Evidence for Electron Transfer from the Nitrogenase Iron Protein to the Molybdenum−Iron Protein without MgATP Hydrolysis: Characterization of a Tight Protein−Protein Complex†

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ABSTRACT: MgATP hydrolysis has been proposed to be absolutely required for electron transfer from the nitrogenase iron (Fe) protein to the molybdenum−iron (MoFe) protein. This work presents evidence for primary electron transfer from the *Azotobacter vinelandii* nitrogenase Fe protein to the MoFe protein in the absence of MgATP hydrolysis. Deletion of an amino acid (Leu 127) in a signal transduction pathway in the Fe protein resulted in an Fe protein conformation resembling the MgATP-bound state. This altered Fe protein (L127Δ) was found to bind to the MoFe protein in the absence of MgATP, forming a tight protein complex. Both steady state and stopped-flow transient kinetic measurements suggest that two L127Δ Fe proteins bind to one MoFe protein with an extremely high affinity. From pre-steady state kinetic determinations of the rate of complex dissociation, the affinity was found to be at least 350 times tighter than that of the wild-type *A. vinelandii* nitrogenase complex and at least 20 times tighter than that of the heterologous *Clostridium pasteurianum* Fe protein−*A. vinelandii* MoFe protein complex. The L127Δ Fe protein−MoFe protein complex was isolated by gel filtration liquid chromatography. Scanning densitometry of an SDS gel of the complex isolated from the gel filtration column revealed a stoichiometry of 1.7 L127Δ Fe proteins bound per MoFe protein. The L127Δ Fe protein was found to transfer a single electron from its [4Fe-4S] cluster to the MoFe protein at a rate of 0.2 s^{-1}. This compares with the MgATP-dependent electron transfer rate of 140 s^{-1} observed for transfer of an electron from the wild-type Fe protein to the MoFe protein. No substrate reduction (H^+ or C_2H_2) was detected when wild-type MoFe protein was complemented with L127Δ Fe protein. The MgATP-independent electron transfer from the L127Δ Fe protein to the MoFe protein required active MoFe protein and was not inhibited by MgADP. EPR spectroscopy of the complex was employed to confirm the electron transfer reaction. These results show that Fe protein in a conformation resembling the MgATP-bound state can transfer at least one electron to the MoFe protein without the need for MgATP hydrolysis.

Biological nitrogen fixation is catalyzed by the two-component metalloenzyme nitrogenase according to the overall reaction

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

The molybdenum−iron protein (MoFe protein) component is an $\alpha_2\beta_2$ tetramer containing two unique molybdenum−iron−sulfur−homocitrate cofactors (FeMoco) (Kim & Rees, 1992), which are the site 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 believed to initially accept the electron 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 a single electron is transferred from the Fe protein to the MoFe protein with the concomitant hydrolysis of two MgATP molecules (Thorneley & Lowe, 1983; Howard & Rees, 1994). The now-oxidized Fe protein, with two molecules of MgADP bound, is released from the MoFe protein, and another reduced Fe protein, with two bound MgATP molecules, binds to the partially reduced MoFe protein for a second round of MgATP hydrolysis and electron transfer (Hageman & Burris, 1978). The dissocia-

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[1] Abbreviations: Fe protein, iron protein of nitrogenase; MoFe protein, molybdenum−iron protein of nitrogenase; FeMoco, iron−molybdenum cofactor; EPR, electron paramagnetic resonance; Av2, *Azotobacter vinelandii* Fe protein; $AvFldII_{HQ}$, hydroquinone form of flavodoxin II from *A. vinelandii*; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; L127Δ, iron protein of nitrogenase with Leu 127 deleted; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography.

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tion of the Fe protein−MoFe protein complex, which occurs after the transfer of each electron, is the rate-limiting step in nitrogenase catalysis. This cycle is repeated until sufficient electrons have been transferred to the MoFe protein to reduce the bound substrate. The Fe protein is the only reductant of the MoFe protein which will support substrate reduction.

MgATP serves at least two functions in the nitrogenase mechanism (Mortenson et al., 1993). First, MgATP binding to the Fe protein induces protein conformational changes which are reflected as changes in the properties of its [4Fe-4S] cluster (Mortenson et al., 1993). Significant among these changes is a lowering of the redox potential of its [4Fe-4S] cluster by 120 mV from −296 to −420 mV (Zumft et al., 1974; Watt et al., 1986). This lowering of the redox potential along with other changes induced in the protein appears to be necessary for docking to the MoFe protein and subsequent electron transfer. The second function for MgATP in the nitrogenase mechanism is the coupling of its hydrolysis to electron transfer from the Fe protein to the MoFe protein and to substrate reduction (Mortenson et al., 1993). Evidence from recent kinetic studies has suggested that MgATP hydrolysis either precedes or follows electron transfer, but in all cases, MgATP hydrolysis is absolutely required for the electron transfer reaction (Thorneley et al., 1989; Mensink et al., 1992; Duyvis et al., 1994).

Recently, we have created an Fe protein by site-directed mutagenesis that demonstrates characteristics of the wild-type Fe protein in the MgATP-bound conformation in the absence of any nucleotides (Ryle & Seefeldt, 1996). This Fe protein was created by deletion of an amino acid (L127) in a MgATP signal transduction pathway (Howard & Rees, 1994), resulting in protein conformational changes characteristic of the MgATP-bound state even in the absence of MgATP. Significantly, the altered Fe protein had a low redox potential (−412 mV) in the absence of nucleotides, which is normally only observed for the wild-type Fe protein in the presence of MgATP (−420 mV). In the present work, we demonstrate that the L127Δ Fe protein binds to the MoFe protein with an extremely high affinity, forming a tight homologous protein complex in the absence of nucleotides. Furthermore, evidence for electron transfer from the altered Fe protein to the MoFe protein in the absence of MgATP hydrolysis is presented.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis, Expression, and Purification of Altered and Wild-Type Fe Proteins. Site-directed mutagenesis of the gene that encodes the Fe protein subunits of *Azotobacter vinelandii* nitrogenase, *nif H*, was performed as previously described (Jacobson et al., 1989; Seefeldt & Mortenson, 1993). Expression and purification of wild-type and altered Fe proteins was carried out as previously described (Seefeldt & Mortenson, 1993), except that buffers for altered Fe proteins contained 20% glycerol. Fe proteins were purified to homogeneity as judged by analysis on SDS gels stained with Coomassie blue (Hathaway et al., 1979). Protein concentrations were determined by the modified biuret method (Chromy et al., 1974), with bovine serum albumin as the standard, and from the absorption at 400 nm using an absorption coefficient of 13.3 $M^{-1}\cdot cm^{-1}$ for the oxidized Fe protein (Lanzilotta et al., 1995b).

Activity Assays. Specific activities of the wild-type nitrogenase component proteins were determined as previously described (Lanzilotta et al., 1995b). Wild-type Fe protein and MoFe protein used in this study had specific activities of 1875 nmol of acetylene reduced$\cdot min^{-1}\cdot$(mg of Fe protein)$^{-1}$ and 1920 nmol of acetylene reduced$\cdot min^{-1}\cdot$(mg of MoFe protein)$^{-1}$ (Seefeldt & Ensign, 1994). When the L127Δ Fe protein was assayed under identical conditions, no detectable acetylene or proton reduction could be detected even after a 24 h incubation period.

For steady state inhibition studies, a variation of the tight binding, irreversible inhibition procedure described by Morrison was employed (Morrison, 1969). The Fe protein was added as a saturating substrate to standard acetylene reduction assay vials. Different quantities of the L127Δ Fe protein (acting as a tight binding inhibitor) were added to each of three sets of assay vials. Various amounts of the MoFe protein (i.e. the enzyme) were added to each vial to initiate the reaction. Reactions were allowed to proceed at 30 °C with shaking for 4 min and were quenched by the addition of 250 μL of 2.5 M H_2SO_4. The rate of acetylene reduction was plotted against the quantity of enzyme (MoFe protein) added for each concentration of inhibitor (L127Δ Fe protein). The extrapolated *x*-intercept from the linear portion of each data set was used to establish the stoichiometry of binding of L127Δ Fe protein to the MoFe protein (Morrison, 1969).

Isolation of a L127Δ Fe Protein−Wild-Type MoFe Protein Complex. The L127Δ Fe protein−MoFe protein complex was isolated by gel filtration column chromatography as follows. A sample of either L127Δ Fe protein or wild-type Fe protein (6.7 or 13.4 nmol) was mixed with a sample of wild-type MoFe protein (3.35 nmol). The total volume of the sample was brought to 100 μL with 50 mM Tris buffer (pH 8.0) containing 2.0 mM sodium dithionite and allowed to incubate for 5 min. A 20 μL aliquot of each mixture was chromatographed on a Spherogel TSK (7.5 mm × 30 cm) size exclusion HPLC column equilibrated with 100 mM MOPS buffer (pH 7.0), containing 0.4 mM dithionite, at a flow rate of 0.4 mL$\cdot min^{-1}$. Protein elution was monitored by the absorbance at 285 nm. Fractions (100 μL) were collected starting at 11 min and until all proteins had eluted. Appropriate fractions were analyzed by SDS gel electrophoresis with Coomassie blue staining (Hathaway et al., 1979). The stoichiometry of the L127Δ Fe protein complex was determined by scanning densitometry of the Coomassie-stained gels. The ratios of each protein were determined by comparison of the relative integrated areas and by comparison to the densitometric scans of gels loaded with known quantities of Fe and MoFe proteins.

Stopped-Flow Spectrophotometry. Absorbance changes occurring during nitrogenase turnover were monitored with a Hi-Tech SF61 stopped-flow spectrophotometer equipped with a data acquisition and curve fitting system (Salisbury, Wilts, U.K.). The SHU-61 sample handling unit was kept anoxic inside an anaerobic chamber operating at less than 0.1 ppm oxygen. The oxygen concentration inside the anaerobic chamber was monitored continually during experimentation using a Teledyne Analytical Instruments series 316 trace oxygen analyzer (Vacuum Atmospheres Co., Hawthorne, CA). Reactants were thermostatted to 23 ± 0.1 °C by the means of closed water circulation with a Techne C-85D circulator (Techne Ltd., Duxford, Cambridge, U.K.) attached to an FC-200 Techne flow cooler. Both the flow

cooler and circulator were external to the anaerobic chamber. Data were collected at either 430 nm for the oxidation/reduction of Fe protein or 580 nm for the oxidation of $AvFldII_{HQ}$. The rate of protein complex dissociation was initially measured by the reduction of oxidized Fe protein in the presence of MoFe protein by dithionite (Thorneley & Lowe, 1983). Fe proteins were oxidized as described (Ashby & Thorneley, 1987), and dithionite was removed from MoFe protein, inside the anaerobic chamber, on a gel filtration column packed with P-6DG (Bio-Rad, Melville, NY) that had been pre-equilibrated with 25 mM HEPES buffer (pH 7.4) containing 150 mM NaCl. In these dissociation experiments, one drive syringe of the stopped-flow spectrophotometer contained oxidized Fe protein and dithionite-free MoFe protein in approximately equal concentrations, while the second drive syringe contained dithionite and Fe protein, at various concentrations. Determination of the wild-type protein complex dissociation rate required the addition of MgADP to both drive syringes. In addition, apparent component protein dissociation rates were calculated by using the oxidation of $AvFldII_{HQ}$ as described (Peters et al., 1995) as an indirect measure of component protein dissociation. The Mo and Fe contents of MoFe protein used in stopped-flow spectrophotometric studies were determined to be 1.5 $\pm$ 0.1 and 25 $\pm$ 2 mol of Mo and Fe per mole of MoFe protein, respectively, by inductively coupled plasma atomic emission spectrometry using a simultaneous spectrometer (Jarrell-Ash ICAP 9000) and a sequential scanning spectrometer (Jarrell-Ash Atomscan 2400). The concentrations of all the components are given in the text or figure legends. All stopped-flow reactions were carried out in 25 mM HEPES buffer (pH 7.4) containing 10 mM $MgCl_2$, unless otherwise stated.

EPR Spectroscopy. All EPR spectra were recorded on a Bruker ESP300E spectrometer equipped with a dual mode cavity and an Oxford liquid helium cryostat. In all cases, 4 mm calibrated quartz EPR tubes (Wilmad, Buena, NJ) were used. Spectra were recorded at 12 K, with a 1.01 mW microwave power and a microwave frequency of 9.64 GHz. A modulation amplitude of 5.64 G, a modulation frequency of 100 KHz, and a time constant and conversion time of 20.48 ms were also used. Five scans were added with 1024 points per scan. All spectra were base line corrected.

RESULTS

Defining a Tight L127Δ Fe Protein−MoFe Protein Complex. While the L127Δ Fe protein would not support substrate reduction when combined with the MoFe protein, kinetic evidence suggested that it could still bind to the MoFe protein (Ryle & Seefeldt, 1996). The L127Δ Fe protein was found to inhibit acetylene reduction activity catalyzed by wild-type Fe protein and MoFe protein, suggesting that the L127Δ Fe protein was binding to the MoFe protein. The affinity and stoichiometry of L127Δ Fe protein binding to the MoFe protein were explored by utilizing a competitive binding activity assay (Morrison, 1969). Figure 1 illustrates the effects of increasing MoFe protein concentration on the acetylene reduction activity at saturating wild-type Fe protein concentrations and fixed concentrations of L127Δ Fe protein. If the L127Δ Fe protein were binding to the MoFe protein as a tight binding inhibitor, then no acetylene reduction activity would be expected until the MoFe protein concentration was increased above half the concentration of the L127Δ

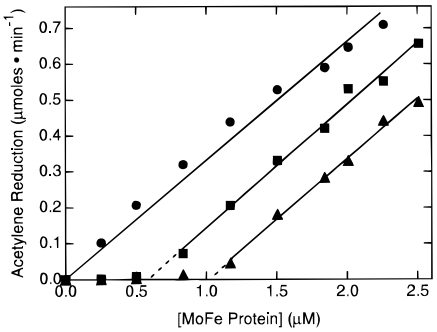

FIGURE 1: Inhibition of wild-type nitrogenase acetylene reduction reactions by L127Δ Fe protein. Acetylene reduction assays were performed as described in Experimental Procedures. Wild-type Fe protein (930 μg, 14 nmol) was added to three sets of standard acetylene reduction assay vials. Each set also contained either 0 μg (0 nmol) (●), 97.5 μg (1.5 nmol) (■), or 156 μg (2.4 nmol) (▲) of L127Δ Fe protein. Reactions were initiated by the addition of increasing amounts (0−690 μg or 0−3 nmol) of wild-type MoFe protein. All reactions were terminated after 4 min of incubation at 30 °C. The rate of acetylene reduction (in micromoles of C_2H_4 formed per minute) was plotted against the concentration of MoFe protein added. The slopes from the linear portion of each data set were 0.30, 0.32, and 0.33 μmol·min^{-1}·(μM MoFe protein)$^{-1}$, respectively, and the corresponding *x*-axis intercepts were 0.09, 0.58, and 1.05 μM, respectively.

Fe protein. Above this concentration, a linear increase in the velocity would be expected as more MoFe protein is added. The results presented in Figure 1 reveal that the L127Δ Fe protein does behave as a tight binding inhibitor of the MoFe protein. When the L127Δ Fe protein concentration was 1.27 μM, a linear increase in acetylene reduction activity was observed only after the MoFe protein concentration was above 0.6 μM. The linear portion of the data was extrapolated back to the *x*-axis, to a MoFe protein concentration of 0.58 μM. The ratio of the L127Δ Fe protein concentration to the extrapolated MoFe protein concentration revealed a binding stoichiometry for the L127Δ Fe protein to the MoFe protein of 2.2. When the L127Δ Fe protein concentration was increased to 2.18 μM, a similar plot was observed, with an extrapolated MoFe protein concentration of 1.05 μM, corresponding to a binding stoichiometry of 2.1 L127Δ Fe proteins to each MoFe protein. Comparison of the slopes from the linear portions of each data set revealed nearly identical values of 0.30, 0.32, and 0.33 μmol·min^{-1}·(μM MoFe protein)$^{-1}$. These results suggest that the L127Δ Fe protein binds to the MoFe protein with high affinity, acting essentially as a tight binding, irreversible inhibitor (Morrison, 1969), and that the L127Δ Fe protein binds to the MoFe protein at an approximate 2:1 stoichiometry.

Isolation of a Tight Nitrogenase Complex. Given the kinetic results suggesting that the L127Δ Fe protein bound tightly to the MoFe protein with a stoichiometry of 2:1, it seemed reasonable to assume that a tight protein complex could be isolated by gel filtration chromatography. Since the MoFe protein has a molecular mass of 230 kDa and the Fe protein a molecular mass of 64 kDa, a 2:1 protein complex would be expected to have a molecular mass of 358 kDa. A calibrated HPLC gel filtration column was used to rapidly separate protein mixtures on the basis of size. When the reduced wild-type Fe protein was mixed with the wild-type MoFe protein, either in the absence or in the presence of MgATP or MgADP, two protein peaks were observed upon chromatography on the HPLC column corresponding to the uncomplexed MoFe protein and the Fe protein. In contrast,

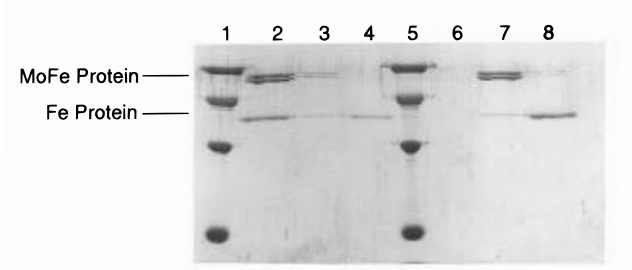

FIGURE 2: SDS gel of the isolated L127Δ Fe protein−wild-type MoFe protein complex. A solution containing 15 μM wild-type MoFe protein and either 30 μM L127Δ Fe protein or 30 μM wild-type Fe protein was prepared, and a 20 μL aliquot was chromatographed on an HPLC gel filtration column. Fractions were collected at defined elution times and were analyzed by SDS gel electrophoresis. The gel was stained for proteins with Coomassie blue. Lanes 2, 3, and 4 correspond to elution times of 12, 13, and 15 min, respectively, for the L127Δ Fe protein−MoFe protein mixture. Lanes 6, 7, and 8 correspond to elution times of 12, 13, and 15 min, respectively, for the wild-type Fe protein−MoFe protein mixture. Lanes 1 and 5 contain the molecular mass standards bovine serum albumin (68 000 Da), ovalbumin (42 000 Da), carbonic anhydrase (29 000 Da), and cytochrome *c* (12 000 Da).

when the reduced L127Δ Fe protein was mixed with the MoFe protein, only a single protein peak eluted from the HPLC column. The molecular mass of this peak was estimated to be about 350 kDa, consistent with a complex consisting of two L127Δ Fe proteins bound to one MoFe protein. The subunit composition of this larger complex was confirmed by SDS gel analysis of the eluted fractions. Figure 2 illustrates a Coomassie blue-stained SDS gel of fractions taken from the HPLC column of either the wild-type Fe and MoFe protein mixture or the L127Δ Fe protein and MoFe protein mixture. As can be seen, the wild-type Fe protein (15 min) and MoFe protein (13 min) were separated and eluted as the individual components. In contrast, the high-molecular mass protein peak observed for the L127Δ Fe protein−MoFe protein mixture (12 min) was found to contain both the Fe protein and the MoFe protein. Scanning densitometry of the SDS gel was used to determine the relative quantities of each protein and thus to calculate the stoichiometry of the Fe protein to MoFe protein in the complex. A ratio of 1.7 Fe proteins to 1 MoFe protein was determined for the L127Δ Fe protein−MoFe protein complex. When the concentration of L127Δ Fe protein was increased to as high as 4 Fe proteins to 1 MoFe protein in the incubation mixture, the stoichiometry of the isolated complex remained 1.7 Fe proteins per MoFe protein, while the excess Fe protein was observed to elute at the time observed for Fe protein alone. A L127Δ Fe protein−MoFe protein complex with the same stoichiometry was isolated whether MgATP or MgADP was included in the initial incubation mixture, revealing that the tight complex formed even without nucleotides.

The L127Δ Fe protein−MoFe protein complex was reversible when the complex was bound to a strong anion exchange column (Mono-Q) and eluted with an NaCl gradient, explaining the observation that the L127Δ Fe protein could be separated and purified away from the MoFe protein from cell extracts. Previous studies have demonstrated that the association of the wild-type Fe protein with the MoFe protein is sensitive to the salt concentration (Deits & Howard, 1990).

Dissociation of the L127Δ Fe Protein from the Wild-Type MoFe Protein. The steady state kinetic experiments and

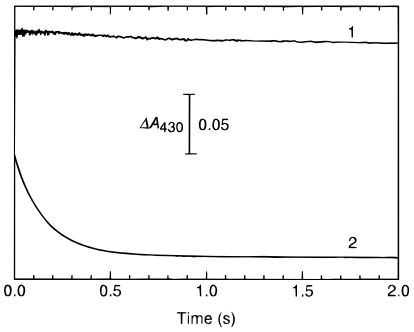

FIGURE 3: Dissociation of Fe protein from the MoFe protein monitored by stopped-flow spectrophotometery. Reduction of the [4Fe-4S] cluster of oxidized Fe protein by sodium dithionite ($Na_2S_2O_4$) upon dissociation from the MoFe protein was monitored by the change in the absorbance at 430 nm (ΔA_{430}). (Trace 1) Absorbance change after mixing a solution containing 40 μM oxidized L127Δ Fe protein, 40 μM MoFe protein (reduced, dithionite-free), and 5 mM MgADP with a solution containing 200 μM wild-type Fe protein and 20 mM sodium dithionite. (Trace 2) Absorbance change after mixing a solution containing 40 μM oxidized wild-type Fe protein, 40 μM MoFe protein (reduced, dithionite-free), and 5 mM MgADP with a solution containing 200 μM wild-type Fe protein and 20 mM sodium dithionite. The rate constants (k_{-3}) for the dissociation were determined from single exponential fits to the data and were found to be 7.0 s^{-1} for the wild-type Fe protein−MoFe protein complex and <0.02 s^{-1} for the L127Δ Fe protein−MoFe protein complex.

isolation of a complex suggested that the L127Δ Fe protein formed a tight complex with the MoFe protein. To quantify the affinity of the L127Δ Fe protein for binding to the MoFe protein, stopped-flow spectrophotometry was employed to define the dissociation constant (k_{-3}) for the complex (Thorneley & Lowe, 1983). In these experiments, Fe protein oxidized with indigo disulfonate (Ashby & Thorneley, 1987) was premixed with dithionite-free MoFe protein (with or without nucleotides) and allowed to react. This mixture was then rapidly mixed with a solution containing reduced Fe protein and dithionite in the stopped-flow spectrophotometer. The decrease in absorbance at 430 nm was monitored continuously after mixing. A series of scans were collected at a variety of concentrations of reduced Fe protein (50−250 μM) in order to obtain reduction data that could be fitted to a single exponential function (Thorneley & Lowe, 1983). The absorbance at 430 nm has been shown to be sensitive to the oxidation state of the Fe protein [4Fe-4S] cluster, with the oxidized state (2+) having an absorption coefficient of 12.0 $mM^{-1}\cdot cm^{-1}$ and the reduced state (1+) having an absorption coefficient of 4.5 $mM^{-1}\cdot cm^{-1}$ (Thorneley & Lowe, 1983). Previous studies with wild-type nitrogenase have shown that only the free, oxidized Fe protein is reduced by dithionite, while the oxidized Fe protein bound to the MoFe protein is not reduced at a detectable rate. The decrease in absorbance at 430 nm (reduction of the [4Fe-4S] cluster) thus provides a measure of the rate of dissociation of the Fe protein from the MoFe protein. Figure 3 illustrates the change in absorbance at 430 nm monitored by stopped-flow spectrophotometry upon the dissociation of the L127Δ Fe protein−MoFe protein complex and a wild-type Fe protein−MoFe protein complex in the presence of MgADP. As can be seen, the wild-type Fe protein−MoFe protein complex showed a rapid, first-order decrease in absorbance with a measured dissociation constant of 7.0 s^{-1}, consistent with previously obtained values of 6.4 ± 0.8 s^{-1} (Thorneley & Lowe, 1983; Peters et al., 1994, 1995). In

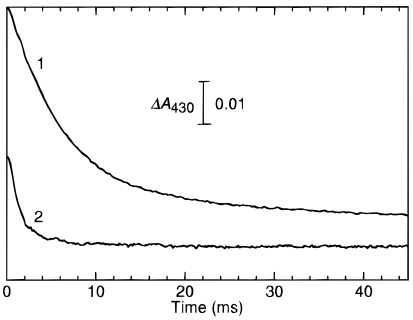

FIGURE 4: Reduction of oxidized Fe proteins by dithionite. Absorbance changes at 430 nm were monitored by stopped-flow spectrophotometry as described in the legend to Figure 3. (Trace 1) Absorbance change after mixing a solution containing oxidized L127Δ Fe protein (40 μM) with a solution containing $Na_2S_2O_4$ (20 mM). (Trace 2) Absorbance change after mixing a solution containing oxidized wild-type Fe protein (40 μM) with a solution containing $Na_2S_2O_4$ (20 mM). Single exponential fits to the data yielded rate constants of 646 s^{-1} for wild-type Fe protein and 168 s^{-1} for L127Δ Fe protein.

contrast, the L127Δ Fe protein−MoFe protein complex showed only a very slow decrease in absorbance. The decrease in absorbance was too slow to be accurately fitted to an exponential function and was estimated to be less than 0.02 s^{-1}.

The rate of oxidation of the reduced hydroquinone form of flavodoxin was used to further investigate dissociation of the L127Δ Fe protein−MoFe protein complex. Upon complex dissociation, the reduced hydroquinone form of flavodoxin becomes oxidized as it transfers its electron to the released, oxidized Fe protein. Because of the very slow release of oxidized L127Δ Fe protein from the complex, it was not possible to obtain rates consistently higher than those obtained for the auto-oxidation of $AvFldII_{HQ}$ detected in the absence of nitrogenase proteins (data not shown). These results indicate that the L127Δ Fe protein−MoFe protein complex is extremely tight, with a binding affinity at least 350 times tighter than that for the wild-type nitrogenase complex.

To confirm that the slow rate of reduction of the oxidized L127Δ Fe protein observed in the dissociation experiments was not the result of a slower rate of dithionite reduction of the oxidized Fe protein, the rates of reduction of oxidized Fe protein by dithionite in the absence of MoFe protein were determined using stopped-flow spectrophotometry. Figure 4 illustrates the rates of dithionite reduction of the $[4Fe\text{-}4S]^{2+}$ cluster of the L127Δ and wild-type Fe proteins. The rate of reduction of oxidized Fe protein is rapid, with observed first-order rate constants of 168 s^{-1} for the L127Δ Fe protein and 646 s^{-1} for the wild-type Fe protein. The observed amplitude for reduction of oxidized wild-type Fe protein is less than the calculated amplitude and the L127Δ Fe protein amplitude as the reaction is partially complete in the mixing time (3 ms) of the stopped-flow spectrophotometer. While the oxidized L127Δ Fe protein was reduced by dithionite at a slower rate than the wild-type Fe protein, it was still much faster than the observed dissociation rate. The effects of added nucleotides on the rate of reduction by dithionite were also established (data not shown). The preincubation of the oxidized Fe proteins with MgATP lowered the rate of reduction for both the wild-type and L127Δ Fe proteins. The wild-type traces showed a biphasic absorbance decrease with an initial first-order rate constant of 7 s^{-1}, followed by a further slow decrease in absorbance of 0.5 s^{-1}; both phases were of approximately equal amplitude. The same experiment with L127Δ Fe protein showed a single exponential reduction with a rate constant of 28 s^{-1}. The biphasic reduction observed for the MgATP-bound state of the wild-type Fe protein is reminiscent of the biphasic chelation of iron from the MgATP-bound Fe protein by the chelator α,α′-dipyridyl (Deits & Howard, 1989). The observed single exponential reduction of the L127Δ Fe protein without or with MgATP suggests that altering the Fe protein has resulted in a rigid conformation. MgADP lowered the rate of reduction for the wild-type Fe protein (14 s^{-1}) while having little effect on the rate of reduction for the L127Δ Fe protein (141 s^{-1}).

Electron Transfer from the L127Δ Fe Protein to the MoFe Protein. Given the observation that the L127Δ Fe protein formed a tight complex with the MoFe protein in the absence of nucleotides and the earlier observation that the L127Δ Fe protein is in a protein conformation resembling the MgATP-bound state (with a lowered redox potential), it was of interest to determine if the L127Δ Fe protein could transfer an electron to the MoFe protein in the absence of MgATP hydrolysis. To test this possibility, the oxidation of the Fe protein [4Fe-4S] cluster from the 1+ to the 2+ oxidation state was monitored by the increase in the absorption at 430 nm upon mixing the Fe protein with the MoFe protein. Figure 5 (panel A) illustrates stopped-flow traces obtained after mixing reduced wild-type Fe protein and MgATP with reduced MoFe protein. As can be seen, a first-order increase in the absorbance at 430 nm was recorded with an apparent rate constant of 140 s^{-1}. No further change in the absorbance at 430 nm was observed for the wild-type Fe protein−MoFe protein complex from 40 ms to 20 s. When MgATP was not included in the mixing reaction, no change in the absorbance was observed (panel B, trace 2). When the reduced L127Δ Fe protein was mixed with the dithionite-reduced MoFe protein in the absence of nucleotides, a first-order increase in absorption at 430 nm was also observed (panel B, trace 1) corresponding to a primary electron transfer rate of 0.2 s^{-1}. When the L127Δ Fe protein was mixed with buffer, no change in the absorbance at 430 nm was observed, ruling out self-oxidation (panel B, trace 3). The electron transfer was dependent upon active MoFe protein, with no change in absorbance observed when the MoFe protein had been inactivated by treatment with O_2 prior to the stopped-flow experiment (data not shown).

The rate of electron transfer to the MoFe protein from both the wild-type and L127Δ Fe proteins was found to be independent of the Fe protein concentration and showed approximately the same total amplitude change (Figure 5, insets). Figure 5 shows typical experimental traces, the amplitudes of which increased to a maximum absorbance with $\Delta A_{430} = 0.1$ at saturation. The insets in Figure 5 show the amplitude data for titrations of MoFe protein with wild-type or L127Δ Fe proteins. The saturation point of the amplitude of the electron transfer reaction was used to determine the competence of the Fe protein to effect the reduction of 10 μM MoFe protein (containing 15 μM active sites). The break points from the amplitude dependence data occurred at 40 μM for the wild-type Fe protein and 20 μM for the L127Δ Fe protein, which corresponded to Fe protein/active site molar ratios for L127Δ of 1.3 and for wild-type of 2.7. These values are consistent with L127Δ and wild-

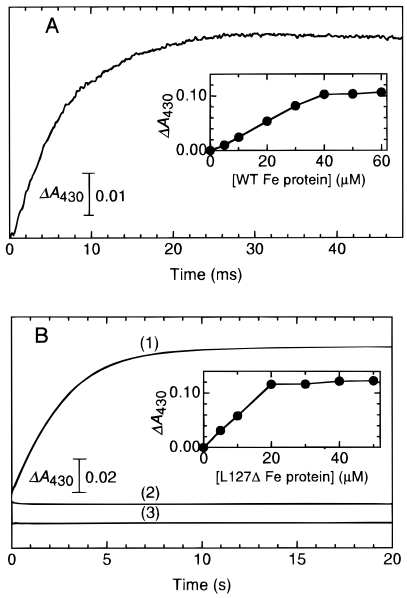

FIGURE 5: Pre-steady state electron transfer from the Fe protein to the MoFe protein. Absorbance changes at 430 nm were monitored by stopped-flow spectrophotometry as described in the legend to Figure 3. (A) Absorbance change as a result of oxidation of the [4Fe-4S] cluster in wild-type Fe protein after mixing a solution containing 75 μM wild-type Fe protein, 10 mM sodium dithionite, and 15 μM MoFe protein with a solution containing 18 mM MgATP and 10 mM sodium dithionite. A rate constant of 140 s^{-1} was determined from fitting the data to a single exponential. (Inset) Absorbance change as a function of the Fe protein concentration. (B) (trace 1) Absorbance change after mixing a solution containing 80 μM L127Δ Fe protein and 10 mM sodium dithionite with a solution containing 20 μM wild-type MoFe protein and 10 mM sodium dithionite (rate constant = 0.2 s^{-1}). (trace 2) Absorbance change after mixing a solution containing 80 μM wild-type Fe protein and 10 mM sodium dithionite against a solution containing 20 μM MoFe protein and 10 mM sodium dithionite. (trace 3) Absorbance change after mixing a solution containing 80 μM L127Δ Fe protein and 10 mM sodium dithionite against 25 mM HEPES buffer (pH 7.4) with 200 mM NaCl and 10 mM sodium dithionite. (Inset) Absorbance change as a function of the L127Δ Fe protein concentration for conditions described in trace 1.

type Fe proteins being 75 and 40% active at MoFe protein reduction. The values for the percentage of wild-type Fe protein active in the electron transfer reaction are comparable to those published (Ashby & Thorneley, 1987) but considerably less than the percentage of L127Δ capable of electron transfer. This is probably a consequence of the formation of the tight binding complex. The observed changes in the absorbance at 430 nm were confirmed by taking full absorbance spectra using a diode array spectrophotometer (data not shown).

Nucleotide Effects on Electron Transfer from the L127Δ Fe Protein to the MoFe Protein. The effects of added nucleotides on the primary electron transfer rate from the wild-type and L127Δ Fe proteins to the MoFe protein were also examined (Figure 6). MgATP was absolutely required for electron transfer from the wild-type Fe protein to the MoFe protein, and MgADP was an inhibitor of electron transfer. In contrast, MgATP was not needed for electron transfer from the L127Δ Fe protein to the MoFe protein. The addition of MgATP did accelerate the electron transfer rate from 0.2 s^{-1} in the absence of nucleotides to 5 s^{-1} when MgATP was included. ATP in the absence of Mg^{2+} is not a substrate for wild-type Fe protein, while ATP alone increased the L127Δ Fe protein electron transfer rate to 8

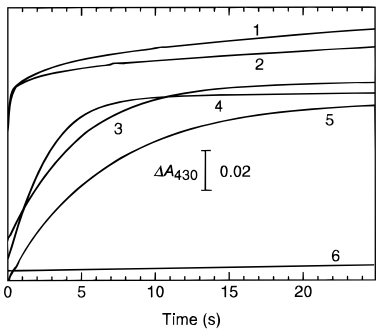

FIGURE 6: Effects of nucleotides on primary electron transfer from the L127Δ Fe protein to the MoFe protein. Absorbance changes at 430 nm were monitored by stopped-flow spectrophotometry following the mixing of a solution containing 20 μM MoFe protein and 10 mM $Na_2S_2O_4$ with a solution containing either 80 μM L127Δ Fe protein or 75 μM wild-type Fe protein and 10 mM $Na_2S_2O_4$. Nucleotides (18 mM) were included with the MoFe protein-containing solution: (trace 1) L127Δ Fe protein and ATP, (trace 2) L127Δ Fe protein and MgATP, (trace 3) L127Δ Fe protein and ADP, (trace 4) L127Δ Fe protein, (trace 5) L127Δ Fe protein and MgADP, or (trace 6) wild-type Fe protein, MgATP, and MgADP.

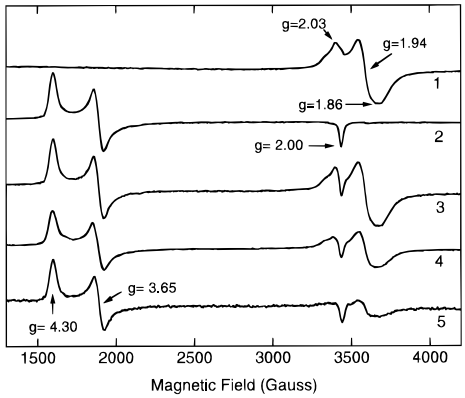

FIGURE 7: EPR spectra of the L127Δ Fe protein−MoFe protein complex over time. EPR spectrum of the L127Δ Fe protein (trace 1) and the MoFe protein (trace 2), the mathematically additive spectrum of traces 1 and 2 (trace 3), and the EPR spectrum of a mixture of L127Δ Fe protein and wild-type MoFe protein 30 s (trace 4) and 1 h (trace 5) after mixing. In all cases, the Fe protein concentration was 120 μM and the MoFe protein concentration was 80 μM. All EPR spectra were recorded at 12 K, with a 9.64 GHz microwave frequency, a 1 mW microwave power, a 100 MHz modulation frequency, and a modulation amplitude of 5.02 G. Unless otherwise noted, *g* values apply to all traces.

s^{-1}. The rate of electron transfer by the L127Δ Fe protein was only partially reduced in the presence of ADP (k_2 = 0.18 s^{-1}) or MgADP (k_2 = 0.16 s^{-1}), while wild-type Fe protein electron transfer was fully inhibited by MgADP.

EPR Spectroscopy. To confirm that the changes in the absorption spectrum that occurred on formation of the L127Δ Fe protein−MoFe protein complex were the result of the one-electron oxidation of the [4Fe-4S] cluster of the Fe protein, EPR spectra of the complex were taken at various times after complex formation. The reduced L127Δ Fe protein ($[4Fe\text{-}4S]^{1+}$ cluster) demonstrated an axial EPR spectrum centered at g = 1.94 (Figure 7, trace 1), while the one-electron oxidized Fe protein ($[4Fe\text{-}4S]^{2+}$ cluster) is EPR silent. The EPR spectrum of reduced MoFe protein demonstrates low-field EPR signals (g = 4.3 and 3.65) and a sharp signal at g = 2.0 (Figure 7, trace 2). These two spectra were added mathematically to give a spectrum that would be predicted for the mixture of the two proteins (trace 3). EPR spectra were recorded for the L127Δ Fe protein−MoFe

protein complex 30 s and 1 h after mixing (Figure 7, traces 4 and 5). As can be seen, after 30 s, an EPR spectrum was recorded which was nearly identical in line shape to the mathematically added spectrum of the L127Δ Fe protein plus MoFe protein, but with a slightly reduced intensity of the $g = 1.94$ signal. After incubation for 1 h, the $g = 1.94$ portion of the EPR spectrum was essentially gone, consistent with the one-electron oxidation of the Fe protein [4Fe-4S] cluster to the EPR silent 2+ oxidation state. The 1 h spectrum looked essentially identical to the MoFe protein spectrum in the absence of Fe protein. In control incubations, when the wild-type Fe protein was mixed with MoFe protein in the absence of MgATP, no changes in the EPR spectrum were observed after 1 h of incubation. Likewise, when either the L127Δ Fe protein alone or the MoFe protein alone was incubated for 1 h, no changes in the EPR line shape or intensity were observed. The inclusion of bovine serum albumin in the control incubation reactions had no effect on the EPR spectra. These results confirm the stopped-flow spectroscopic results indicating that the L127Δ Fe protein is oxidized when incubated with the MoFe protein.

DISCUSSION

Elucidating the function of MgATP binding to the Fe protein and MgATP hydrolysis by the Fe protein−MoFe protein complex is essential to defining a detailed mechanism for nitrogenase substrate reduction reactions. The results of the present study provide insights into both the role that MgATP binding to the Fe protein plays in complex formation with MoFe protein and the role that MgATP hydrolysis plays in intercomponent electron transfer.

MgATP Conformational Change. MgATP binding to the nitrogenase Fe protein has been shown by several techniques to induce protein conformational changes which are reflected as changes in the spectroscopic (Zumft et al., 1974; Lindahl et al., 1987; Chen et al., 1994; Lanzilotta et al., 1995a; Ryle et al., 1996) and redox properties of the Fe protein [4Fe-4S] cluster (Zumft et al., 1974; Watt et al., 1986). While it has been suggested that these changes in the properties of the [4Fe-4S] cluster of the Fe protein were necessary for Fe protein docking to the MoFe protein, the need for only these conformational changes and not MgATP hydrolysis has not been possible to test. Our recent characterization of an altered Fe protein (L127Δ) which appears to be in a protein conformation resembling the MgATP-bound state in the absence of any bound nucleotides (Ryle & Seefeldt, 1996) provided a unique opportunity to address the role of MgATP-induced Fe protein conformational changes in interaction with the MoFe protein. The data presented in this work demonstrating that the L127Δ Fe protein forms a tight complex with the MoFe protein clearly suggest that the MgATP-bound conformation of the Fe protein is sufficient to promote docking with the MoFe protein. The observation that the L127Δ Fe protein forms a tight complex with the MoFe protein with an affinity at least 300 times tighter than that of the wild-type *A. vinelandii* Fe protein−MoFe protein complex further suggests that a possible function for MgATP hydrolysis is to promote the release of the Fe protein from the MoFe protein (Howard & Rees, 1994). The observed stoichiometry of binding of approximately two L127Δ Fe proteins bound to each MoFe protein in the tight complex is consistent with stoichiometries suggested from both kinetic studies and models prepared from the known X-ray structures for both the Fe protein (Georgiadis et al., 1992) and the MoFe protein (Kim & Rees, 1992).

Tight Fe protein−MoFe protein complexes have previously been observed for a few cases where nitrogenase components from different bacterial sources were mixed (Emerich & Burris, 1976; Smith et al., 1976). For example, a mixture of the Fe protein from *Clostridium pasteurianum* and the MoFe protein from *A. vinelandii* was found to form a tight protein complex (Emerich & Burris, 1976). This complex could be isolated by gel filtration chromatography and was found to contain two Fe proteins bound to one MoFe protein. The affinity of the heterologous complex was about 20 times tighter than that of the homologous complex of the *A. vinelandii* nitrogenase proteins (Emerich & Burris, 1976). Interestingly, the heterologous complex was formed both in the absence and in the presence of nucleotides as was found for the homologous tight complex described in the present work. Earlier work has shown that the wild-type Fe protein can associate with the MoFe protein for chemical cross-linking even in the absence of nucleotides (Willing et al., 1989). Recently, it has been reported that the addition of aluminum fluoride and MgADP to *A. vinelandii* Fe protein and MoFe protein results in a tight protein complex (Duyvis et al., 1996). It was suggested that AlF_4^- and MgADP bind to the Fe protein, forming a transition state conformation, which allows the Fe protein to form a tight complex with the MoFe protein.

Several suggestions have been made to explain the heterologous tight nitrogenase complex (Jacobson et al., 1990; Peters et al., 1994). Amino acid residues near the C-terminus of the *C. pasteurianum* Fe protein were suggested to contribute to the tight complex formation (Sundaresan & Ausubel, 1981; Chen et al., 1986). Altering the C-terminal region of the *A. vinelandii* Fe protein to resemble that of the *C. pasteurianum* Fe protein did not, however, result in a tight protein complex with the MoFe protein, suggesting that other regions of the protein were involved (Jacobson et al., 1990). Another region of the Fe protein that is involved in docking to the MoFe protein (Fe protein amino acid residues 59−67) was also altered by site-directed mutagenesis to resemble the corresponding region of the *C. pasteurianum* Fe protein (Peters et al., 1994). While the altered Fe protein was found to have a 2-fold-increased affinity for binding to the MoFe protein, a tight protein complex was not observed. These results suggest that regions of the Fe protein other than the two regions previously examined must be involved in tight complex formation. Unlike the homologous tight protein complex described in the present work, which showed no detectable MgATP hydrolysis, the heterologous *C. pasteurianum* Fe protein−*A. vinelandii* MoFe protein tight complex was found to catalyze MgATP hydrolysis at a low rate, suggesting in this case that the MgATP being hydrolyzed is not functioning in promoting component release. Thus, it would seem that the factors that contribute to our homologous tight complex are different from those which result in the previous heterologous tight complex. The principal difference between these two tight complexes may be that the L127Δ Fe protein cannot achieve the MgADP-bound state, which has been suggested to be required for component release (Howard & Rees, 1994).

Electron Transfer without MgATP Hydrolysis. Previous studies have shown that MgATP hydrolysis is absolutely required for all substrate reduction reactions catalyzed by

nitrogenase with a minimum of two MgATP molecules hydrolyzed for each electron transferred from the Fe protein to the MoFe protein. The hydrolysis of MgATP does not necessarily lead to a single subsequent event, and its role in nitrogenase catalysis is by no means clear. Pre-steady state studies with stopped-flow spectrophotometry using pH indicators to measure proton production (Mensink et al., 1992; Duyvis et al., 1994) and stopped-flow calorimetry to measure heat changes that occur during turnover (Thorneley et al., 1989) have provided contradictory data on the order of MgATP hydrolysis and electron transfer. This conflict likely results from a combination of the different experimental conditions employed and the difficulty in unambiguously assigning heat and pH changes to a particular step in the turnover reaction. Consistent with the results of the pH study, recent results obtained from a real-time assay system, using a fluorescent probe to monitor phosphate release from nitrogenase, indicate that electron transfer occurs prior to the hydrolysis of MgATP (Lowe et al., 1995). The MgATP-independent electron transfer observed in the present work from the L127Δ Fe protein to the MoFe protein provides evidence that at least one electron can be transferred from the Fe protein to the MoFe protein without the need for MgATP hydrolysis. However, the results do not provide data about the temporal relationship of electron transfer and MgATP hydrolysis during substrate reduction. It should be noted that in the present case only a single electron is transferred from the Fe protein to the MoFe protein and that no substrate reduction has been observed. The absence of substrate reduction is probably due to the formation of the tight protein complex, which would block subsequent binding of reduced Fe proteins for a second electron transfer to the MoFe protein.

The results presented here suggest that one important function of MgATP binding to the Fe protein is to lower the redox potential of the [4Fe-4S] cluster, providing more favorable thermodynamics for electron transfer to the MoFe protein. It is worth noting, however, that MgADP also results in a lowering of the redox potential of the Fe protein but does not stimulate electron transfer (Zumft et al., 1974). This observation clearly suggests that the function of MgATP binding to the Fe protein is more complex than a simple lowering of the redox potential. The EPR spectrum of the L127Δ Fe protein−MoFe protein complex following electron transfer shows only a change in the Fe protein EPR spectrum. No changes in the FeMoco EPR signal were observed. This suggests that the first electron transferred from the Fe protein to the MoFe protein might go to the 8Fe cluster (Orme-Johnson et al., 1972; Smith et al., 1973; Fisher et al., 1991). Several studies have suggested that the 8Fe (or P) cluster of the MoFe protein receives up to two electrons from the Fe protein and probably mediates the transfer of these electrons to the FeMoco for substrate reduction (Lowe et al., 1993; Peters et al., 1995). Our current results would be consistent with the first electron transferred from the Fe protein going to the 8Fe cluster of the MoFe protein. One problem with this model is that all of the iron atoms of the 8Fe cluster in the dithionite-reduced state are in the 2+ state, suggesting that electrons tranferred to the 8Fe cluster must be going somewhere other than on the Fe atoms (Smith & Lang, 1974; Münck et al., 1975; Smith, 1983). Thus, it has been suggested that reduction of the 8Fe cluster results in the reduction of the proposed corner-to-corner disulfide bridge between the two subclusters (Kim & Rees, 1994). It should be noted that in the present work no additional EPR signals were observed for the one-electron reduced MoFe protein, suggesting that the electron was delocalized over the cluster or that a possible sulfur radical was spin coupled to another paramagnetic center. Mössbauer spectroscopy of the one-electron reduced MoFe protein should now prove useful in determining the site of the first electron transferred to the MoFe protein.

MgATP Hydrolysis. What then is the role of MgATP hydrolysis in the nitrogenase mechanism? One model (Lowe et al., 1995) has suggested that the MgATP-bound conformation of the Fe protein is the only requirement for electron transfer and that MgATP hydrolysis is coupled to some other energy transfer event. The results of the present study are consistent with such a model, demonstrating that MgATP hydrolysis is not required for transfer of a single electron. It is noteworthy, however, that the MgATP-independent electron transfer observed here is slower than that observed for wild-type Fe protein under turnover conditions. It is possible that the slower rate of electron transfer is a consequence of a change in the protein−protein association rate or that MgATP hydrolysis functions to accelerate the rate of electron transfer from the Fe protein to the MoFe protein. As mentioned earlier, the observation that an Fe protein that is locked into a MgATP-bound conformation that forms a tight complex with the MoFe protein suggests that one significant function of MgATP hydrolysis may be in dissociation of the Fe protein from the MoFe protein (Howard & Rees, 1994). Another possible role for MgATP hydrolysis comes from the EPR results presented here which suggested that the first electron transferred to the MoFe protein did not go to FeMoco. This might suggest that MgATP hydrolysis functions to promote electron transfer from the 8Fe cluster to FeMoco for substrate reduction. The present results would support an earlier "gating" model proposed for MgATP hydrolysis in the nitrogenase mechanism (Howard & Rees, 1994).

In summary, the results presented in this work have (i) characterized a homologous tight Fe protein−MoFe protein complex using an Fe protein engineered into a MgATP-like protein conformation and (ii) demonstrated, for the first time, primary electron transfer between the Fe protein and the MoFe protein in the absence of MgATP hydrolysis. These results now provide the opportunity to dissect the initial steps of intramolecular electron transfer within the MoFe protein. The tight complex characterized here should also allow the opportunity to gain structural insights into the nitrogenase complex by X-ray crystallography.

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