 |
| F135H | 190 | 3.1 | 320 | 1.4 | 100 | 70 |

[a] The apparent dissociation constants ($K_d$) represent the product of the two true dissociation constants with strong cooperativity. All data were fit to the Hill equation as previously described (Ryle et al., 1995). In each case, the maximum number of nucleotides bound per Fe protein ranged from 1.8 to 2.2, with a cooperativity of 1.9-2.2. [b] The fold increase in affinity compared to the wild-type Fe protein under the same conditions.

135 did not disrupt nucleotide binding to the Fe protein. To assess this, apparent equilibrium dissociation constants ($K_d$) for MgATP or MgADP binding to both reduced and oxidized states of the Phe 135 altered Fe proteins were determined by using a modified equilibrium column binding technique (Ryle et al., 1995). As is evident from the data in Table 1, all of the Phe 135 altered Fe proteins (oxidized or reduced) showed similar affinities for binding MgADP to those of the wild-type Fe protein. In contrast, the affinities for MgATP binding to the Phe 135 altered Fe proteins (oxidized or reduced) were different from the wild-type Fe protein. A significant increase in affinity for binding MgATP compared to the wild-type Fe protein was observed for all of the Phe 135 altered Fe proteins in the oxidized state. Likewise, the reduced states of the F135W and F135H Fe proteins showed higher affinities for binding MgATP when compared to the wild-type Fe protein.

*MgATP-Induced Conformational Changes in Wild-Type and Phe 135 Altered Fe Proteins.* Given that each of the Phe 135 altered Fe proteins bound nucleotides with equal or greater affinity compared to wild-type Fe protein, it was important to determine if MgATP or MgADP binding could induce changes in the properties of the [4Fe-4S] cluster as observed for wild-type Fe protein. One sensitive assay to monitor the MgATP-induced changes in the properties of the [4Fe-4S] cluster is the rate of chelation of Fe from the cluster by Fe specific chelators such as α,α′-dipyridyl or bathophenanthrolinedisulfonic acid. As observed for the wild-type Fe protein, none of the Phe 135 altered Fe proteins showed Fe chelation in the absence of added MgATP. When MgATP was added to the wild-type Fe protein, a time-dependent chelation of $Fe^{2+}$ by α,α′-dipyridyl was observed, with an apparent first order rate constant of 0.006 $s^{-1}$. A total of 3.3 ± 0.2 nmol of Fe (nmol of Fe protein)$^{-1}$ was chelated from the wild-type Fe protein. The addition of MgATP to the Phe 135 altered Fe proteins also resulted in apparent first order rates of Fe chelation, although the rates were slower than for the wild-type Fe protein. An apparent first order rate constant of 0.004 $s^{-1}$ was measured for the F135Y Fe protein, with a total of 2.9 ± 0.1 nmol of Fe (nmol of Fe protein)$^{-1}$. Rate constants could not be determined for the F135I, F135W, and F135H Fe proteins because of incomplete chelation even after 30 min. This suggested that the F135W, F135I, and F135H altered Fe proteins did not undergo a full conformational change upon the binding of MgATP.

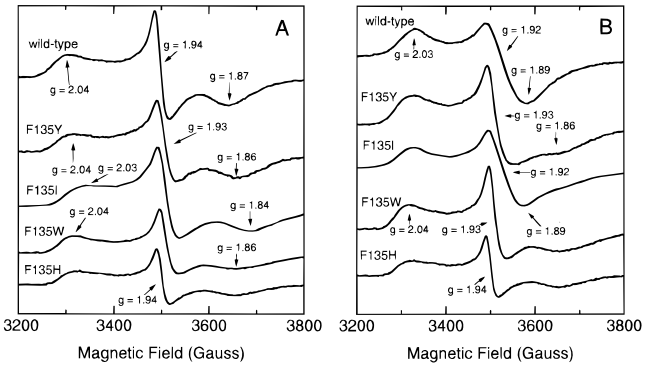


FIGURE 2: EPR spectra of wild-type and Phe 135 altered Fe proteins in the presence or absence of MgATP. Wild-type (30 mg $mL^{-1}$), F135Y (28 mg $mL^{-1}$), F135I (29 mg $mL^{-1}$), F135W (30 mg $mL^{-1}$), and F135H (29 mg $mL^{-1}$) Fe protein samples were prepared and spectra were acquired as described in Experimental Procedures. All samples contained 2 mM dithionite, and spectra were recorded at 12 K, at a microwave frequency of 9.51 GHz, and at a microwave power of 0.5 milliwatts. (Panel A) The EPR spectra of wild-type (wild-type) and Phe 135 altered Fe proteins in the absence of nucleotides. (Panel B) The EPR spectra of wild-type (wild-type) and Phe 135 altered Fe proteins with a 10-fold molar excess of MgATP.

The chelation of iron from the Fe protein by bathophenanthrolinedisulfonic acid (BPS) provides an alternative method to monitor the MgATP-dependent conformational change. It appears that this chelator has a different mechanism of chelation of Fe from the Fe protein when compared to α,α′-dipyridyl since it shows considerably higher rates of chelation (Ljones & Burris, 1978). Like the results from the α,α′-dipyridyl assay, BPS did not chelate iron from wild-type or Phe 135 altered Fe proteins in the absence of MgATP. The addition of MgATP to all of the proteins resulted in an apparent first-order rate of Fe chelation with measured rate constants of 0.020 $s^{-1}$ for wild-type, 0.018 $s^{-1}$ for F135Y, 0.017 $s^{-1}$ for F135I, 0.016 $s^{-1}$ for F135W, and 0.017 $s^{-1}$ for F135H Fe proteins. The total Fe released was comparable to the Fe content determined earlier for each protein. The observation of complete Fe chelation from the Phe 135 altered proteins in the BPS assay was in contrast to the incomplete chelation observed in the α,α′-dipyridyl assay. These results reveal that the signal transduction pathway from the nucleotide binding site to the [4Fe-4S] cluster is intact in the Phe 135 altered Fe proteins.

*Spectroscopic Properties of the [4Fe-4S] Cluster in Altered Fe Proteins.* It was of interest to establish the effects of changing Phe 135 on the properties of the [4Fe-4S] cluster and the nature of the MgATP and MgADP induced changes in the properties of the cluster. Three spectroscopic techniques that have been used to monitor the [4Fe-4S] cluster of Fe protein and the nucleotide-induced changes are EPR, CD, and proton NMR.

The EPR spectrum of the wild-type Fe protein in the reduced state provides a way to monitor the environment of the cluster, with the spectrum changing from rhombic to axial upon MgATP binding (Zumft et al., 1973; Lindahl et al., 1985). The EPR spectra of Phe 135 altered Fe proteins were recorded in the absence or presence of MgATP (Figure 2). In the absence of nucleotides, wild-type and all of the Phe 135 altered Fe proteins showed rhombic EPR line shapes (Figure 2, panel A), indicating that the Phe 135 altered Fe proteins contained an intact [4Fe-4S] cluster, and that no major changes in the electronic properties of the cluster

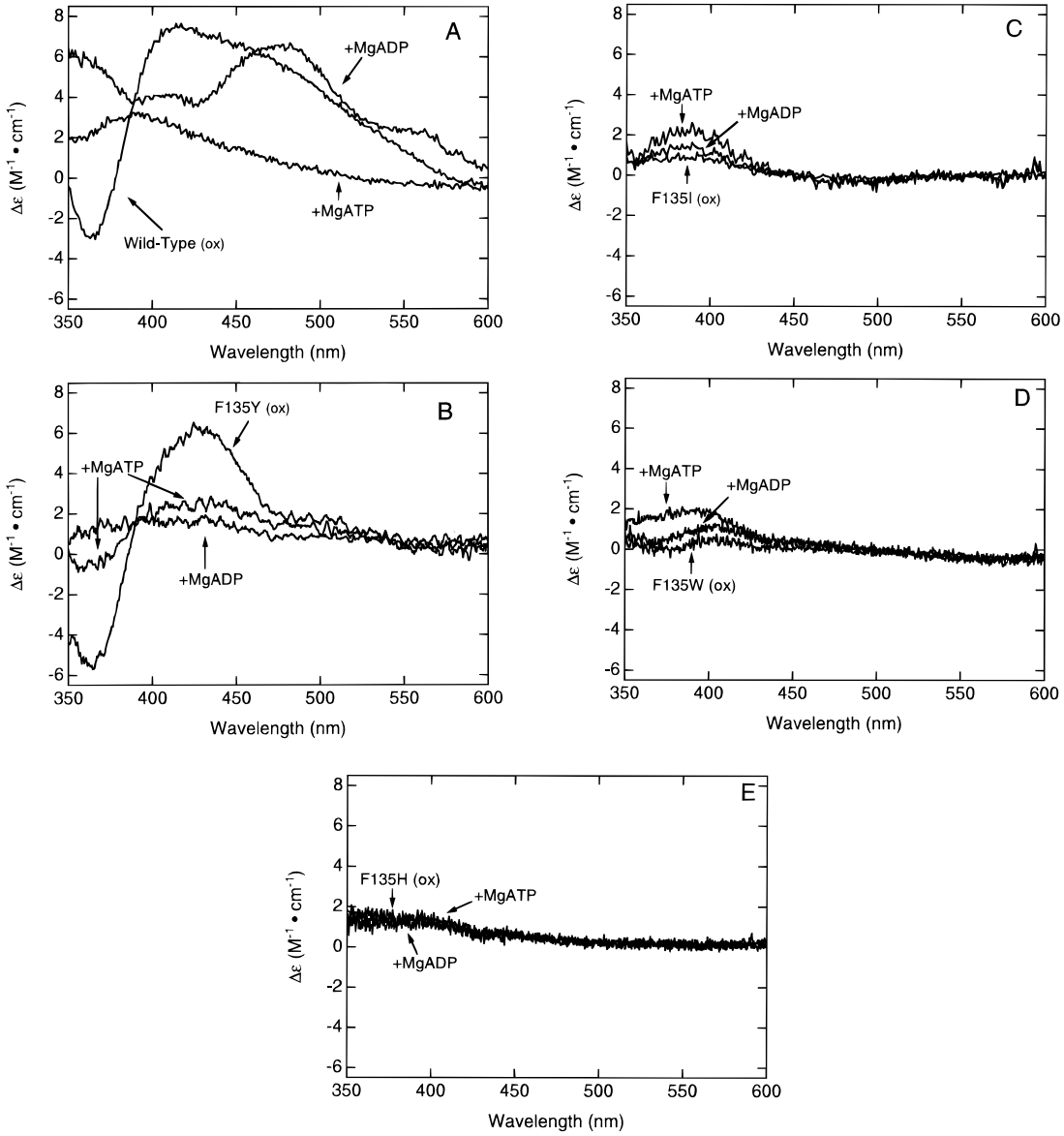


FIGURE 3: Circular dichroism spectra of wild-type and Phe 135 altered Fe proteins in the presence or absence of nucleotides. The visible region circular dichroism (CD) spectra of oxidized Fe proteins were recorded as described in Experimental Procedures. Spectra were recorded for wild-type (Panel A), F135Y (Panel B), F135I (Panel C), F135W (Panel D) and F135H (Panel E) Fe proteins in the absence of added nucleotides or following the addition of MgATP (+MgATP) or MgADP (+MgADP) to a final concentration of 1 mM.

resulted from the amino acid substitutions. Subtle shifts in the *g* values were apparent, however, suggesting that changing Phe 135 did have minor effects on the cluster or its protein environment. The EPR signal intensities for the F135W and F135H Fe proteins were somewhat lower than for the wild-type Fe protein, consistent with the lower total Fe content found for these proteins. The effects of the addition of MgATP on the EPR line shapes of the wild-type and Phe 135 altered Fe proteins are shown in Figure 2 (panel B). The wild-type Fe protein showed a change in line shape to axial upon the addition of MgATP. The addition of saturating quantities of MgATP to the F135I and F135Y altered Fe proteins resulted in subtle changes in the EPR line shape, but neither protein demonstrated an axial-like spectrum. Both the F135W and F135H altered Fe proteins showed no change from the rhombic line shape in the presence of MgATP.

The visible region circular dichroism (CD) spectrum of the oxidized Fe protein has been shown to provide a sensitive way to monitor changes in the [4Fe-4S] cluster environment (Stephens et al., 1979; Ryle et al., 1996). In particular, MgATP or MgADP binding to the Fe protein result in very different CD spectra, clearly indicating different changes in the protein environment around the cluster (Ryle et al., 1996). The CD spectra of the oxidized Phe 135 altered Fe proteins are compared to wild-type Fe protein in Figure 3, both in the absence of nucleotides and in the presence of MgATP or MgADP. In the absence of nucleotides, the F135Y Fe protein showed a CD spectrum similar to the wild-type Fe protein, with a slightly more negative feature around 370 nm. The addition of either MgATP or MgADP to the F135Y Fe protein resulted in CD spectra similar to that observed for the MgATP-bound state of the wild-type Fe protein. Surprisingly, the F135I, F135W, and F135H Fe proteins all showed little chiroptical activity in the visible region with or without added nucleotides. The presence of intact $[4Fe\text{-}4S]^{2+}$ clusters in these altered proteins was confirmed from their UV/visible absorption spectra. These results clearly indicate that changing Phe 135 has had a significant impact

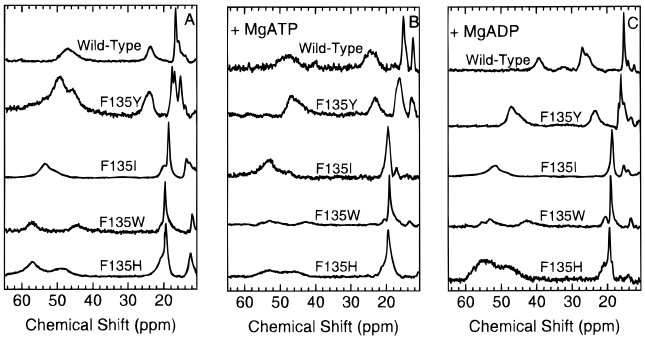


FIGURE 4: $^1$H NMR spectra of wild-type and Phe 135 altered Fe proteins in the absence or presence of nucleotides. Fe protein samples (60−95 mg mL$^{-1}$) were prepared in Chelex-treated, Tris-buffered $D_2O$ solution as described in Experimental Procedures. The downfield portion of the 400 MHz $^1$H NMR spectrum at 320 K for wild-type, F135Y, F135W, F135I, and F135H Fe proteins were recorded in the absence of nucleotides (Panel A), in the presence of 5 mM MgATP (Panel B), or in the presence of 5 mM MgADP (Panel C).

on the chiroptical properties of the [4Fe-4S] cluster of the Fe protein.

Finally, the downfield portion of the proton NMR spectrum of the reduced Fe protein contains resonances from eight $\beta$-$CH_2$ and four α-CH protons of the four cysteinyl ligands to the [4Fe-4S], which appear as four isotropically shifted peaks between 13 and 50 ppm (Lanzilotta et al., 1995a). These proton resonances are primarily shifted by through bond interactions with the paramagnetic $[4Fe\text{-}4S]^{1+}$ cluster and are thus sensitive to distance and orientation relative to the cluster. Both MgATP and MgADP binding to the wild-type Fe protein have been shown to result in changes in the chemical shifts of these proton resonances, indicating changes induced in the protein environment around the cluster (Meyer et al., 1988; Lanzilotta et al., 1995a). The downfield portion of the 400.17 MHz $^1$H NMR spectrum for each of the Phe 135 altered Fe proteins is compared with that of the wild-type Fe protein, without or with nucleotides, in Figure 4. Each of the Phe 135 altered Fe proteins revealed downfield NMR spectra which were generally similar to the wild-type Fe protein spectrum but with shifts in the resonances. The assignment of the resonances for each altered protein to the corresponding resonance in the wild-type Fe protein was confirmed from the temperature dependence of the chemical shift. A pattern for the shifts in these resonances emerges from an examination of the spectra for the altered proteins indicating changes from the wild-type Fe protein chemical shifts in the order F135Y < F135I < F135W < F135H. The resonances assigned to the α-CH and $\beta$-$CH_2$ protons of the two cysteinyl ligands bound to the mixed valence iron pair ($Fe^{2+}$−$Fe^{3+}$) of the [4Fe-4S] cluster (47 and 17 ppm for wild-type Fe protein) were shifted progressively downfield with F135Y < F135I < F135W < F135H. In addition, the $\beta$-$CH_2$ resonances for these cysteinyl ligands were split into two resonances for the F135W and F135H altered proteins. The resonances assigned to α-CH and $\beta$-$CH_2$ protons of the two cysteinyl ligands bound to the all ferrous iron pair ($2Fe^{2+}$) of the cluster (23 and 13 ppm for wild-type Fe protein) were shifted progressively upfield with F135Y < F135I < F135W < F135H. These data indicate that changing Phe 135 to Tyr, Ile, Trp, and His resulted in progressively larger changes in the protein environment around the cluster as revealed by changes in the chemical shifts of the ligand protons.

MgADP or MgATP binding to the wild-type Fe protein has been shown to result in changes in the chemical shifts of the isotropically shifted protons (Meyer et al., 1988; Lanzilotta et al., 1995a). These observations suggest that nucleotide binding by the Fe protein induces changes in the protein environment around the [4Fe-4S] cluster. The downfield portion of the NMR spectrum for each of the Phe 135 altered Fe proteins in the presence of MgATP or MgADP is compared to that of the wild-type Fe protein in Figure 4 (panels B and C). MgATP binding to the wild-type and Phe 135 altered Fe protein was generally found to have minimal effects on the downfield-shifted resonances, with the most significant effect on the α-CH protons equivalent to the 14 ppm peak of the wild-type Fe protein. The binding of MgADP to wild-type Fe protein resulted in the most dramatic shifts in the downfield peaks. Smaller, but noticeable shifts were observed upon MgADP binding to the Phe 135 altered Fe proteins. Taken together, the $^1$H NMR spectra of the altered Fe proteins confirmed that altering Phe 135 resulted in subtle changes in the geometry of the cluster ligands and affected both the MgATP, and MgADP-induced changes in the proton NMR spectrum.

*Redox Potentials of Fe Proteins.* Given the observed changes in the CD and NMR spectra of the Fe proteins when Phe 135 was changed to Tyr, Trp, Ile, and His, it was important to examine the effects of these changes on the redox potential of the [4Fe-4S] cluster. Potentiometric titrations of the $[4Fe\text{-}4S]^{2+/1+}$ couple for the Phe 135 altered Fe proteins and the wild-type Fe protein are presented in Figure 5. As previously shown (Watt et al., 1986), the wild-type Fe protein had a redox potential of −300 mV in the absence of bound nucleotides (Table 2). In contrast, the redox potentials for the Phe 135 altered Fe proteins were found to be shifted significantly more positive compared to the wild-type Fe protein, with redox potentials of −250 mV for the F135Y, −240 mV for the F135I, −210 mV for the F135W, and −180 mV for the F135H altered Fe protein (Figure 5 and Table 2). MgATP binding to the wild-type Fe protein is known to result in a −120 mV shift in the redox potential to −420 mV. The addition of MgATP to the Phe 135 altered Fe proteins was also found to result in approximately −100 mV shifts in the redox potentials compared to the no nucleotide bound states (Table 2). In contrast, the addition of MgADP to the Phe 135 altered Fe proteins was found to result in much smaller shifts in the redox potential ($\Delta E_m$ = −30 to −50 mV) compared to the observed −160 mV shift in the wild-type Fe protein (Table 2). These results indicate that changing Phe 135 has profound effects on the redox potential of the [4Fe-4S] cluster as well as on the MgADP-induced changes in the redox potential. In contrast, little effect on the MgATP-induced changes in the redox potential was observed.

## DISCUSSION

The results of the present study can be discussed in the context of four important questions about nitrogenase Fe protein function: (i) how is the redox potential of the [4Fe-4S] cluster of the Fe protein controlled by the protein, (ii) how do nucleotides induce changes in the redox potential of the [4Fe-4S] cluster, (iii) what is the role of Phe 135 in defining the spectroscopic (i.e., CD) properties of the Fe protein, and (iv) what is the role of Phe 135 in nitrogenase catalysis?

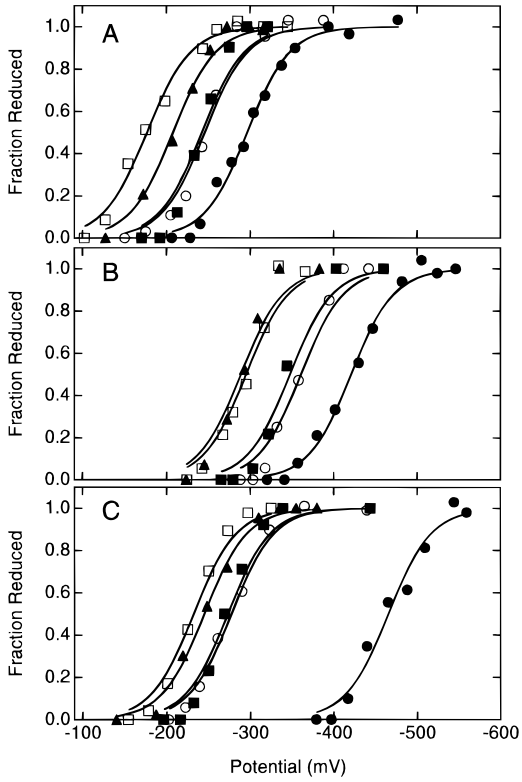


FIGURE 5: Redox titrations of wild-type and Phe 135 altered Fe proteins. Potentiometric redox titrations using EPR were performed as outlined in Experimental Procedures. Wild-type (●), F135Y (○), F135I (■), F135W (▲), and F135H (□) Fe protein samples (10−15 mg mL$^{-1}$) were prepared in 50 mM Tricine buffer, pH 8.0, with 250 mM NaCl, 50 $\mu$M methyl viologen, 50 $\mu$M benzyl viologen, and 50 $\mu$M flavin mononucleotide. Titrations are presented for Fe protein samples in the absence of added nucleotides (Panel A), in the presence of 2 mM MgATP (Panel B), or in the presence of 2 mM MgADP (Panel C).

Table 2: Redox Potentials of Wild-Type and Phe 135 Altered Fe Proteins

| | $E_m$ (mV)[a] | | | | | |
|---|---|---|---|---|---|---|
| | no addition | | +MgATP | | +MgADP | |
| Fe protein | $\Delta E_m$[b] (vs wild type) | | $\Delta E_m$[c] (vs no nucleotides) | | $\Delta E_m$[c] (vs no nucleotides) | |
| wild type | −300 | | −420 | −120 | −460 | −160 |
| F135Y | −250 | +50 | −360 | −110 | −280 | −30 |
| F135Y | −240 | +60 | −350 | −110 | −270 | −30 |
| F135Y | −210 | +90 | −290 | −80 | −240 | −30 |
| F135Y | −180 | +120 | −300 | −120 | −230 | −50 |

[a] Redox potentials ($E_m$) were measured by EPR titrations as described in the Experimental Procedures in 100 mM Tricine buffer, pH 8.0. Potentials (mV) are reported relative to the normal hydrogen electrode (NHE). [b] The change in the redox potential relative to the wild-type Fe protein under the same conditions. [c] The change in the redox potential upon the addition of the noted nucleotide.

*The Redox Potential of the [4Fe-4S] Cluster of Fe Protein.* [4Fe-4S] clusters occur widely in proteins and are usually classified according to the oxidation states typically accessed upon oxidation or reduction. Ferredoxin type [4Fe-4S]$^{2+/1+}$ clusters[2] have been found to have redox potentials ranging from +50 to −650 mV, while the high-potential iron protein (HiPIP) type [4Fe-4S]$^{3+/2+}$ clusters have redox potentials ranging from +50 to +450 mV (Johnson, 1994). Several models have been proposed to explain the differences in redox potential between ferredoxin and HiPIP type [4Fe-4S] clusters and the differences observed within a single group (Jensen et al., 1994). These models have been developed from analysis of X-ray crystal structures, from analysis of proteins modified by site-directed mutagenesis, and from theoretical calculations. While there is still considerable debate, it is now generally believed that three factors probably define the redox potential of [4Fe-4S] clusters in proteins, namely (i) protein control of solvent access to the cluster, (ii) side chain (e.g., aromatic residue) interactions with the cluster, and (iii) protein amide dipole (including, but not limited to hydrogen-bonding) interactions with the cluster (Jensen et al., 1994).

The [4Fe-4S] cluster of the nitrogenase Fe protein is of the ferredoxin type, with the physiological oxidation states being 2+ and 1+. Unlike most other ferredoxin type [4Fe-4S] clusters, however, it is bound at the interface between two identical subunits and near the surface (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 protein ligands to the cluster (Howard et al., 1989; Georgiadis et al., 1992). An approximate two-fold axis of symmetry through the [4Fe-4S] cluster provides nearly identical protein environments on each side of the cluster. The two Phe 135 side chains are located symmetrically below the [4Fe-4S] cluster and thus might be expected to influence the properties of the cluster. The results from the present study reveal that changing both of these Phe 135 residues to several different amino acids did have dramatic effects on the properties of the [4Fe-4S] cluster. The mechanism by which the side chains of Phe 135 affect the cluster properties in the Fe protein can be discussed in the context of prevailing models for how proteins might control the properties of [Fe-S] clusters.

The simplest model might suggest that the Phe 135 side chains interact directly with the [4Fe-4S] cluster. Aromatic amino acids have been observed near [4Fe-4S] clusters in several proteins and have been suggested to modulate the physicochemical properties of these clusters (Carter et al., 1974; Carter, 1977; Sheridan et al., 1981; Krishnamoorthi et al., 1986; Breiter et al., 1991). The two Phe 135 residues are the only aromatic residues in the immediate environment of the [4Fe-4S] cluster in the Fe protein. If the side chains of these residues interacted directly with the cluster, then changing the side chains would be expected to dramatically influence the properties of the [4Fe-4S] cluster. Changing Phe to Tyr, Trp and His would be expected to provide side chains with greater hydrogen-bonding potential to the cluster, which would be predicted to stabilize the reduced state of the cluster, thereby increasing the redox ($E_m$) potential. The observation that the substitution of Phe 135 to Tyr, Trp, Ile, and His resulted in large positive shifts in the redox potential (+50 to + 120 mV) of the [4Fe-4S] cluster tend to support such a mechanism. The observed positive shift in the redox potential upon changing Phe 135 to Ile, however, does not fit the model. The hydrophobic side chain of Ile would be predicted to destabilize the reduced state of the cluster, thereby lowering the redox potential. This would suggest that the influence of Phe 135 on the [4Fe-4S] cluster is not by direct interactions. Similar conclusions have been drawn recently regarding the possible role of aromatic residues in

[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)$_4$], where the $2^+/1^+$ couple is defined as $2^-/3^-$.

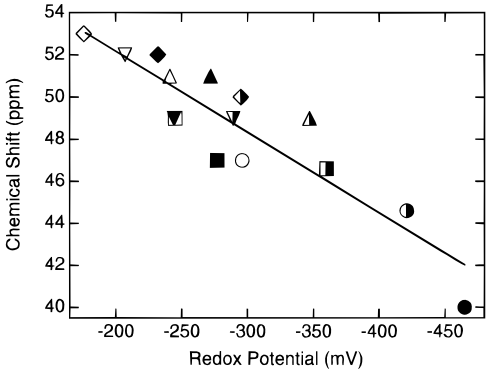


FIGURE 6: Correlation between redox potential and NMR chemical shift of the downfield resonances of Fe proteins. For each altered Fe protein, the NMR chemical shift (ppm) for the average of the most downfield-shifted resonances corresponding to cysteinyl $\beta$-$CH_2$ protons bound to the mixed valence iron pair of the [4Fe-4S] cluster (Lanzilotta et al., 1995a) are plotted against the measured redox potentials ($E_m$). Data are presented in the absence of added nucleotides (open symbols), in the presence of MgADP (closed symbols), and in the presence of MgATP (half-closed symbols), for the wild-type (○, ●, ◑), F135Y (□, ■, ◨), F135I (△, ▲, ◭), F135W (▽, ▼, ▾), and F135H (◇, ◆, half-closed tilted squares) Fe proteins.

defining the properties of [4Fe-4S] clusters in two HiPIP proteins. Changing a Tyr at position 12 located near the [4Fe-4S] cluster in the HiPIP from *Ectothiorhodospira halophila* had minimal effects on the redox potential of the cluster (Iwagami et al., 1995). Likewise, changing a Tyr at position 19 located near the [4Fe-4S] cluster in the HiPIP from *Chromatium vinosum* had little effect on the redox potential of the cluster (Agarwal et al., 1995).

Recent calculations have revealed that protein amide dipole (including, but not limited to hydrogen-bonding) interactions probably dominate protein control of $E_m$ for ferredoxin [4Fe-4S] clusters (Jensen et al., 1994). Several lines of evidence presented in this work would suggest that such a mechanism may be the dominant factor in defining the properties of the [4Fe-4S] cluster in the nitrogenase Fe protein. As mentioned earlier, at least six amide hydrogen-bonds between the Fe protein and the [4Fe-4S] cluster are suggested from the X-ray structure. Specifically, the NH groups of Ala 98 and Gly 134 appear to interact with inorganic S atoms in the cluster, while the NH group of Gly 99 appears to interact with the S$\gamma$ of Cys 97, the cluster ligand (Georgiadis et al., 1992). These amide interactions might be expected to control $E_m$ for the [4Fe-4S] cluster, and changes in Phe 135 could result in changes in orientation of these amide dipole bonds. This model is supported by the observation that the redox potential observed when Phe 135 was changed to Tyr, Trp, Ile, and His appeared to correlate with the chemical shifts of the proton NMR resonances of the $\beta$-$CH_2$ protons of the cluster cysteinyl ligands. As can be seen in Figure 6, a plot of the chemical shift of the $\beta$-$CH_2$ protons of the cysteinyl ligands bound to the mixed valence iron pair of the [4Fe-4S] cluster against the measured redox potential of the altered Fe proteins revealed a correlation between these two parameters. It is interesting that these cysteinyl ligands were tentatively assigned to the Cys 132 residues (Lanzilotta et al., 1995a), which would be close to the Phe 135. This observation appears to support the notion that changing Phe 135 results in slight changes in the orientation of the protein environment around the cluster, reflected as changes in the orientation, and hence chemical shift, of the $\beta$-$CH_2$ protons of the cluster ligands. Such changes might be expected to change the orientation of amide dipole interactions with the cluster as well, which could change the redox potential of the cluster. An average value for the contribution of a first shell, properly oriented amide dipole interaction with a [4Fe-4S] cluster has been predicted to be approximately 16 kJ/mol, which would be equivalent to a 170 mV change in $E_m$ (Jensen et al., 1994). Thus, the observed +50 to +120 mV changes in $E_m$ upon substitution of Phe 135 could easily be accounted for by the reorientation of a single amide dipole with respect to the cluster. A more likely model would suggest minor reorientation of several such amide interactions.

*Nucleotide-Induced Changes in the Redox Potential of the Fe Protein.* How does MgATP or MgADP binding to the Fe protein induce −120 or −160 mV changes in the redox potential of the [4Fe-4S] cluster? Since nucleotides bind ~19 Å away from the cluster, nucleotide-induced changes in the spectroscopic and redox properties of the [4Fe-4S] cluster must be communicated through the protein backbone and must be reflected as changes in the protein environment around the cluster (Ryle & Seefeldt, 1996). EXAFS results suggest that the Fe−Fe and Fe−S bond distances of the [4Fe-4S] cluster do not change upon nucleotide binding, further supporting the idea that changes in the protein environment around the cluster must account for the changes in the cluster properties (Lindahl et al., 1987a; Ryle et al., 1996). One transduction pathway from the nucleotide binding sites to the [4Fe-4S] cluster has recently been defined as the protein domain from Asp 125, in the nucleotide binding site, to Cys 132, a ligand to the cluster (Ryle & Seefeldt, 1996). While the nature of the protein changes in the environment of the cluster is not clear, the results of this study appear to provide some insights. The binding of MgATP or MgADP to the Fe protein causes a significant lowering of the redox potential of the cluster, along with significant upfield shifts in the $\beta$-$CH_2$ cysteinyl proton NMR resonances. This contrasts with the significant increase in the redox potential and downfield shift of the same proton resonances observed for substitution of Phe 135. Thus, the chemical shift of the protons nearest the cluster appears to report subtle changes in the protein backbone around the cluster. This seems to support a model in which the redox potential of the cluster is defined by the changes in the protein backbone around the cluster.

It is interesting that changing Phe 135 to Tyr, Trp, His, and Ile resulted in proteins which still underwent an approximately −100 mV shift in $E_m$ upon MgATP binding but showed a dramatically smaller change in $E_m$ for MgADP binding. This would suggest that Phe 135 is not essential for stabilizing the MgATP-induced changes in the environment of the cluster which result in the lowering of the redox potential, but that it is critical for the MgADP-induced changes. Thus, MgATP- and MgADP-mediated changes in the properties of the cluster probably occur through different mechanisms, with Phe 135 being essential only for the MgADP mechanism.

*Circular Dichroism Spectrum of the Fe Protein.* Phe 135 appears to also play a role in defining the chiroptical properties of the [4Fe-4S] cluster in the Fe protein. Very little is known about the factors which contribute to the visible region CD spectra of proteins containing [4Fe-4S] clusters, except that S → Fe charge transfer transitions are being measured (Stephens et al., 1979). Changing Phe 135

to Trp, His, and Ile resulted in Fe proteins with relatively featureless CD spectra in the oxidized state, even though the UV/vis spectra suggested that the cluster electronic transitions were not detectably changed. The lack of chiroptical activity for these altered Fe proteins when compared to the wild-type Fe protein clearly suggests that Phe 135 contributes to the rotational strength of the [4Fe-4S] cluster. The simplest model would suggest that changing Phe 135 has resulted in a more symmetrical [4Fe-4S] cluster, which might be expected to lessen the rotation of polarized light. This model does not seem likely, however, given the observation from NMR that changing Phe 135 probably resulted in a less symmetrical environment around the cluster as noted by the splitting of several resonances. An alternative view comes from models developed for heme containing proteins which suggests that aromatic residues 10−15 Å away from the heme strongly influence the rotational strength of the heme center (Hsu & Woody, 1971). The mechanism by which aromatic residues might influence the chiroptical properties of heme proteins has been proposed to be through interactions between heme $\pi-\pi^*$ transitions and $\pi-\pi^*$ transitions of the nearby aromatic side chains (Hsu & Woody, 1971). Recent studies have also suggested that interactions between aromatic side chains and [4Fe-4S] clusters might influence the visible region CD spectrum. Changing Tyr 12 of *E. halophila* HiPIP (which is near the [4Fe-4S] cluster) to Trp or His resulted in a significant reduction of the visible CD spectrum (Iwagami et al., 1995). Since Phe 135 is the only aromatic residue in the Fe protein near the [4Fe-4S] cluster, and removing this residue resulted in a dramatic reduction in the chiroptical properties of the cluster, it seems possible that Phe 135 in the Fe protein contributes to these chiroptical properties. The mechanism of this coupling is not clear, however, as the mixing of aromatic $\pi-\pi^*$ transitions with [Fe-S] charge transfer bands has not been previously reported.

*Communication from the Cluster to the Nucleotide Binding Site.* An important finding of the present work is the observation that alteration of Phe 135 to Trp or His resulted in an Fe protein with a greater affinity for binding MgATP. Since the distance from Phe 135 to the nucleotide binding site is approximately 10 Å, it seems highly unlikely that this effect is due to a direct interaction between residue 135 and the nucleotide binding site. Both Phe 135 and the nucleotide binding sites are located at the subunit interface (Georgiadis et al., 1992). It, therefore, seems possible that the alteration of Phe 135 could subtly affect the spacing between the subunits and thus influence the affinity of the Fe protein for nucleotides. Again, this is supported by proton NMR studies which reveal changes in the orientation of the protons of the cysteinyl ligands upon binding of MgATP. A similar mechanism may explain the observation that the reduced and oxidized states of wild-type Fe protein demonstrate different affinities for nucleotides. The difference in nucleotide affinities observed for the reduced and oxidized states of Fe protein may also be a result of a change in subunit orientation.

*The Role of Phe 135 in Nitrogenase Catalysis.* It is interesting to note that despite the fact that all of the Phe 135 altered Fe proteins contained a [4Fe-4S] cluster and bound nucleotides, these proteins did not catalyze substrate reduction or demonstrate MgATP hydrolysis when combined with the MoFe protein. A possible explanation for this observation would be that changing Phe 135 has reduced the affinity of Fe protein binding to the MoFe protein. This would be consistent with the observed lack of inhibition by these altered proteins for wild-type Fe protein−MoFe protein complex formation in an activity assay. It is becoming apparent that the productive docking of the Fe protein to the MoFe protein probably involves a complex series of events, including protein conformational changes within each protein. The present data would suggest that Phe 135 plays a key role in productive component docking required to activate MgATP hydrolysis, electron transfer, and substrate reduction.

In summary, the present work has provided evidence that the conserved Phe at position 135 in the nitrogenase Fe protein plays an important role in defining the physicochemical properties of the [4Fe-4S] cluster. It appears that Phe 135 contributes to the chiroptical properties of the Fe protein in the visible region. Furthermore, Phe 135 appears to participate in the MgADP-induced modulation of the redox potential, but not in the MgATP-induced changes. It will now be interesting to probe the role of other amino acids within van der Waals contact of the [4Fe-4S] cluster in defining the electronic and redox properties, including nucleotide-induced changes, of the nitrogenase Fe protein.

## ACKNOWLEDGMENT

We thank Professors L. E. Mortenson, V. D. Parker, and G. M. Swain and Drs. G. M. Jensen and M. E. Rasche for helpful discussions.

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BI9608572