Evidence for Electron Transfer-dependent Formation of a Nitrogenase Iron Protein-Molybdenum-Iron Protein Tight Complex

THE ROLE OF ASPARTATE 39*

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Nitrogenase-catalyzed substrate reduction reactions require the association of the iron (Fe) protein and the molybdenum-iron (MoFe) protein, electron transfer from the Fe protein to the MoFe protein coupled to the hydrolysis of MgATP, followed by protein-protein complex dissociation. This work examines the role of MgATP hydrolysis and electron transfer in the dissociation of the Fe protein-MoFe protein complex. Alteration of aspartate 39 to asparagine (D39N) in the nucleotide binding site of Azotobacter vinelandii Fe protein by site-directed mutagenesis resulted in an Fe protein-MoFe protein complex that did not dissociate after electron transfer. While the D39N Fe protein-MoFe protein complex was inactive in all substrate reduction reactions, the complex catalyzed both reductant-dependent and reductant-independent MgATP hydrolysis. Once docked to the MoFe protein, the D39N Fe protein was found to transfer one electron to the MoFe protein requiring MgATP hydrolysis, with an apparent first order rate constant of 0.02 s\(^{-1}\) compared with 140 s\(^{-1}\) for the wild-type Fe protein. Only following electron transfer to the MoFe protein did the D39N Fe protein form a tight complex with the MoFe protein, with no detectable dissociation rate. This was in contrast with the dissociation rate constant of the wild-type Fe protein from the MoFe protein following electron transfer of 5 s\(^{-1}\). Chemically oxidized D39N Fe protein with MgADP-bound did not form a tight complex with the MoFe protein, showing a dissociation rate similar to chemically oxidized wild-type Fe protein (3 s\(^{-1}\) for D39N Fe protein and 6 s\(^{-1}\) for wild-type Fe protein). These results suggest that electron transfer from the Fe protein to the MoFe protein within the protein-protein complex normally induces conformational changes which increase the affinity of the Fe protein for the MoFe protein. A model is presented in which Asp-39 participates in a nucleotide signal transduction pathway involved in component protein-protein dissociation.

Nitrogenase-catalyzed substrate reduction involves a complex series of reactions including the association of the two nitrogenase metalloproteins, MgATP hydrolysis coupled to interprotein electron transfer, followed by the dissociation of the two metalloproteins (1–3). The two metalloproteins that constitute nitrogenase are the iron (Fe)\(^{1}\) protein, which contains two nucleotide binding sites and a [4Fe-4S] cluster bridging the two identical subunits (4) and the molybdenum-iron (MoFe) protein, an \(\alpha_2\beta_2\) tetramer with two unique [8Fe-(7–8)S] clusters (P-clusters) and two molybdenum-iron-sulfur-homocitrate clusters (FeMoco) (5, 6). The generally accepted mechanism for nitrogenase-catalyzed reduction reactions is that two MgATP molecules bind to the Fe protein, priming this protein for docking to the MoFe protein. After complex formation, a single electron is transferred from the Fe protein [4Fe-4S] cluster to a MoFe protein FeMoco, with a P-cluster as a probable intermediate. Two molecules of MgATP bound to the Fe protein are hydrolyzed to two MgADP molecules and two Pi molecules in a reaction that appears to be coupled to electron transfer (7–9). Following electron transfer, the oxidized Fe protein, with two bound MgADP molecules, dissociates from the one-electron reduced MoFe protein in what is considered to be the reaction rate-limiting step (10, 11). The oxidized Fe protein is reduced by low potential electron transfer proteins (e.g. flavodoxin or ferredoxin), and the two MgADP molecules are replaced with two MgATP molecules, preparing the Fe protein for a second round of docking to the MoFe protein and electron transfer. This cycle is repeated until sufficient electrons have accumulated to reduce the substrate bound to FeMoco (12, 13).

MgATP appears to serve at least three important functions in the proposed reaction mechanism, namely (i) MgATP binding induces protein conformational changes in the Fe protein necessary for docking to the MoFe protein (14); (ii) MgATP hydrolysis is coupled to intercomponent electron transfer; and (iii) MgATP hydrolysis is coupled to the dissociation of the Fe protein from the MoFe protein (15). One MgATP molecule binds to each subunit of the Fe protein, about 19 Å away from the bridging [4Fe-4S] cluster and MoFe protein docking interface (4). MgATP binding to the Fe protein is known to result in changes in the properties of the [4Fe-4S] cluster, suggesting long range communication through protein conformational changes. Nucleotide-induced changes in the properties of the Fe protein [4Fe-4S] cluster have been monitored as changes in EPR (16), circular dichroism (CD) (17, 18) and \(^{1}H\) NMR (19, 20) spectra, along with a −120 mV shift in the redox potential of the [4Fe-4S]\(^{2+/3+}\) couple (21, 22). At least one signal transduction pathway from the nucleotide binding site to the [4Fe-4S] cluster was proposed (23).

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cluster has recently been identified as the protein chain from Asp-125 to Cys-132, designated as switch II (14). It appears that MgATP binding induces changes in the position of this switch, resulting in changes in the protein environment around the [4Fe-4S] cluster. Thus, this has been suggested to ready the Fe protein for interaction with the MoFe protein (14). Upon hydrolysis of MgATP to MgADP and P\textsubscript{i}, the Fe protein is proposed to undergo additional protein conformational changes (2). This is deduced from the observation that the MgADP-bound state of the Fe protein exhibits distinct spectroscopic properties for the [4Fe-4S] cluster compared with the MgATP-bound state (15, 20, 23). Thus, it has been suggested that the conformational changes induced within the Fe protein in going from the MgATP-bound state to the MgADP-bound state represent a molecular switch, which could be coupled to electron transfer and dissociation of the protein-protein complex (2). The status of the nucleotide bound to the protein would have to be communicated to the [4Fe-4S] cluster and the MoFe docking interface approximately 19 Å away. Switch II participates in this communication event, both in changes to the cluster and in defining the affinity for association with the MoFe protein (15).

Related to these issues is the important observation that the MgADP state of the Fe protein exhibits distinct spectroscopic properties for the [4Fe-4S] cluster compared with the MgATP-bound state (18, 20, 23). Thus, it has been suggested that the conformational changes induced within the Fe protein in going from the MgATP-bound state to the MgADP-bound state represent a molecular switch, which could be coupled to electron transfer and dissociation of the protein-protein complex (2). The status of the nucleotide bound to the protein would have to be communicated to the [4Fe-4S] cluster and the MoFe docking interface approximately 19 Å away. Switch II participates in this communication event, both in changes to the cluster and in defining the affinity for association with the MoFe protein (15).

Recently, Asp-129 on the switch II pathway has been suggested to be a possible general base that could activate a water molecule for nucleophilic attack on the γ-phosphate of MgATP, and (ii) Asp-39 could function in switch I nucleotide signal transduction to the MoFe protein docking interface. To probe these possible functions of Asp-39, we have changed this residue to the amino acids asparagine (D39N) and glutamic acid (D39E) and characterized the properties of the purified, altered Fe proteins. The D39N altered Fe protein was still active in MgATP hydrolytic activity when combined with the MoFe protein, ruling out an essential role for Asp-39 as a general base in the MgATP hydrolysis mechanism. The D39N Fe protein was able to transfer a single electron to the MoFe protein. However, following electron transfer, the D39N Fe protein did not dissociate from the MoFe protein, resulting in a tight protein-protein complex. These results suggest that Asp-39 participates in the signal for component protein dissociation following electron transfer and that electron transfer induces protein conformational changes within the nitrogenase protein-protein complex.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis, Expression, and Purification of Altered and Wild-type Fe Proteins—Site-directed mutagenesis of the nitrogenase Fe protein gene from Azotobacter vinelandii, nif H, was carried out as described previously (28, 29). Expression of the altered Fe proteins in A. vinelandii and their purification were performed essentially as described previously (29), except that 20% glycerol (v/v) was included in buffers used for purification of altered Fe proteins. Prior to freezing the proteins in liquid nitrogen, the glycerol concentration was decreased to 5% by dilution with 50 mM Tris buffer, pH 8.0. Protein concentrations were determined by a modified biuret method (30), with bovine serum albumin as the standard. SDS-polyacrylamide gel electrophoresis with Coomassie staining (31) was used to confirm that all proteins were homogeneous. Protein manipulations for CD spectroscopy were performed in an oxygen-free atmosphere glove box, with an oxygen concentration of less than 1 part per million. Stopped-flow spectroscopic studies were done in a nitrogen-filled glove box. Unless noted, all buffers were saturated with argon and contained 2 mM sodium dithionite (Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}).

Activity Assays, Nucleotide Binding, and MgATP Hydrolysis—Acetylene reduction assays were performed as described previously (32) under argon in 100 mM MOPS buffer, pH 7.0, with 10 mM sodium dithionite as the reductant. The rates of MgATP hydrolysis were determined in assay solutions without the MgATP-regenerating system as described previously (29). The stoichiometry of MgATP molecules hydrolyzed per electron transferred was determined as described previously (29). The binding of MgATP and MgADP to the wild-type, D39E, and D39N Fe proteins were performed by an equilibrium column binding technique as described earlier (33).

Binding of the Asp-39 Altered Fe Proteins to the MoFe Protein—The ability of the D39N or D39E Fe proteins to bind to the MoFe protein was tested by a competition assay described earlier (24). Increasing quantities of the altered Fe protein (from 0 to 230 μg) were added to fixed concentrations of wild-type Fe protein (115 μg or 1.8 nmol) and MoFe protein (216 μg or 0.9 nmol). If the altered Fe protein binds to the MoFe protein, then a decrease in the rate of acetylene reduction should be observed. The percent of acetylene reduction activity remaining was plotted versus the ratio of altered Fe protein to wild-type Fe protein, and the data were fitted to the Hill equation.

UV-visible Absorption Spectra of Wild-type Fe Protein and Altered Fe Proteins—Absorption spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer in 2.0-ml quartz cuvettes fitted with serum stoppers. Anaerobicity was obtained by purging the cuvettes with O\textsubscript{2}-free argon for 6 min. The buffer used for these experiments was 50 mM MOPS, pH 7.0, unless otherwise noted. A MgATP-regenerating system was included during nitrogenase catalysis to prevent the formation of MgADP, which is a known inhibitor of MgATP binding to the Fe protein (34).

Stopped-flow Spectrophotometry—Absorbance changes were monitored...
tored with a Hi-Tech SP61 stopped-flow spectrophotometer equipped with a data acquisition and curve fitting system (Salisbury, Wilts, UK) as described previously (15). Electron transfer rates, and the rates of reduction of the Fe protein by sodium dithionite, were determined as described previously (15). The rate of dissociation of the chemically oxidized Fe protein from the MoFe protein (15) and the dissociation rate of the turnover-oxidized Fe protein from the MoFe protein (11) were also determined. All protein concentrations are reported in the appropriate figure legend.

**Circular Dichroism Spectra of Wild-type and Asp-39 Altered Fe Proteins—**Fe protein samples (20 mg or 312 nmol) containing 2 mol dithio- dinitrobenzene were dissolved in a passage through a Sephadex G-25 column (0.5 × 10 cm) equilibrated with 100 mM Tris buffer, pH 8.0, without dithionite. The Fe protein was then oxidized by the addition of 25 μl of a 20 mM indigo disulfonate (IDS) solution. The IDS was separated from the Fe protein on a Dowex 1 column (0.5 × 5 cm) equilibrated with 100 mM Tris buffer, pH 8.0, without dithionite. The oxidized Fe protein, free of small molecules, was then split into two equal aliquots and placed into two 4-ml sealed quartz cuvettes. The total volume of each cuvette was then brought to 2 ml by the addition of 100 mM Tris buffer, pH 8.0. Full absorption spectra were recorded to confirm the oxidation state of the Fe protein and to determine the protein concentration. CD spectra were then recorded on an Aviv model 62DS spectropolarimeter and were base-line subtracted (18).

**Proton NMR Spectra of Isotopically Shifted Protons for Fe Proteins—**Proton NMR spectra of reduced Fe proteins were recorded as described previously (20). All spectra were recorded at 305 K in anaerobic glass NMR tubes fitted with degassed Teflon stoppers (Wilmad, Buena, NJ). Fe protein samples were exchanged into Chelex 100-treated Tris-buffered D2O solution by passage through a Sephadex G-25 column (20). The reduced Fe protein samples were concentrated to between 0.85 and 1.1 mg using a Centricon-30 concentrator (Amicon, Beverly, MA).

**Electron Transfer Monitored by EPR Spectroscopy—**Electron transfer from the Fe protein to the MoFe protein was monitored by following the disappearance of the EPR signal of the Fe protein in going from the [4Fe-4S]1+ to the [4Fe-4S]3+ oxidation states. Samples were prepared essentially as described previously (15). Wild-type or D39N Fe protein (60 μg) was mixed with MoFe protein (112 μg), MgATP (1 mM), and a MgATP-regenerating system and allowed to react for 1 h. Samples were removed before the addition of MgATP and following the 1-h incubation and frozen in EPR tubes. All EPR spectra were recorded on a Bruker ESP300E spectrometer equipped with a dual mode cavity and an Oxford liquid helium cryostat. A total of 10 scans were added for each spectrum, and all other parameters were as described (15).

**RESULTS**

**Alteration of Asp-39 of the Fe Protein—**Changing Asp-39 by site-directed mutagenesis to the amino acids glutamic acid (D39E) and asparagine (D39N) provided a useful way to probe the function of this amino acid. Changing aspartic acid to glutamic acid represents a conservative amino acid change while maintaining the carboxylate side chain. Changing Asp to Asn would remove the negatively charged carboxylate side chain and is predicted to be a conservative structural change (35). The altered Fe proteins (D39E and D39N) were expressed in A. vinelandii cells in place of the wild-type Fe protein. This was accomplished by replacing the chromosomal wild-type Fe protein gene, nif H, with the mutated gene by a homologous recombination procedure (28). The A. vinelandii cells expressing the altered Fe protein were tested for their ability to grow under nitrogen-fixing conditions in order to analyze the functional consequences of the amino acid changes. A. vinelandii cells expressing either the D39E or D39N altered Fe proteins were unable to grow under nitrogen-fixing conditions, revealing that changing Asp-39 to Glu or Asn renders the Fe protein inactive in nitrogen fixation. It was of interest to investigate the properties of the purified, altered Fe proteins to further define the functional consequences of the amino acid change and thus the function of Asp-39. The D39E and D39N Fe proteins were overexpressed in A. vinelandii cells using a derepression protocol previously described (29). The D39N Fe protein could be purified with yields similar to the wild-type Fe protein, whereas the D39E Fe protein was purified with slightly lower yields.

**Substrate Reduction and MgATP Hydrolysis—**Given the observation that the D39N and D39E Fe proteins were inactive in nitrogen fixation in whole cells, it was important to ascertain any activities of the purified Fe proteins when combined with purified MoFe protein. Three activities were monitored, namely the two-electron reduction of acetylene to ethylene, the two-electron reduction of protons to H2, and the hydrolysis of MgATP. The last is particularly important to determine because the wild-type Fe protein will hydrolyze MgATP to MgADP at a considerable rate when combined with the MoFe protein in the absence of reductant, in a reaction termed reductant-independent MgATP hydrolysis (36–38). Neither the D39N nor the D39E Fe proteins were found to support acetylene or proton reduction activities when combined with MoFe protein. Interestingly, the D39N Fe protein did show a significant MgATP hydrolysis activity (53 nmol of MgADP/min for mg of Fe protein) when combined with MoFe protein in the presence of reductant. The rate was much lower than that catalyzed by the wild-type nitrogenase complex (8772 nmol of MgADP/min for mg Fe protein) but significantly above background rates determined for each protein alone at 1.0 nmol of MgADP/min for mg of Fe protein. The D39N Fe protein also catalyzed reductant-independent MgATP hydrolysis (5 nmol of MgADP formed/min for mg Fe protein) when combined with the MoFe protein, which was catalyzed by the wild-type rate of 817 nmol of MgADP formed/min for mg of Fe protein. For both the wild-type and D39N Fe proteins, the reductant-independent rates of MgATP hydrolysis were about 10% the reductant-dependent rates, consistent with previously published results for wild-type nitrogenase (36–38). The MgATP hydrolysis catalyzed by the D39N Fe protein-MoFe protein complex clearly indicates that Asp-39 is not absolutely required in the mechanism of MgATP hydrolysis. In addition, the requirement for MoFe protein suggests that both the oxidized and reduced states of the D39N Fe protein can dock with the MoFe protein, forming a complex capable of MgATP hydrolysis, even in the absence of substrate reduction.

**Nucleotide Binding—**Given the location of Asp-39 in the Fe protein near the probable binding site of the phosphate portion of a bound nucleotide, it was important to determine how changing Asp-39 affected the affinity of the Fe protein for binding nucleotides. Both the stoichiometry of binding and the affinity for binding MgATP or MgADP to the Fe protein can be determined by an equilibrium column binding procedure (33). The reduced states of the D39N and D39E Fe proteins were found to bind a maximum of 2 nucleotides, with apparent dissociation constants (Kd) similar to those found for the wild-type Fe protein (Table I). The D39E Fe protein bound both MgATP and MgADP with a slightly lower affinity compared to MgATP.
The addition of 115 Fe protein were also added to each vial. The reaction was initiated by the Hill equation, where the cooperativity factor (two D39NF e protein to two wild-type F e protein) was also added to each vial. The reaction was initiated by the Hill equation, where the cooperativity factor (two D39NF e protein to two wild-type F e protein). The maximum uninhibited activity of the maximum acetylene reduction activity (nmol of ethylene produced per min) was plotted against the ratio of altered Fe protein (D39N or D39E) to MoF e protein. The maximum uninhibited activity of wild-type Fe protein was 1872 nmol of ethylene formed per min (D39N or D39E). The data for the D39N Fe protein were fitted to the inverse Hill equation, for the cooperativity factor (n) was found to be 2.1. The relative ratios of the component proteins at 50% inhibition were two D39N Fe proteins to two wild-type Fe proteins to one MoF e protein.

with wild-type Fe protein. In contrast, the D39N Fe protein bound MgATP with a slightly greater affinity and MgADP with a slightly lower affinity when compared with wild-type Fe protein. These results clearly show that both the D39N and D39E Fe proteins bind 2 nucleotides with similar affinity when compared with the wild-type Fe protein and thus that Asp-39 is not essential to nucleotide binding to the Fe protein. In addition, both the D39N and D39E Fe proteins demonstrated strong positive cooperativity in binding both MgATP and MgADP, consistent with the results for the wild-type Fe protein, indicating that Asp-39 does not participate in this cooperative mechanism.

Affinity of Fe Protein for Binding MoFe Protein—The MoFe protein-dependent MgATP hydrolysis activity of the D39N Fe protein suggested that this altered protein could bind to the MoFe protein. To probe this possible interaction, the Asp-39 altered Fe proteins were used in a competitive binding activity assay (24). In this assay, the inactive altered Fe protein is titrated against wild-type Fe and MoFe proteins to determine competition for binding of Fe proteins to the MoFe protein. The formation of a D39N Fe protein-MoFe protein complex would be inactive and thus would reduce the total substrate reduction activity. Fig. 2 shows that while the D39E Fe protein did not inhibit the wild-type acetylene reduction activity, the D39N Fe protein did show strong inhibition. A fit of the D39N Fe protein inhibition data to the Hill equation revealed that 50% inhibition of activity was achieved at a ratio of two D39N Fe proteins to one MoF e protein. A similar pattern of inhibition has recently been observed for another altered Fe protein (L127Δ), which was shown to form a tight, irreversible complex with the MoFe protein (15). The D39N Fe protein clearly binds to the MoFe protein, possibly forming a tight protein-protein complex.

Electron Transfer—Given the fact that the D39N Fe protein bound to the MoFe protein and still hydrolyzed MgATP, but did not reduced substrates, it was of interest to determine if a single electron could be transferred to the MoFe protein. A simple way to monitor electron transfer from the Fe protein to the MoFe protein is to follow the increase in absorbance in the 430 nm region as a result of the oxidation of the Fe protein [4Fe-4S][MoFe] cluster upon electron transfer (Fig. 3, panel A). Under these conditions, protons are reduced to H2. When mixed with Fe protein, MgATP, and reductant, a rapid increase in absorbance in the 430 nm region of the UV-visible spectrum is observed, corresponding to the oxidation of MoF e protein(MoFe) Cl 1 oxidation state (15). When the wild-type Fe protein is combined with the MoFe protein, MgATP and reductant, a rapid increase in absorbance in the 430 nm region of the UV-visible spectrum is observed, corresponding to the oxidation of MoF e protein(MoFe) Cl 1 oxidation state (15). Under these conditions, protons are reduced to H2. When mixed with MoFe protein, MgATP, and reductant, the D39N Fe protein [4Fe-4S][MoFe] cluster was also oxidized to the 2+ state (panel B). By following this oxidation at 430 nm using a stopped-flow spectrophotometer, apparent first order rate constants for primary electron transfer from the Fe protein to the MoFe protein were determined (15). A rate constant of 140 s−1 was determined for the
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To 900 μl of anaerobic 50 mM MOPS buffer, pH 7.0, in a 2.0-ml quartz cuvette, various amounts of MoFe protein were added: 0 nmol (trace 1), 2.4 nmol (trace 2), 3.6 nmol (trace 3), 4.9 nmol (trace 4), 6.2 nmol (trace 5), 7.3 nmol (trace 6), 9.8 nmol (trace 7), and 10.1 nmol (trace 8) and the spectrophotometer was blanked. D39N Fe protein (17 nmol) was added to each cuvette, and the reaction was initiated by the addition of MgATP to a final concentration of 1 mM. Absorption spectra were recorded until no further change was observed, and the final absorption spectrum is shown for each MoFe protein concentration. Inset, plot of the change in absorbance at 430 nm versus the ratio of MoFe protein to D39N Fe protein.

Additional support for the observation of a single electron transfer from the Fe protein to the MoFe protein was observed, ruling out wild-type Fe protein mediation of electron transfer.

Finally, the transfer of an electron from the D39N Fe protein to the MoFe protein was confirmed by monitoring with EPR spectroscopy (data not shown). In these studies, the reduced D39N or wild-type Fe proteins was mixed with MgATP and the MoFe protein and allowed to react for 1 h before being frozen in EPR tubes. Both the D39N and wild-type Fe proteins showed a MgATP-dependent disappearance of the S = 1/2 EPR signal consistent with the oxidation of the Fe protein (4Fe-4S) cluster from the 1+ to the 2+ oxidation state. There was also a small decrease in the intensity of the S = 3/2 region EPR signal, consistent with the reduction of FeMoco.

Affinity of Fe Protein Binding to the MoFe Protein—To quantify the affinity of binding of oxidized D39N Fe protein to the MoFe protein, stopped-flow spectrophotometry was used to define the dissociation rate constants for Fe protein-MoFe protein complexes. In this experiment, Fe protein is oxidized with a mediator (e.g. indigo disulfonate) prior to the addition of reduced, dithionite-free MoFe protein and MgADP. This solution is rapidly mixed in the stopped-flow apparatus against a solution containing reduced Fe protein (4Fe-4S)1+, dithionite, and MgADP. As the oxidized Fe protein dissociates from the MoFe protein, it is reduced by the dithionite, resulting in a decrease in absorbance at 430 nm. Thus, the decrease in absorbance at 430 nm can be directly correlated with the dissociation of the Fe protein from the MoFe protein (11). The results of such an experiment with IDS-oxidized wild-type or D39N Fe proteins are shown in Fig. 5 (panel A). As can be seen, both the wild-type and D39N Fe proteins showed similar rates of dissociation from the MoFe protein, with apparent first order rate constants of 6 s⁻¹ for the wild-type Fe protein and 3 s⁻¹ for the D39N Fe protein. These experiments were also performed over a range of Fe protein concentrations from 50 to 250 μM as described previously (11).

These results suggest that the IDS-oxidized D39N Fe protein with bound MgADP does not bind to the MoFe protein with a significantly higher affinity than does the wild-type Fe protein. This is in contrast to the suggestion of a tight complex between the oxidized D39N Fe protein and the MoFe protein from the earlier steady-state spectroscopic data. A significant difference between the steady-state data and the stopped-flow spectroscopic data is the method of oxidation of the D39N Fe protein. In the stopped-flow experiment, the Fe proteins were oxidized first to the 2+ state with a dye (i.e. IDS) and then incubated with MgADP and the MoFe protein. In the steady-state experiments, the Fe proteins were oxidized by electron transfer directly to the MoFe protein under turnover conditions. It seemed possible that the electron transfer from the Fe protein to the MoFe protein as part of a complex might result in a tighter protein-protein complex, explaining the different results between the two experiments. To test this possibility, the stopped-flow dissociation experiment was repeated, except that the Fe proteins were oxidized under turnover conditions by incubation with MgATP and the MoFe protein for 30 min prior to rapidly mixing with dithionite and the reduced Fe protein. As can be seen in Fig. 5 (panel B), the wild-type Fe protein showed an apparent first order dissociation rate constant from the MoFe protein of 5 s⁻¹. This is essentially identical to the rate constant observed in the experiment where the Fe protein was IDS-oxidized. In contrast, the D39N Fe protein showed essentially no dissociation from the MoFe protein when turnover oxidized. This contrasts with the rapid dissociation of the D39N Fe protein from the MoFe protein when the D39N Fe protein was oxidized by IDS. It is important to note that in both
cases, the Fe protein is oxidized by one electron to the 2+ state and that MgATP hydrolysis continues. In a control reaction, incubation of the IDS-oxidized D39N Fe protein with MgATP and the MoFe protein did not result in a tight Fe protein-MoFe protein complex, indicating that MgATP hydrolysis was not sufficient to allow D39N Fe protein-MoFe protein tight complex formation. These results provide compelling evidence that the D39N Fe protein forms a tight, essentially irreversible complex with the MoFe protein only following electron transfer to the MoFe protein.

Conformational Changes in the Fe Proteins—To understand why the D39N Fe protein formed a tight complex with the MoFe protein only under conditions of electron transfer and MgATP hydrolysis, it was important to understand how nucleotide-induced conformational changes were affected in the D39N altered Fe protein. A number of spectroscopic techniques have been used to monitor the MgATP- or MgADP-induced protein conformational changes in the environment of the [4Fe-4S] cluster. The visible region CD spectra of the oxidized Fe protein provides the only spectroscopic technique that gives distinct spectra for the three states of the oxidized Fe protein, namely the nucleotide-free, the MgATP-bound, and the MgADP-bound states (17, 18).

Fig. 5. Dissociation of IDS-oxidized and turnover-oxidized Fe proteins from the MoFe protein monitored by stopped-flow spectrophotometry. Reduction of the [4Fe-4S] cluster of oxidized Fe proteins was monitored by changes in absorbance at 430 nm (ΔA$_{430}$) by stopped-flow spectrophotometry. IDS-oxidized Fe proteins were prepared as described under “Experimental Procedures.” Panel A, a solution containing 40 μM IDS-oxidized D39N Fe protein (trace 1) or wild-type Fe protein (trace 2), 40 μM dithionite-free MoFe protein, and 5 mM MgADP was allowed to incubate for 30 min and then mixed with a solution containing 200 μM wild-type Fe protein and 20 mM dithionite. Apparent first order rate constants of 3 s$^{-1}$ and 6 s$^{-1}$ were determined for dissociation of the D39N and wild-type Fe protein from the MoFe protein, respectively. Panel B, turnover-oxidized D39N Fe protein (trace 1) or wild-type Fe protein (trace 2) were prepared by allowing a solution of Fe protein (60 μM), MoFe protein (60 μM), and MgATP (5 mM) to incubate in one drive syringe of the stopped-flow system for 30 min. Dissociation of the turnover-oxidized Fe protein from the MoFe protein was then observed by monitoring the change in absorbance at 430 nm after mixing the turnover-oxidized solution with a solution containing wild-type Fe protein (200 μM) and sodium dithionite (20 mM). A rate constant of 5 s$^{-1}$ for the wild-type Fe protein was determined. No dissociation rate constant could be determined for the D39N Fe protein.

Fig. 6. Circular dichroism (CD) spectra of the wild-type, D39N, and D39E Fe proteins with and without nucleotides. The visible region CD spectra of oxidized Fe proteins were recorded as described under “Experimental Procedures.” CD spectra are shown for the oxidized wild-type, D39E, and D39N Fe proteins in the absence of nucleotides (panel A), in the presence of MgATP (panel B), and in the presence of MgADP (panel C). Fig. 6 shows the CD spectra for the wild-type, D39N, and D39E Fe proteins in the absence of nucleotides (panel A), in the presence of MgATP (panel B), and in the presence of MgADP (panel C). As can be seen, all three proteins showed very similar CD spectra in the absence of nucleotides, indicating that changing Asp-39 to Asn or Glu did not have a significant impact on the [4Fe-4S] cluster environment. Likewise, the addition of MgATP to all three proteins resulted in similar CD spectra (panel B), consistent with previously reported spectra for the MgATP-bound state of wild-type Fe protein (18). This revealed that all three proteins underwent similar MgATP-induced protein conformational changes around the [4Fe-4S] cluster, suggesting that Asp-39 is not critical for communicating the MgATP-induced conformational change to the [4Fe-4S] cluster. In marked contrast, the wild-type and Asp-39 altered Fe proteins showed very different CD spectra when MgADP was added. Notably, the CD spectrum of the D39N Fe protein was most similar to the spectra observed for the Fe protein with MgATP bound. These results indicate that changing Asp-39 to Asn has had a significant impact on the MgADP-induced conformational changes communicated to the [4Fe-4S] cluster.

As another, more global way to monitor the nucleotide-induced conformational changes in the Fe protein, the proton NMR spectra for the wild-type and Asp-39 altered Fe proteins were compared (Fig. 7). The downfield portion of the proton NMR spectrum of the reduced Fe protein contains resonances from eight β-CH$_2$ and four α-CH protons of the four cysteinylligands to the [4Fe-4S] cluster, which appear as four isotropically shifted peaks between 13 and 50 ppm (20). These proton resonances are primarily shifted by through bond interactions with the paramagnetic [4Fe-4S]$^{1+}$ cluster and are thus sensi-
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FIG. 7. $^1$H NMR spectra of the wild-type, D39N, and D39E Fe proteins with and without nucleotides. The paramagnetic region of the 400-MHz proton NMR spectrum for Fe proteins was recorded as described under "Experimental Procedures." Spectra at 305 K are shown for reduced wild-type (WT), D39N, and D39E Fe proteins in the absence of nucleotides (panel A), in the presence of MgATP (panel B), and in the presence of MgADP (panel C).

As mentioned earlier, an important reaction in the catalytic mechanism of nitrogenase is the dissociation of the Fe protein from the MoFe protein following MgATP hydrolysis and electron transfer. This has been suggested to be the overall reaction rate-limiting step. The results from the present study can be discussed in the context of two important questions relating to the signal for dissociation of the nitrogenase proteins. (i) What is the role of MgATP hydrolysis in signaling protein-protein dissociation, and (ii) what is the role of electron transfer from the Fe protein to the MoFe protein in signaling protein-protein dissociation?

**MgATP Hydrolysis**—It is becoming clear that MgATP serves several important functions in the nitrogenase reaction mechanism (2, 3). MgATP binding to the Fe protein clearly induces protein conformational changes that are communicated from the nucleotide binding sites at the center of the protein to the [4Fe-4S] cluster and MoFe protein docking surface approximately 19 Å away (2, 14). These conformational changes dramatically alter both the physicochemical properties of the [4Fe-4S] cluster and the binding equilibrium in favor of the Fe protein-MoFe protein complex (15). In addition to influencing the initial binding of the Fe protein to the MoFe protein, it has been shown that MgATP hydrolysis is somehow coupled to the transfer of an electron from the Fe protein [4Fe-4S] cluster to the MoFe protein (3). This model has been challenged recently by the observation that an altered Fe protein locked in the MgATP-like state (L127Δ) could transfer one electron to the MoFe protein in the absence of MgATP hydrolysis, thus suggesting that only the MgATP-bound protein conformation of the Fe protein was required for electron transfer, not necessarily MgATP hydrolysis (15). It is important to note that the rate of electron transfer from the L127Δ Fe protein was significantly slower than that observed from the wild-type Fe protein with MgATP hydrolysis, suggesting that one possible role for MgATP hydrolysis is to accelerate electron transfer. How then is MgATP hydrolysis initiated? Since the Fe protein alone shows no MgATP hydrolysis activity, it can be concluded that MoFe protein docking to the Fe protein initiates MgATP hydrolysis. Several models have been suggested to explain this MoFe protein dependence of MgATP hydrolysis, the most likely being that residues within the Fe protein nucleotide binding site are moved into position to catalyze the hydrolysis reaction (2, 8). It would seem reasonable that this would occur by long range protein conformational changes from the MoFe protein docking surface back to the nucleotide binding sites. The results of the present work clearly indicate that Asp-39 cannot be an essential residue (e.g. general base) in the MgATP hydrolysis mechanism as changing the Asp to Asn did not stop MgATP hydrolysis activity. The significantly slower rate of MgATP hydrolysis catalyzed by the D39N Fe protein-MoFe protein complex suggests that Asp-39 could participate either directly or indirectly in positioning residues critical for MgATP hydrolysis.

Taken together, these observations suggest that an important function for MgATP hydrolysis in the nitrogenase mechanism might be to induce protein conformational changes that would be coupled to protein-protein dissociation. The results from the present study support the notion (2) that the change in protein conformation in the Fe protein in going from the MgATP-bound state to the MgADP-bound state during MgATP hydrolysis could dramatically alter the interactions (and hence affinity) between the Fe protein and the MoFe protein by altering the position of a series of amino acid residues involved in protein-protein interaction. Altering Asp-39 to Asn resulted in an Fe protein that could not access the MgADP-bound protein conformation as detected by changes in the CD and proton NMR spectra of the [4Fe-4S] cluster. The resulting tight complex between the D39N Fe protein and the MoFe protein could be the result of the lack of the Fe protein attaining the MgADP-bound protein conformation, even though MgATP is hydrolyzed to MgADP as part of the complex. Together with the observa-
tion of a tight complex between the L127Δ modified Fe protein and the MoFe complex, this suggests that the MgADP-bound state of the Fe protein has a significantly reduced affinity for binding to the MoFe protein, essentially functioning as a trigger for protein-protein dissociation. This idea is supported by the recent observation that if AlF₄⁻ was included with nitrogenase under turnover conditions, a tight, protein-protein complex was formed (25, 40). AlF₄⁻ is thought to be a phosphate analog and to bind to the Fe protein nucleotide site as an ADP-AlF₄⁻ complex, preventing the formation of the MgADP-bound state required for complex dissociation. Hence, the Fe protein in the ADP-AlF₄⁻ bound conformation cannot achieve the correct conformation for dissociation (25, 40). A reasonable model would be that one important function of MgATP hydrolysis is as a trigger to alter the Fe protein-MoFe protein equilibrium to favor protein-protein dissociation.

Electron Transfer—Whereas the above model for the function of MgATP hydrolysis in protein-protein dissociation is reasonable, results from the present studies suggest that it is not that simple. Rather, it appears that electron transfer is also somehow coupled to protein-protein dissociation. The results from the present work with the D39N altered Fe protein clearly point to a significant role for electron transfer in defining the affinity for Fe protein binding to the MoFe protein. This comes from the observation that only following electron transfer from the D39N Fe protein to the MoFe protein did a tight, irreversible protein-protein complex form. MgATP hydrolysis to MgADP and P, continued in both the reduced and oxidized D39N Fe protein-MoFe protein complexes, suggesting that MgATP hydrolysis is not a sufficient signal to initiate protein-protein dissociation. Instead, it would appear that electron transfer is linked to the nucleotide-bound status of the Fe protein to trigger dissociation. This suggests a complex mechanism for protein dissociation in which electron transfer induces protein conformational changes within the Fe protein (and possibly the MoFe protein), which are linked to conformational changes induced by MgATP hydrolysis. There is growing precedent for oxidation or reduction (i.e., electron transfer) of redox active proteins resulting in significant protein conformational changes. For example, flavodoxins (41, 42), cytochrome c (43), and the [Fe-S] cluster containing adrenodoxin (44) have all been observed to undergo significant protein conformational changes upon oxidation or reduction. In addition, extended x-ray absorption fine structure data for the nitrogenase Fe protein has revealed a 0.02-Å shortening of the Fe-S bond distance in the [4Fe-4S] cluster upon oxidation from the 1 to the 2+ state (45). This change in the [4Fe-4S] cluster could easily be translated into changes in the protein environment around the cluster, thus affecting the affinity of the Fe protein for the MoFe protein.

So, a reasonable model would suggest that electron transfer from the Fe protein to the MoFe protein results in the oxidation of the Fe protein [4Fe-4S] cluster, inducing protein conformational changes affecting the affinity for MoFe protein docking. This event might also initiate the signal for MgATP hydrolysis, consistent with recent data suggesting that MgATP hydrolysis follows electron transfer (9). Once in the [4Fe-4S] cluster oxidized state, MgATP hydrolysis then initiates the nucleotide-induced protein conformational changes as the protein goes to the MgADP-bound state, which then favor protein-protein dissociation. Asp-39, being located near the MgATP binding site, may initiate a communication pathway that then proceeds to a region of the Fe protein involved in MoFe protein interactions. This communication pathway may be a second such pathway identified in the Fe protein, going from Asp-39 to Val-67. This putative domain in the Fe protein is highly homologous with similar domains in other nucleotide-binding proteins, which have been designated as switch I (26). Thus, as has been suggested for several other nucleotide switch proteins, the nucleotide status of the protein results in different movements in the protein chain switch I, which are communicated over a distance to the binding site for another protein (26). In the Fe protein, it seems reasonable that the MgADP- or MgATP-bound state could be communicated to amino acid residues in the 59 to 67 region critical for docking with the MoFe protein. The 59 to 67 region of the Fe protein has recently been suggested to participate in MoFe protein interactions (27).

In summary, the present work provides evidence that the conserved Asp at position 39 of the nitrogenase Fe protein plays an important role in defining a shift in equilibrium toward Fe protein-MoFe protein dissociation. This could be communicated through a protein domain designated as switch I, which might originate at Asp-39. In addition, evidence was presented suggesting that electron transfer from the Fe protein to the MoFe protein within the complex induces protein conformational changes that are critical in defining the protein-protein binding equilibrium.

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