Electron Transfer from the Nitrogenase Iron Protein to the [8Fe-(7/8)S] Clusters of the Molybdenum−Iron Protein†

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ABSTRACT: The reduction of substrates catalyzed by nitrogenase requires electron transfer between the iron (Fe) protein and the molybdenum−iron (MoFe) protein in a reaction that is coupled to the hydrolysis of MgATP. The [4Fe-4S] cluster of the Fe protein transfers one electron ultimately to the M-clusters (FeMoco) of the MoFe protein for substrate reduction, with the P-clusters ([8Fe-(7/8)S]) of the MoFe protein as proposed electron transfer intermediates. This work presents direct EPR evidence for primary electron transfer from the [4Fe-4S] cluster of the Fe protein to the P-clusters of the MoFe protein in a reaction that requires the MgATP-bound state of the Fe protein. An oxidized state of the MoFe protein was prepared in which the P-clusters were oxidized by 2 equiv of electrons to the P^{2+} state. In this oxidation state, the M-clusters ($S = {}^3/_2$) and the P^{2+}-clusters ($S \geq 3$) are paramagnetic and can be observed by perpendicular and parallel mode EPR, providing the opportunity to follow electron transfer from the Fe protein to either cluster type in the MoFe protein. Electron transfer from the reduced $[4Fe\text{-}4S]^{1+}$ cluster of two different Fe proteins to the P^{2+} clusters of the MoFe protein was observed by the disappearance of the $[4Fe\text{-}4S]^{1+}$ cluster EPR signal and the conversion of the MoFe protein P-clusters from the P^{2+} to the P^{1+} oxidation state. In the first case, stoichiometric quantities of the wild-type Fe protein transferred one electron to the P-clusters only in the presence of MgATP. MgADP would not support this electron transfer reaction. In the second case, an altered Fe protein (L127Δ) that is in a conformation resembling the MgATP-bound state was found to transfer an electron to the P-clusters in the absence of MgATP. These results suggest that the first electron transferred from the Fe protein goes to the P-cluster and that the MgATP-bound protein conformation of the Fe protein, not MgATP hydrolysis, is required for this electron transfer reaction.

Nitrogenase-catalyzed substrate reduction involves a complex series of reactions, including the association of the Fe protein[1] with the MoFe protein and electron transfer from the Fe protein to the MoFe protein coupled to the hydrolysis of MgATP, followed by dissociation of the oxidized Fe protein from the one-electron-reduced MoFe protein (Mortenson et al., 1993; Howard & Rees, 1994; Peters et al., 1995a). This cycle is repeated until enough electrons have accumulated in the MoFe protein for substrate reduction (Hageman & Burris, 1978). Electron transfer reactions in nitrogenase appear to involve three different mixed metal clusters (Dean et al., 1993). The $[4Fe\text{-}4S]^{1+}$ cluster of the Fe protein transfers a single electron to the MoFe protein in a reaction that is coupled to the hydrolysis of MgATP to MgADP (Mortenson et al., 1993). The electrons transferred to the MoFe protein ultimately end up on a molybdenum−iron−sulfur−homocitrate cofactor (FeMoco or M-cluster), which appears to be the site of substrate binding and reduction (Shah & Brill, 1977; Hawkes et al., 1984). The [8Fe-(7/8)S] or P-clusters of the MoFe protein have been suggested to be electron transfer intermediates between the [4Fe-4S] cluster of the Fe protein and the M-clusters of the MoFe protein (Lowe et al., 1993; Peters et al., 1995b; Ma et al., 1996), although conclusive evidence for a role of the P-clusters in electron transfer is lacking.

The suggestion that the P-clusters function as electron transfer mediators in nitrogenase charge transfer reactions has evolved from several observations. Models for the docking site of the Fe protein to the MoFe protein place the P-clusters between the Fe protein [4Fe-4S] cluster and the M-cluster within the MoFe protein, with an edge-to-edge distance of 15 Å between the [4Fe-4S] cluster and the P-cluster (Kim & Rees, 1992a). In addition to this structural evidence, recent studies with altered MoFe proteins also suggest that the P-clusters act as primary electron acceptors from the Fe protein. In one study, a catalytically inactive form of the MoFe protein was created by substitution of the M-clusters with a modified FeMoco derivative (Ma et al., 1996). The modified MoFe protein was inactive in substrate reduction but was found to accept electrons from the Fe protein. Following electron transfer from the Fe protein, an increase in the intensity of an EPR signal assigned to the P-clusters was observed. This $g = 2$ region EPR signal integrated to one spin per P-cluster, suggesting that the electron transferred from the Fe protein went to the P-clusters (Ma et al., 1996). In a separate study, amino acids within the MoFe protein located between the P-clusters and the

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[1] Abbreviations: Fe protein, iron protein of nitrogenase; MoFe protein, molybdenum−iron protein of nitrogenase; L127Δ Fe protein, Fe protein with Leu 127 deleted; EPR, electron paramagnetic resonance; P-cluster, [8Fe-(7/8)S] cluster of nitrogenase; M-cluster, iron−molybdenum cofactor of nitrogenase; IDS, indigo disulfonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; E_m, midpoint potential.

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M-clusters were altered by site-directed mutagenesis to test the possible role of these residues in electron transfer from the P-clusters to the M-clusters (Peters et al., 1995b). Stopped-flow spectroscopic results indicated that electrons transferred from the Fe protein accumulated on the P-clusters before proceeding to the M-clusters. It was suggested that an electron transfer pathway from the P-clusters to the M-clusters had been disrupted and that the P-clusters were accumulating electrons transferred from the Fe protein (Peters et al., 1995b). Finally, stopped-flow and EPR spectroscopic results for nitrogenase during the reduction of dinitrogen revealed that the P-clusters were oxidized following the reduction of bound nitrogen with 4 equiv of electrons (Lowe et al., 1993). It was suggested that the P-clusters transferred electrons to the M-clusters to provide the last two electrons required for the reduction of bound N_2.

Two important points in the mechanism of nitrogenase that remain to be elucidated in detail are the following. (i) What is the exact mechanism for coupling MgATP hydrolysis to electron transfer from the Fe protein to the MoFe protein? (ii) What are the function and relevant oxidation states of the P-clusters as electron transfer mediators? Recently, we engineered an Fe protein (L127Δ) by site-directed mutagenesis that is locked into a conformation resembling the MgATP-bound state even in the absence of bound MgATP (Ryle & Seefeldt, 1996). The L127Δ Fe protein was found to form a tight complex with the MoFe protein and to transfer a single electron to the MoFe protein in the absence of MgATP hydrolysis (Lanzilotta et al., 1996). These results provided evidence that the MgATP-bound state of the Fe protein was sufficient to allow the transfer of a single electron to the MoFe protein and that MgATP hydrolysis was not absolutely required for this electron transfer reaction. The transfer of a single electron into the MoFe protein from the Fe protein provided a unique opportunity for capturing the MoFe protein in a single-electron-reduced transition state, possibly allowing localization of the first electron acceptor within the MoFe protein. In the reduced or purified state, the MoFe protein exhibits EPR signals arising from the $S = {}^3/_2$ spin state of the two M-clusters (Münck et al., 1975), while the two P-clusters are in an $S = 0$ spin state and are thus EPR silent (Zimmermann et al., 1978). When the MoFe protein was reduced by one electron from the L127Δ Fe protein, no differences in the EPR signals arising from the M-clusters were observed, suggesting that the P-clusters might be the initial electron acceptor for this first electron transferred from the L127Δ Fe protein (Lanzilotta et al., 1996). Unfortunately, the reduced state of the P-clusters (P^N) are EPR silent (Zimmermann et al., 1978), and no state more reduced than P^N has ever been observed, thus precluding assignment of a role for the P-clusters in this electron transfer reaction.

In the present work, we have taken advantage of the fact that the P-clusters of the MoFe protein can be reversibly oxidized by two electrons from the P^N oxidation state to the P^{1+} and P^{2+} oxidation states, the latter being EPR active (Zimmermann et al., 1978; Watt et al., 1980; Johnson et al., 1981; Pierik et al., 1993; Tittsworth & Hales, 1993). Using the oxidized (P^{2+}) form of the MoFe protein as a substrate and EPR spectroscopy, it was found that the L127Δ Fe protein could reduce the P-clusters of the MoFe protein from the P^{2+} to the P^{1+} oxidation states in the absence of MgATP hydrolysis. The wild-type Fe protein was also found to reduce the P-clusters from the P^{2+} to the P^{1+} state, but only when MgATP was present. MgADP did not support wild-type Fe protein reduction of the P^{2+} clusters of the MoFe protein. These results suggest that the Fe protein transfers the first electron to the P-clusters of the MoFe protein and that the MgATP-bound protein conformation is the trigger for this electron transfer.

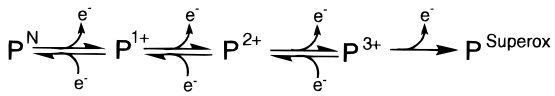
EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis, Expression, and Purification of Nitrogenase. Wild-type Fe and MoFe proteins were purified from *Azotobacter vinelandii* cells essentially as described (Seefeldt & Mortenson, 1993). Site-directed mutagenesis of the gene that encodes the subunits of the Fe protein of *A. vinelandii*, *nif H*, was performed as described (Seefeldt & Mortenson, 1993), and the L127Δ Fe protein was expressed and purified as before (Seefeldt & Mortenson, 1993; Ryle & Seefeldt, 1996). All proteins were homogeneous as determined by coomassie blue staining of SDS gels (Hathaway et al., 1979). Protein concentrations were determined by a modified biuret method (Chromy et al., 1974), with bovine serum albumin as the standard. MgATP hydrolysis rates, H_2 evolution rates, and acetylene reduction rates were determined as described (Seefeldt & Mortenson, 1993; Seefeldt & Ensign, 1994). The MoFe protein had a specific activity of 2260 nmol of acetylene reduced·min^{-1}·(mg of MoFe protein)$^{-1}$, while the wild-type Fe protein had a specific activity of 1975 nmol of acetylene reduced·min^{-1}·(mg of Fe protein)$^{-1}$. No acetylene reduction activity was detected for the L127Δ Fe protein when combined with the MoFe protein. All sample manipulations were carried out in an argon-filled glovebox (Vacuum Atmospheres, Hawthorne, CA) with less than 1 ppm oxygen. All buffers were purged with argon.

Oxidation of the MoFe Protein. MoFe protein was oxidized by 4 equiv of electrons by the addition of an excess of oxidized indigo disulfonate (IDS) (Christiansen et al., 1995). This was done by exchanging dithionite-reduced MoFe protein into anaerobic, dithionite-free 100 mM MOPS buffer (pH 7.0) with 250 mM NaCl by passage through a Sephadex G-25 column in an anaerobic glovebox. Enough IDS was added to the MoFe protein eluted from the column until a blue color remained and the mixture was allowed to react for 15 min. IDS was then separated from the MoFe protein by passage through a Dowex-1 (Sigma Chemical Co., St. Louis, MO) column equilibrated with 50 mM MOPS buffer (pH 7.0). The MoFe protein (P^{2+}) oxidized with 4 equiv of electrons was collected, and the concentration was determined from the absorption spectrum and the known absorption coefficient of 73 mM^{-1}·cm $^{-1}$ at 400 nm (Watt et al., 1980). Reduced, dithionite-free Fe protein was prepared by passage of the protein through a Sephadex G-25 column equilibrated with 50 mM MOPS buffer (pH 7.0). The concentration of Fe protein was determined from the absorption spectrum and the known absorption coefficient of 11.1 mM^{-1}·cm^{-1} at 430 nm (Lanzilotta et al., 1995b).

EPR Spectroscopy. EPR spectra were recorded on a Bruker ESP300E spectrometer equipped with a dual-mode cavity and an Oxford ESR 900 liquid helium cryostat. In all cases, 4 mm calibrated quartz EPR tubes (Wilmad, Buena, NJ) were used. All spectra were recorded at 12 K with 1024 points per scan and are either the sum of 20 scans for

Scheme 1



perpendicular mode spectra or the sum of 31 scans for parallel mode spectra. All other parameters are noted in the figure legends.

UV−Visible Absorption Spectra. Absorption spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer using 2.0 mL quartz cuvettes fitted with serum stoppers. Anaerobicity was obtained by purging the cuvettes with O_2-free argon for 6 min. The buffer used for these experiments was 50 mM MOPS (pH 7.0) unless noted otherwise. A MgATP-regenerating system (Seefeldt & Mortenson, 1993) was included with the wild-type Fe protein−MoFe protein mixture to prevent the formation of MgADP.

RESULTS

MgATP-Independent Electron Transfer from the L127Δ Fe Protein to the P-Clusters of the MoFe Protein. The P-clusters of the MoFe protein have been prepared in at least five different oxidation states as summarized in Scheme 1 (Pierik et al., 1993; Tittsworth & Hales, 1993). The MoFe protein is normally purified in the presence of the reductant dithionite, with the P-clusters in the reduced (P^N) state. It has been found that each P-cluster of the MoFe protein can be reversibly oxidized by at least 2 equiv of electrons to oxidation states designated as P^{1+} and P^{2+} without oxidation of the M-clusters (Surerus et al., 1992; Tittsworth & Hales, 1993; Christiansen et al., 1995). Further oxidation of the MoFe protein results in the oxidation of the P-clusters to irreversibly oxidized states ($P^{superox}$) and the reversible oxidation of the M-clusters by 6−9 equiv of electrons (Watt et al., 1980). Thus, by controlled oxidation of the MoFe protein, the P-clusters can be poised in the P^{2+} oxidation state. While the P^N state of the P-clusters is diamagnetic ($S = 0$) and therefore EPR silent, both the P^{1+} and P^{2+} oxidation states are paramagnetic and EPR active (Tittsworth & Hales, 1993). The P^{1+} state of the P-clusters has been characterized as a mixed integer spin state with an S of $^1/_2$ and $^5/_2$ and EPR signals in the $g = 2$ and 5 regions of the perpendicular mode EPR spectrum (Tittsworth & Hales, 1993). The P^{2+} state of the P-clusters is an integer spin state with an S of ≥ 3 and a parallel mode EPR signal near $g = 12$ (Pierik et al., 1993). We have prepared the MoFe protein in the P^{2+} oxidation state by controlled oxidation with oxidized indigo disulfonate (IDS) (Christiansen et al., 1995). This state of the MoFe protein was used as a substrate for electron transfer from the L127Δ Fe protein to determine if the P-clusters could accept electrons from the Fe protein.

Figure 1 (trace 1) illustrates the perpendicular mode EPR spectrum for the P^{2+} state of the MoFe protein. The observed inflections at $g = 4.27$, 3.63, and 2.00 have been assigned to the $S = {}^3/_2$ spin state of the two reduced M-clusters (Münck et al., 1975). The perpendicular mode spectrum of the reduced state of the L127Δ Fe protein is shown in Figure 1 (trace 2) with the $S = {}^1/_2$ spin state of the [4Fe-4S] cluster having an axial signal with inflections at $g = 2.03$, 1.93, and 1.89 (Ryle & Seefeldt, 1996). The spectrum that would be predicted for a mixture of the P^{2+} state of the MoFe protein and the reduced L127Δ Fe protein (assuming no electron transfer) is taken to be the additive spectrum of traces 1 and 2 and is shown in trace 3. Trace 4 shows the actual EPR spectrum recorded for a sample in which the P^{2+} state of the MoFe protein was mixed with the reduced, dithionite-free state of the L127Δ Fe protein. The signals assigned to the reduced state of the Fe protein ($[4Fe\text{-}4S]^{1+}$ cluster) disappeared, consistent with the oxidation of the [4Fe-4S] cluster to the 2+ oxidation state upon electron transfer to the MoFe protein. The signal assigned to the M-clusters with $g = 4.27$, 3.63, and 2.00 did not appear to change, suggesting no reduction. Interestingly, however, a new EPR signal was observed with inflections at $g = 5.06$, 2.05, 1.94, and 1.81. These signals are consistent with the signals previously assigned to the mixed-integer spin state ($S = {}^5/_2$, $S = {}^1/_2$) of the P-clusters oxidized (P^{1+}) with 1 equiv of electrons of the MoFe protein (Tittsworth & Hales, 1993). These data suggest that the L127Δ Fe protein transferred an electron to the P^{2+}-clusters of the MoFe protein, reducing them to the P^{1+} oxidation state.

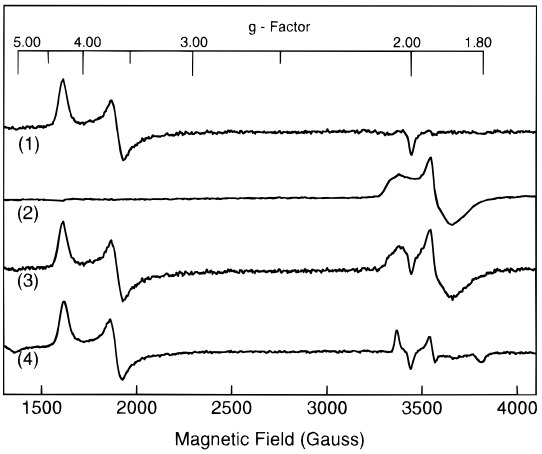


FIGURE 1: Electron transfer from the L127Δ Fe protein to the P-clusters of the MoFe protein monitored by perpendicular mode EPR spectroscopy. MoFe protein, with each P-cluster oxidized by two electrons (P^{2+} state), and the reduced but dithionite-free L127Δ Fe protein were prepared as described in Experimental Procedures. All samples were incubated for 2 min prior to freezing in liquid nitrogen, and the buffer was 50 mM MOPS (pH 7.0). Perpendicular mode EPR spectra are shown for the oxidized (P^{2+}) state of the MoFe protein (54 μM) (trace 1), the reduced state of the L127Δ Fe protein (91 μM) (trace 2), the mathematical additive spectrum for traces 1 and 2 (trace 3), and the mixture of L127Δ Fe protein (91 μM) and the P^{2+} state of the MoFe protein (54 μM) (trace 4). All spectra were recorded at 12 K with a microwave frequency of 9.64 GHz, a modulation frequency of 100 kHz, a modulation amplitude of 5.028 G, a time constant of 20.48 ms, and a microwave power of 10.1 mW.

To confirm electron transfer to the P^{2+}-clusters of the MoFe protein from the L127Δ Fe protein, parallel mode EPR was employed. Parallel mode EPR allows the observation of integer spin states, such as the $S \geq 3$ spin state of the P^{2+} oxidation state of the P-clusters (Pierik et al., 1993). Half-integer spin states such as those found for the other oxidation states of the P-clusters and the two oxidation states of the [4Fe-4S] cluster of the Fe protein (2+ and 1+) do not give parallel mode EPR signals. Figure 2 shows the $g = 11.8$ EPR inflection assigned to the $S \geq 3$ spin state of the P^{2+}-clusters (Figure 2, trace 1). The reduced Fe protein did not demonstrate any parallel mode EPR spectrum (Figure 2, trace 2). Incubation of the L127Δ Fe protein with the P^{2+} state of the MoFe protein resulted in the disappearance of the

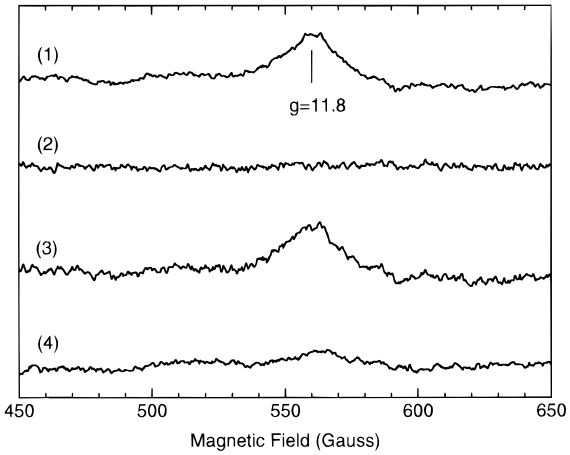


FIGURE 2: Electron transfer from the L127Δ Fe protein to the P-clusters of the MoFe protein monitored by parallel mode EPR spectroscopy. MoFe protein, with each P-cluster oxidized by two electrons (P^{2+}), and reduced L127Δ Fe protein were prepared as described in Experimental Procedures. All samples were incubated for 2 min prior to freezing in liquid nitrogen, and the buffer was 50 mM MOPS (pH 7.0). Parallel mode EPR spectra are shown for the oxidized (P^{2+}) state of the MoFe protein (54 μM) (trace 1), the reduced state of the L127Δ Fe protein (91 μM) (trace 2), the mathematical additive spectrum of traces 1 and 2 (trace 3), and a mixture of L127Δ Fe protein (91 μM) and the P^{2+} state of the MoFe protein (54 μM) (trace 4). All spectra were recorded at 12 K with a microwave frequency of 9.35 GHz, a modulation frequency of 100 kHz, a modulation amplitude of 12.63 G, a time constant of 20.48 ms, and a microwave power of 50.5 mW.

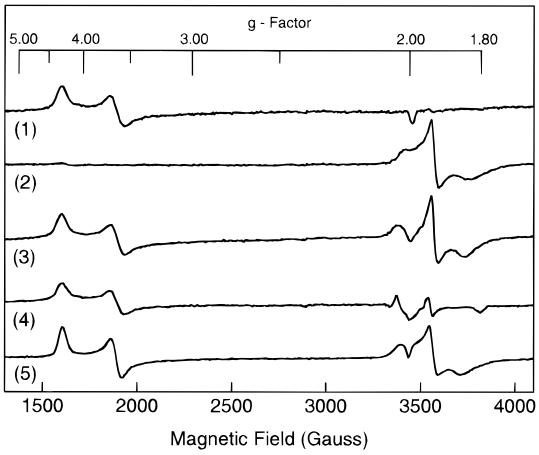


FIGURE 3: Electron transfer from the wild-type Fe protein to the P-clusters of the MoFe protein monitored by perpendicular mode EPR spectroscopy. MoFe protein, with each P-cluster oxidized by two electrons (P^{2+}), and the reduced but dithionite-free state of the wild-type Fe protein were prepared as described in Experimental Procedures. All samples were incubated for 2 min prior to freezing in liquid nitrogen, and the buffer was 50 mM MOPS (pH 7.0). Perpendicular mode EPR spectra were recorded for the oxidized (P^{2+}) state of the MoFe protein (52 μM) (trace 1), the reduced state of the wild-type Fe protein (96 μM) (trace 2), a mixture of oxidized (P^{2+}) MoFe protein (52 μM) and wild-type Fe protein (96 μM) (trace 3), the same mixture as in trace 3 with 1 mM MgATP (trace 4), and the same mixture as in trace 3 with 1 mM MgADP (trace 5). EPR parameters were the same as those described in the legend to Figure 1.

parallel mode EPR signal at $g = 11.8$ (Figure 2, trace 4). Again, this is consistent with the reduction of the P^{2+} state of the P-clusters to the P^{1+} oxidation state (Tittsworth & Hales, 1993). These results, coupled with the appearance of perpendicular mode EPR signals assigned to the P^{1+} state, reveal primary electron transfer from the L127Δ Fe protein in the absence of MgATP to the P-clusters of the MoFe protein.

MgATP-Dependent Electron Transfer from the Wild-Type Fe Protein to the P-Cluster of the MoFe Protein. It seemed possible that the reduced Fe protein might transfer an electron to the P^{2+} oxidization state of the P-clusters by nonspecific electron transfer, as would be expected for small electron transfer mediators like dithionite (Watt et al., 1980). To determine the specificity of electron transfer from the Fe protein to the P^{2+}-clusters in the MoFe protein, the reduced wild-type Fe protein was tested for electron transfer to the P^{2+}-clusters of the MoFe protein. A hallmark of the electron transfer from the wild-type Fe protein to the reduced state of the MoFe protein (P^N) is the absolute need for MgATP (Mortenson, 1964). Therefore, possible electron transfer from the wild-type Fe protein to the P^{2+} state of the MoFe protein was monitored in the absence of nucleotides, in the presence of MgATP, or in the presence of MgADP.

The perpendicular mode EPR spectra of the oxidized (P^{2+}) MoFe protein and the reduced, dithionite-free, wild-type Fe protein are shown in Figure 3 (traces 1 and 2) for reference. The EPR spectrum for a sample of wild-type Fe protein incubated with the P^{2+} oxidation state of the MoFe protein in the absence of added nucleotides is shown in trace 3. There were no significant changes in the EPR spectrum of the Fe protein or the MoFe protein, suggesting that no electron transfer had occurred between the proteins. Similarly, the inclusion of MgADP in the incubation mixture of the P^{2+} state of the MoFe protein and the Fe protein did not result in any significant changes in the line shape or intensity of the signals in the perpendicular mode EPR spectrum (trace 5). The subtle change in the line shape of the $S = {}^1/_2$ signal of the Fe protein is the result of MgADP binding-induced conformational changes (Lindahl et al., 1987). In contrast, the addition of MgATP to the Fe protein−MoFe protein mixture (trace 4) resulted in the formation of new EPR signals with inflections at $g = 2.05$, 1.94, and 1.81. These signals are consistent with the signals observed for the P^{1+} state of the MoFe protein (Tittsworth & Hales, 1993) and are the same as those observed for the L127Δ Fe protein−MoFe protein samples without MgATP. Interestingly, a small decrease in the intensity of the inflections for the $S = {}^3/_2$ signal of the M-cluster was also observed, suggesting some reduction of the M-cluster by the wild-type Fe protein, which was not seen for the L127Δ Fe protein−MoFe protein complex.

The above EPR results were confirmed by the changes in the parallel mode EPR spectrum, as shown in Figure 4, with reduction of the P^{2+} oxidation state of the MoFe protein only when MgATP was included in the wild-type Fe protein−MoFe protein mixture. In total, the parallel and perpendicular mode EPR spectra show that stoichiometric quantities of the wild-type Fe protein will reduce the P^{2+} clusters of the MoFe protein to the P^{1+} oxidation state only in the presence of MgATP. This establishes that the reduction of the P^{2+} state of the MoFe protein by the Fe protein is specific to the MgATP-bound state and is not simply the result of nonspecific electron transfer. It is also important to note that the function of MgATP in triggering this electron transfer is not simply to lower the redox potential of the Fe protein [4Fe-4S] cluster since MgADP binding to the Fe protein also lowers the redox potential by a comparable amount (Zumft

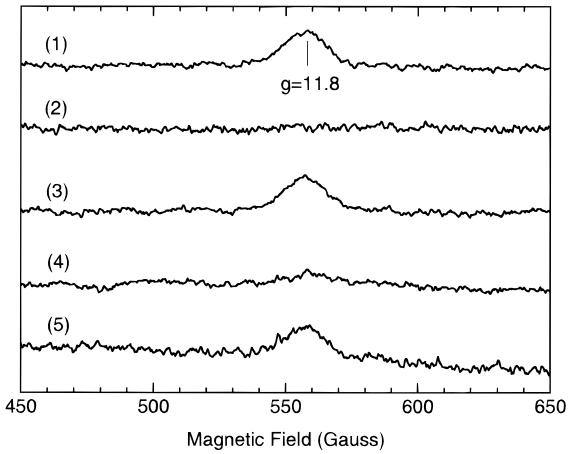


FIGURE 4: Electron transfer from the wild-type Fe protein to the P-clusters of the MoFe protein monitored by parallel mode EPR spectroscopy. MoFe protein, with each P-cluster oxidized by two electrons (P^{2+}), and reduced wild-type Fe protein were prepared as described in Experimental Procedures. All samples were incubated for 2 min prior to freezing in liquid nitrogen, and the buffer was 50 mM MOPS (pH 7.0). Parallel mode EPR spectra are shown for the oxidized (P^{2+}) state of the MoFe protein (52 μM) (trace 1), the reduced state of the wild-type Fe protein (96 μM) (trace 2), a mixture of wild-type Fe protein (96 μM) and the P^{2+} state of the MoFe protein (52 μM) (trace 3), the same mixture as in trace 3 with 1 mM MgATP (trace 4), and the same mixture as in trace 3 with 1 mM MgADP (trace 5). EPR parameters were the same as those described in the legend to Figure 2.

et al., 1974), and yet MgADP did not stimulate electron transfer to the P-clusters.

A Tight Complex between the L127Δ Fe Protein and the Oxidized MoFe Protein. Finally, we previously established that either the reduced or the oxidized states of the L127Δ Fe protein would bind tightly with the reduced MoFe protein, forming an essentially irreversible protein−protein complex (Lanzilotta et al., 1996). Given the observation of electron transfer from the reduced L127Δ Fe protein to the P^{2+}-oxidized MoFe protein, it was important to investigate if an irreversible complex was formed between the reduced L127Δ Fe protein and the MoFe protein (P^{2+}) oxidized with 4 equiv of electrons. This was determined by monitoring changes in the visible absorption spectrum of the Fe protein [4Fe-4S] cluster upon oxidation or reduction. After electron transfer to the MoFe protein, the L127Δ Fe protein is left in an oxidized state. The assumption is that, if the oxidized Fe protein is bound tightly to the MoFe protein, then the $[4Fe\text{-}4S]^{2+}$ cluster will be protected from reduction by the low-molecular weight reductant dithionite (Thorneley & Lowe, 1983). The absorption spectrum of the oxidized wild-type Fe protein, following electron transfer to the MoFe protein, is shown in Figure 5 (panel A, trace 1). A significant decrease in the absorption spectrum is observed 15 s after addition of excess dithionite (Figure 5, panel A, trace 2). This shows that the wild-type Fe protein is rapidly reduced by dithionite and thus is not forming a tight complex with the MoFe protein. In contrast, the L127Δ Fe protein was found to be protected from dithionite reduction following electron transfer to the oxidized MoFe protein (Figure 5, panel B). These results indicate the formation of a tight complex between the L127Δ Fe protein and the oxidized (P^{2+}) state of the MoFe protein.

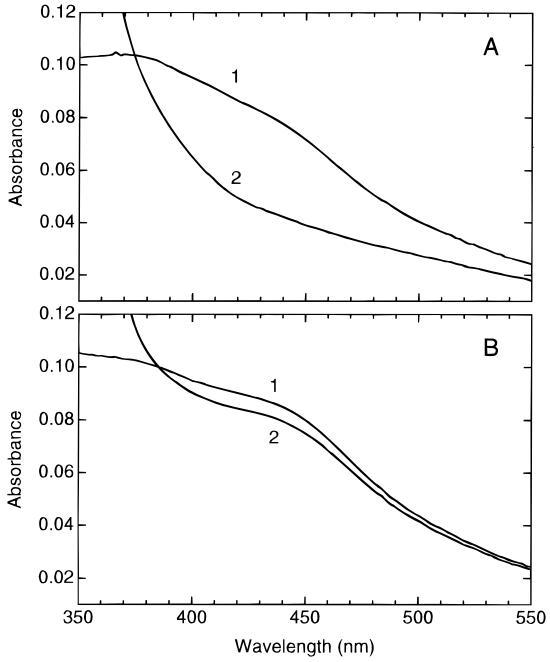


FIGURE 5: Fe protein−MoFe protein electron transfer and dissociation monitored by UV−visible absorption spectroscopy. MoFe protein, with each P-cluster oxidized by two electrons (P^{2+}), and reduced Fe proteins were prepared as described in Experimental Procedures. The buffer used in all cases was 50 mM MOPS (pH 7.0). Oxidized (P^{2+}) MoFe protein was added to a final concentration of 3.8 μM in a 1 mL total volume to a sealed cuvette, and the spectrometer was blanked. (A) Reduced, dithionite-free wild-type Fe protein (8 μM) and MgATP (1 mM) were added, and an absorption spectrum was recorded after no further change (trace 1). Dithionite was added to a final concentration of 2 mM, and an absorption spectrum was recorded 15 s later (trace 2). (B) Reduced, dithionite-free L127Δ Fe protein (8 μM) and MgATP (1 mM) were added, and an absorption spectrum was recorded after no further change (trace 1). Dithionite was added to a final concentration of 2 mM, and an absorption spectrum was recorded 5 min later (trace 2).

DISCUSSION

The results of the present study can be discussed in the context of two important questions concerning electron transfer within nitrogenase. (i) What is the role of the P-clusters in intercomponent electron transfer? (ii) What is the role of MgATP hydrolysis in triggering electron transfer?

Primary Electron Transfer and the Role of the P-Clusters. The possible functions of the P-clusters in the nitrogenase mechanism have been the focus of attention for many years (Lowe et al., 1993; Rees et al., 1993; Peters et al., 1995a). The results of the present study clearly demonstrate that the Fe protein can transfer electrons directly to the P-clusters in a reaction that only requires the MgATP-bound Fe protein conformation. This observation builds on earlier work suggesting that the P-clusters mediate electron transfer from the [4Fe-4S] cluster of the Fe protein to the M-cluster of the MoFe protein as part of the substrate reduction mechanism. In one of the earlier studies, the roles of amino acid side chains in electron transfer between the P-clusters and the M-clusters of the MoFe protein were examined (Peters et al., 1995b). Changing Tyr α91 or Tyr β98 to several other amino acids was observed to alter substrate reduction rates, without altering the rate of primary electron transfer from the Fe protein to the MoFe protein (Peters et al., 1995b). This provided circumstantial evidence that the P-clusters

accepted electrons from the Fe protein prior to electron transfer to the M-clusters.

Additional data that suggest that the P-clusters accept electrons from the Fe protein prior to mediating electron transfer to the M-clusters come from the recent construction of an altered form of the MoFe protein (Ma et al., 1996). To construct this altered form, homocitrate was removed from isolated FeMoco and the modified cofactor was inserted into the apo-MoFe protein. The resulting modified MoFe protein did not reduce substrates but did accept a single electron from the Fe protein (Ma et al., 1996). This altered form of the MoFe protein also displayed an axial EPR signal at $g = 1.93$ that was assigned to the P-clusters. The primary evidence that the P-clusters accepted the electron from the Fe protein was an increase in the intensity of this EPR signal upon electron transfer from the Fe protein. The oxidation state of the P-clusters in this latter work was not determined.

While these studies suggest a likely role for the P-clusters in mediating electron transfer, the relevant oxidation states and number of electrons mediated by the P-clusters remain unclear. Part of the problem with clearly assigning the P-clusters as participants in electron transfer reactions results from the fact that, in the as purified state, the P-clusters have all eight iron atoms in the 2+ oxidation state (Zimmermann et al., 1978), suggesting that the cluster is fully reduced and cannot accept more electrons. In fact, no observation of P-clusters in a state more reduced than the P^N state has yet been made. A possible explanation for how the P-clusters might be reduced beyond the P^N state has come from a structural model of the cluster deduced from the X-ray crystal structure of the MoFe protein. There is general agreement that the P-clusters are composed of two bridged [4Fe-4S] subclusters (Kim & Rees, 1992b; Bolin et al., 1993). There is, however, some disagreement on the nature of the bridge between the subclusters. One model suggests a disulfide bond between one inorganic sulfur in each subcluster (Kim & Rees, 1992b). The other model suggests that the two subclusters share a common six-coordinate inorganic sulfur from one corner of each subcluster (Bolin et al., 1993). The nature of the bridge between the subclusters is important for understanding the redox properties of the P-clusters. Since nitrogenase reduces a range of substrates in units of two electrons (Burgess, 1985), it has been suggested that the P-clusters might mediate the transfer of two electrons to the M-clusters for substrate reduction. In the one model of the P-clusters, the disulfide bond between the subclusters could be reduced by two electrons (Rees et al., 1993). This model has the attractive feature that the disulfide bridge could serve as a two-electron redox mediator. There are problems with this model, however. First, another model for the P-clusters also derived from the X-ray crystal structure of a MoFe protein does not have a disulfide bridge (Bolin et al., 1993), which would make the former reduction model impossible. In addition, a sulfur radical species would be expected for the one-electron-reduced P-cluster, and no evidence has been observed to suggest the formation of such a radical, which should be observable by EPR. Finally, a state of the P-clusters further reduced than P^N has not been observed in rapid freeze EPR experiments (Zumft et al., 1974).

This leads to what now appears to be a more likely model for redox states of the P-clusters in which the relevant oxidation states are P^N to P^{2+}. It might be imagined that the reduced (P^N) state of the P-clusters transfers two electrons to the M-clusters, resulting in the formation of the oxidized P^{2+} clusters. The Fe protein might then transfer electrons to the P^{2+}-clusters, one at a time, to reduce the P-clusters back to the P^N state. Hence, in this model, the P^N state of the P-clusters would be the most reduced state, with more oxidized states of the P-clusters being relevant to the electron transfer mechanism. Three observations would support such a redox model for the P-clusters. First, the midpoint potentials (E_m) for the $P^N \leftrightarrow P^{1+}$ and $P^{1+} \leftrightarrow P^{2+}$ couples have been reported to be −307 mV (Pierik et al., 1993). This E_m value would be very near the E_m for the Fe protein with bound nucleotides of around −440 mV (Ryle & Seefeldt, 1996). Thus, electron transfer from the Fe protein to the oxidized states of the P-clusters would be thermodynamically favorable, with a ΔE_m of approximately −133 mV. Indeed, the results of the current work clearly demonstrate that the transfer of electrons from the Fe protein to the oxidized P-clusters is favorable as observed by the rapid electron transfer. We are in the process of establishing kinetic constants for these electron transfer reactions. Stopped-flow kinetic studies coupled with EPR spectroscopy have suggested that the P-clusters become oxidized during the reduction of N_2 (Lowe et al., 1993). After the four-electron reduction step of N_2, the P-clusters became oxidized. This supports the proposal that the P-clusters might transfer electrons to the M-clusters to provide electrons for substrate reduction (Lowe et al., 1993). Likewise, intramolecular electron transfer from the M-clusters to the oxidized P-clusters has been observed to occur at a rate similar to the measured turnover rate for the enzyme, supporting a role for the oxidized P-clusters in nitrogenase electron transfer (Smith et al., 1983). While the current work, coupled with these other published works, clearly supports a role for the P-clusters in electron transfer, a detailed description of the relevant oxidation states of the P-clusters remains to be established.

Role of MgATP Interactions. So a question that remains is the following. What is the function of MgATP in triggering electron transfer within nitrogenase? It is clear from the results with the L127Δ Fe protein that MgATP hydrolysis is not required for primary electron transfer from the Fe protein to the reduced (Lanzilotta et al., 1996) or oxidized P-clusters (this work). Rather, it appears that the MgATP-bound protein conformation of the Fe protein is all that is necessary for primary electron transfer to the P-clusters (Lowe et al., 1995; Lanzilotta et al., 1996). It is tempting to assign the function of MgATP to simply lowering the redox potential of the Fe protein [4Fe-4S] cluster from −290 to −420 mV, thus making electron transfer to the P-clusters thermodynamically favorable. The observation that the MgADP-bound state of the Fe protein, however, does not transfer electrons to the MoFe protein even though it lowers the E_m of the Fe protein to −460 mV (Ryle et al., 1995) argues against this simple model. This suggests that the MgATP-bound state of the Fe protein is critically different from the MgADP-bound state in the protein conformation required for electron transfer. These differences in the environment of the [4Fe-4S] cluster have been confirmed by several spectroscopic techniques (Chen et al., 1994; Lanzilotta et al., 1995a; Ryle et al., 1996a) and could well be associated with complex association and electron transfer.

It is also possible that MgATP hydrolysis could be coupled in some way to triggering electron transfer from the

P-clusters to the M-clusters. Results presented in this work might provide some support for such a mechanism. While electron transfer from the L127Δ Fe protein to the oxidized P-clusters (P^{2+}) of the MoFe protein had no affect on the $S = {}^3/_2$ signal of the M-clusters, there is clearly a slight decrease in the intensity of that signal when the wild-type Fe protein with MgATP was the reductant. One possible explanation for this result would be that the wild-type Fe protein is free to dissociate after primary electron transfer and therefore some of the oxidized (P^{2+}) MoFe protein may be reduced by more than one electron. The presence of EPR signals assigned to the P^{1+} clusters would argue against this interpretation. Instead, it seems possible that the hydrolysis of MgATP and phosphate release catalyzed by the wild-type Fe protein might be coupled to transfer of electrons from the P-cluster to the M-cluster. More conclusive evidence supporting this model is necessary.

In summary, the results presented in this work provide direct EPR evidence for electron transfer from the [4Fe-4S] cluster of the Fe protein to the P-clusters of the MoFe protein. This electron transfer reaction required the MgATP-bound conformation of the Fe protein but not MgATP hydrolysis.

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