BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Nucleotide-Assisted $[Fe_4S_4]$ Redox State Interconversions of the *Azotobacter vinelandii* Fe Protein and Their Relevance to Nitrogenase Catalysis

Deloria Jacobs and Gerald D. Watt*

Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah 84602, United States

ABSTRACT: The $[Fe_4S_4]$ cluster of the nitrogenase Fe protein from *Azotobacter vinelandii* can exist in three redox states: oxidized $[Fe_4S_4]^{2+}$, dithionite reduced $[Fe_4S_4]^{1+}$, and two forms of the all ferrous $[Fe_4S_4]^0$, $S = 4$ and 0. Operation of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple transfers one electron to the MoFe protein during catalysis with hydrolysis of two MgATPs. In contrast, the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ couple transfers two electrons per binding event, accompanied by hydrolysis of only two MgATPs. Both reactions occur at nearly identical rates even though the number of electrons transferred differs by a factor of 2. MgATP and MgADP facilitate interconversion of the three redox states: $2[Fe_4S_4]^{1+} + 4\ MgATP = [Fe_4S_4]^{2+}(MgATP)_2 + [Fe_4S_4]^0(MgATP)_2$, as demonstrated by the MgATP reaction. This reaction was investigated as a possible precursor reaction to provide two electrons in the form of $[Fe_4S_4]^0(MgATP)_2$ for delivery to the MoFe protein to then conduct a two-electron substrate reduction. However, experiments showed that this disproportionation reaction, which readily occurs, was not viable during nitrogenase catalysis utilizing the $[Fe_4S_4]^{1+}$ cluster state. The known cooperative behavior of the Fe protein in the $[Fe_4S_4]^{1+}$ state taken together with a measured turnover potential of −460 mV with an $n = 2$ value, suggest a gating process on the MoFe protein involving a two electron step.

$$2[Fe_4S_4]^{1+} = [Fe_4S_4]^{2+} + [Fe_4S_4]^0$$

Nitrogenase is a protein complex consisting of two dissimilar metalloproteins that operates in the input leg of the earth's nitrogen cycle to catalyze the formation of ammonia from atmospheric N_2.[1,2] The smaller Fe protein (α_2, M_r = 64 kda) has two ATP binding sites and a redox-active $[Fe_4S_4]$ cluster. The protein structure changes in the presence of nucleotides[3,4] and the $[Fe_4S_4]$ cluster redox potential becomes more negative. The larger MoFe protein ($\alpha_2\beta_2$, M_r = 230 kda) contains two pairs of complex metal clusters: the P-clusters (Fe_8S_7) bridging $\alpha\beta$ subunits and the FeMoco centers ($MoFe_7S_9$-homocitrate) located in the α subunits.[5,6] During enzymatic catalysis, the Fe protein reduction cycle transfers low potential electrons produced during ATP hydrolysis to the P-clusters, which in turn transfers electrons to FeMoco centers. Multiple electrons are either initially stored in the MoFe protein metallo centers or accumulate there during the Fe protein cycle in preparation for reduction of the natural substrate N_2 and alternate substrates such as acetylene, H^+, and others.[7,8]

Nitrogenase catalysis is initiated by ATP binding to the reduced Fe protein, which shifts the redox potential of the $[Fe_4S_4]^{1+}$ cluster to −460 mV followed by specific binding of the activated Fe protein to the MoFe protein. An electron is transferred to the metal clusters of the MoFe protein coupled with the hydrolysis of two MgATPs.[7,8] However, this process is more complicated than outlined because the number of electrons transferred by the Fe protein cycle can be one or two depending upon the reductant.[9,10] Dithionite is the most commonly used artificial reductant and during catalysis only supports the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple resulting in a one-electron transfer to the MoFe protein per cycle. As two, one-electron Fe protein cycles are required for each two-electron substrate-reduction step, 4−5 ATPs are typically hydrolyzed.

If reductants are used that support the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox state, including the natural flavoprotein reductant, an apparent two-electron Fe protein cycle operates and the ATP requirement is halved to values near 2.0.[10−12] At specific Fe/MoFe protein ratios and reductant concentrations, both redox couples can operate either separately or concurrently, resulting in ATP/2e values ranging from 2.0 to 5.0.[10] What is unusual about the use of either redox couple (or a combination of both) is that the rate of substrate reduction is independent of whether two one-electron transfer reactions occur during which two ATPs are hydrolyzed at each step or if a two-electron transfer reaction occurs with hydrolysis of only two ATPs.[11,12] Measuring nearly identical rates using the two different redox states of the Fe protein is not consistent with the well-accepted view that dissociation of the oxidized Fe protein from the MoFe protein is the rate-limiting step in nitrogenase catalysis.[7,8] If Fe protein dissociation is rate limiting, the rates should differ by a factor of 2 between these two Fe protein redox couples instead of being invariant. This important result shifts focus on how single and double electron transfers are accommodated and accumulate at the MoFe protein. An electron gating process has been proposed[13,14] to be operative in the MoFe protein that is initiated by the Fe protein binding to the MoFe protein. This gating process is likely the controlling factor of electron flow into the MoFe protein from either the one- or two-electron reduced Fe protein.

Received: March 4, 2013
Revised: June 8, 2013
Published: June 18, 2013

dx.doi.org/10.1021/bi301547b | *Biochemistry* 2013, 52, 4791−4799

The process by which electrons are transferred to the MoFe protein becomes an important mechanistic issue because the turnover potential of the MoFe protein was shown by four independent studies to occur as a rate limiting step near −460 mv with an $n = 2$ value.[15−18] What is significant about these results is that all four reports used the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple for single-electron transfers to the MoFe protein. An $n = 2$ value for nitrogenase turnover requires that two single-electron transfers from Fe protein cycles occur in rapid succession before a two-electron substrate reduction ($2H^+ + 2e = H_2$) occurs at the MoFe protein. A cooperative interaction of the Fe protein with the MoFe protein was demonstrated from steady-state kinetic measurements and is consistent with an $n = 2$ value.[18,19] The recent gating proposal[13,14] is applicable if two sequential Fe protein interactions occur and two electrons are transferred in the gating process.

The results just outlined form a quite different mechanistic view of how nitrogenase catalysis is conducted. To further evaluate and possibly extend this view, we have considered an alternate possibility for delivering two electrons to the MoFe cofactor center to satisfy both the $n = 2$ requirement and the operating turnover potential of −460 mV observed during nitrogenase turnover.

The $[Fe_4S_4]^0$ form of the Fe protein (either $S = 4$ or $S = 0$) is now well established as an effective catalytic form of the Fe protein, which functions during nitrogenase catalysis to deliver two electrons per binding event.[9−12] Its formation was previously proposed to occur by Reaction 1.[9] The hypothesis we wish to explore arises from Reaction 1 and examines the possibility that the $[Fe_4S_4]^0$ form of the Fe protein first forms either by Reaction 1 or a nucleotide-assisted variation of it shown as Reaction 1a.

$$2\text{Fer} = \text{Feo} + \text{Ferr} \quad (1)$$

$$2\text{Fer(ATP)2} = \text{Ferr(MgATP)}_2 + \text{Feo(MgATP)}_2 \quad (1a)$$

$[Fe_4S_4]^0$ would then function by transferring two electrons to the MoFe protein at its known redox potential of −460 mV in a single $n = 2$ step consistent with the measured turnover potential of −460 mv.[15−18] In what follows, Feo, Fer, and Ferr are used as convenient abbreviated forms for reactions of the Fe protein for the respective cluster states $[Fe_4S_4]^{2+}$, $[Fe_4S_4]^{1+}$, and $[Fe_4S_4]^0$.

Evaluation of the above-discussed hypothesis would not only examine an alternate mechanistic view of electron transfer during nitrogenase catalysis, but also provide a better understanding of how the $[Fe_4S_4]$ cluster states of the iron protein are interconverted. Here, we report three developments regarding the interaction of the Fe protein during nitrogenase catalysis and their mechanistic implications. The first is the use of formadine sulfinic acid to form the all ferrous $S = 0$ cluster spin state of the Fe protein. The second is the nucleotide assisted interconversion reactions of Feo, Fer, and Ferr shown in Reaction 1. Finally, the rates for electron transfer to the MoFe protein from both Fer and Ferr are compared during nitrogenase catalysis.

MATERIALS AND METHODS

Formadine sulfinic acid (FSA) was obtained from Sigma at 98% purity. Ti(III)-citrate (Ti(III)) solutions were prepared from $TiCl_3$ and standardized as previously described.[10] Conditions were chosen where inhibition by Ti(IV) and citrate were minimized.[10] *Azotobacter vinelandii* Fe and MoFe proteins with specific activities of 1700−2100 nmol H_2 $mg^{-1}min^{-1}$ were obtained as previously described.[12] Fe protein was further purified by passing it 3−4 times through a 2.5 × 150 cm Sephacryl column equilibrated with 0.05 M Tris, 0.05 M NaCl, pH 7.5, containing 1.0 mM sodium dithionite (DT). After this purification, Fe protein contained no contaminating MoFe protein as evidenced by adding MgATP to Fer in the absence of added reductant and measuring no H_2 or ethylene formation from acetylene. Coulometry on the various forms of the Fe protein was carried out as previously described.[9]

Fer in the absence of reductant was prepared for kinetic and equilibrium measurements by reduction with excess DT followed by size-exclusion chromatography on Sephadex G-25 columns equilibrated in 0.05 M Tris, 0.05 M NaCl, pH 7.5, free of DT. Ferr was prepared in a similar way after reduction with 0.1 M FSA. In some experiments, Ferr$(MgATP)_2$ was obtained by Reaction 1a as described later. EPR and vis−UV spectra were obtained as previously described.[9−12] All preparations were conducted in a Vacuum Atmospheres Glove Box under N_2 or argon with O_2 levels <1.0 ppm.

Two types of kinetic measurements were conducted that compared the rates of Fer and Ferr utilization during nitrogenase catalysis by using an HP 8453 or a Cary 118 spectrophotometer with the cell compartments controlled at 30.0 °C. The first followed the rate of utilization of DT and Ti(III) at a Fe/MoFe ratio of 5.0 in 0.05 M MOPS pH 7.4 in the presence of the MgATP regenerating system under both argon and nitrogen atmospheres. Anaerobic buffer containing the nitrogenase proteins was equilibrated at 30 °C and then a concentrated MgATP regeneration system was added to a final MgATP concentration of 5 mM to initiate the reaction. The absorbance change was monitored as a function of time and in identical but separate experiments MgADP was determined by HPLC analysis.[11] In the second type of experiment, 100−1000-fold excess Fer or Ferr over MoFe protein, all free of DT, were prepared as described above and were equilibrated at 30 °C. MgATP was then added to 5 mM to initiate the reaction and the absorbance change for Fer or Ferr→Feo was monitored at 400, 430, or 460 nm as the Fe proteins were oxidized and produced H_2 (argon) or NH_3 (N_2). In identical but separate experiments, MgADP formation was determined by HPLC.[11]

RESULTS

MV at 0.1 mM, Ti(III) at <3 mM and DT at 20 mM only support the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple of the Fe protein, giving ATP/2e values near 5.0.[10] In contrast, *Azotobacter vinelandii* flavoprotein, Ti(III), and MV at higher concentrations support the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple giving ATP/2e values of 2.0.[9,12] Ti(III) is unusual in that it forms a catalytically functional $S = 4$ spin state[20] of the $[Fe_4S_4]^0$ cluster, whereas the other reductants exclusively form an $S = 0$ state.[12] The use of these three reductants for $[Fe_4S_4]^0$ formation is complicated because they or their products have strong $g = 2.0$ EPR signals or strong optical absorption in the UV−visible region, which interfere with the observation of $[Fe_4S_4]^0$.

Formation of Ferr ($S = 0$). Figure 1 shows the optical spectrum of Ferr formed by reduction of Fer by FSA ($E_{1/2} = -1.2$ V). The reaction is slow (~1 h, 40 °C) as shown in the inset and the visible spectrum does not show a peak at ~520 nm characteristic of the $S = 4$ Fe protein but instead shows an absorbance decrease from 400 to 800 nm and an increase between 350 and 400 nm. The rate of reduction of Fer to Ferr by FSA is first order in both Fer and FSA. The $g = 2$

dx.doi.org/10.1021/bi301547b | *Biochemistry* 2013, 52, 4791−4799

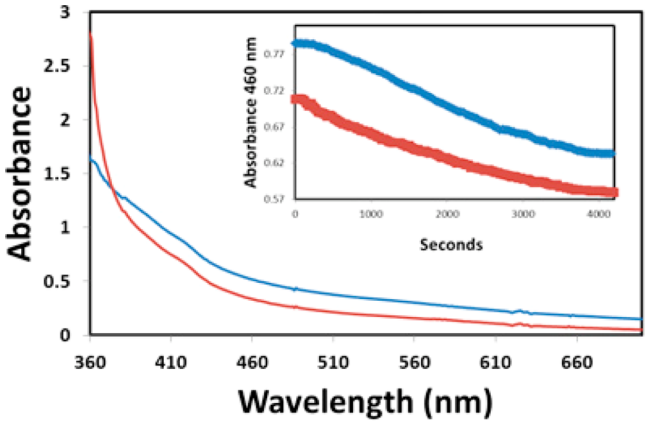

Figure 1. Reduction of Fer (blue spectrum) by 0.10 M formadine sulfinic acid at 40 °C at pH 7.5 in 0.05 M Tris buffer containing 0.05 M NaCl to form Ferr (red spectrum). The inset shows separate progress curves for reduction of Fer to Ferr monitored at 460 nm in 0.10 M FSA (upper curve) and the same reaction in the presence of 5 mM MgADP (lower curve).

perpendicular EPR signal of Fer is eliminated and no new EPR signals are observed, results consistent with formation of $S = 0$ Ferr.[12] The inset shows that the overall rate of Fer reduction is similar with or without added MgADP but the initial small lag phase noted in the absence of nucleotides is eliminated with MgADP.

The addition of MgATP to 5 mM does not perturb the absorption spectrum of Ferr, so MgATP binding cannot be confirmed by this method. However, by analogy to Fer, whose spectrum is not perturbed by MgATP but binding is known to occur, MgATP binding to Ferr is expected (see later).[22]

Figure 2a shows that the addition of Ti(III) to Fer initially forms stable Ferr ($S = 4$). The addition of DT causes an increase in absorbance at 360 nm but the absorbance remains constant indicating no reaction with DT occurs. In contrast, the addition of DT to Ferr ($S = 0$) formed by reduction with MV (or FSA) causes immediate loss of DT monitored at 360 nm. For this latter reaction, a higher concentration of DT was used to clearly demonstrate DT loss. The difference in background absorbance in Figure 2a and b arises from the presence of Ti(III) and MV in the solution. An all-ferrous cluster forms in both experiments but in different spin states. The ability of the all ferrous cluster to catalyze DT decomposition is therefore determined by specific reactivity of its $S = 0$ spin state.

Disproportionation of Fer. An earlier report suggested that Ferr could be formed from Fer by three Reactions 1−3 and might be relevant to nitrogenase catalysis.[9] Each of these reactions has now been examined and their properties and viability as catalytic species during nitrogenase catalysis have been evaluated.

$$2\text{Fer} = \text{Feo} + \text{Ferr} \qquad K = 0.0035 \tag{1}$$

$$2\text{Fer(ATP)2} = \text{Ferr(MgATP)}_2 + \text{Feo(MgATP)}_2 \qquad K = 7.4 \tag{1a}$$

$$2\text{Fer} + 4\text{ATP} = \text{Feo(MgADP)}_2 + \text{Ferr(MgATP)}_2 + 2\text{Pi} \qquad K = 5.5 \times 10^{18}\text{M}^{-2} \tag{2}$$

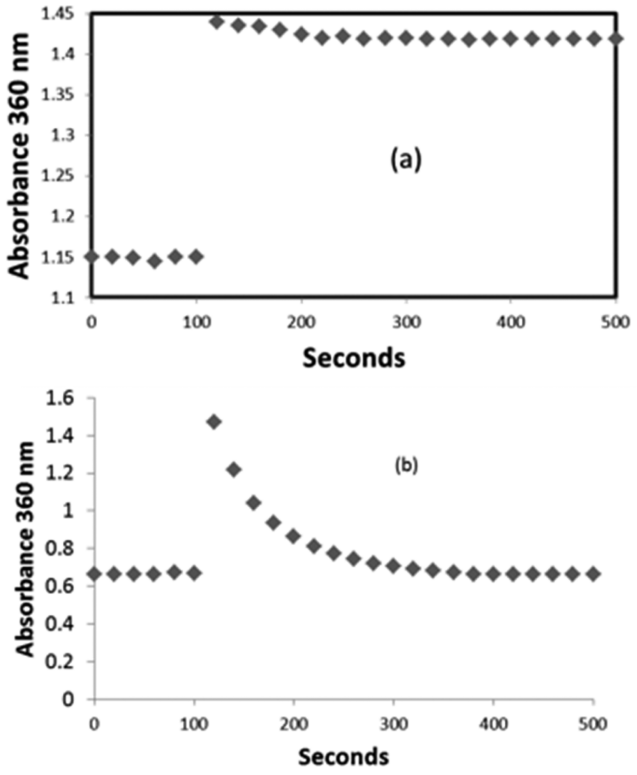

Figure 2. DT reactivity of Fe protein with spin states of $S = 4$ and $S = 0$. (a) 65 μ Fe protein in 0.05 M Tris containing 0.05 M NaCl pH 7.5 was incubated with 105 μM Ti(III) and DT added to 0.70 mM at 100 s and monitored at 360 nm (ε = 425 M^{-1} cm^{-1}. (b) Same as (a) except MV replaced Ti(III) forming the $S = 0$ EPR spin state and DT was added to 2.1 mM to initiate the reaction at 100 s.

$$2\text{Fer} + 4\text{ATP} = \text{Feo(MgATP)}_2 + \text{Ferr(MgATP)}_2 \qquad K = 1.5 \times 10^{12}\text{M}^{-4} \tag{3}$$

Reaction 1. Reaction 1 is the spontaneous disproportionation of Fer into Feo and Ferr. The free energy change was calculated[9] as +14.5 kJ/mol ($K = 0.0035$), which predicts that the equilibrium for Reaction 1 lies the left. Reaction 1a is a variation of Reaction 1 and represents what would occur in the cell where ATP is present at 5.0−10.0 mM. This reaction shows that the equilibrium is shifted to the right as a consequence of binding of MgATP to both Feo and Ferr.

Making DT-free Fer and then conducting three separate experiments confirms the prediction of Reaction 1. Optical and EPR samples of DT-free Fer were first prepared and their spectra recorded. Excess DT was added to each and the spectra compared. No differences in signal intensities were observed indicating that Fer in the absence of added reductant was fully present due to the equilibrium being to the left and inadvertent oxidation not having occurred. Coulometry also verified that Feo was not present. Reduction at −800 mV demonstrated a one-electron reduction and the formation of Ferr as previously described.[9] Fer prepared in the absence of external reductant was used in Figure 3 and other experiments described below.

Reaction 2. This reaction represents the highly spontaneous disproportionation of 2Fer to Feo and Ferr assisted by ATP hydrolysis to form ADP and the subsequent binding of ADP and ATP to the latter two species, respectively. This reaction would account for the formation of Feo(ADP)_2 formed during nitrogenase catalysis and would provide for the formation of

dx.doi.org/10.1021/bi301547b | Biochemistry 2013, 52, 4791−4799

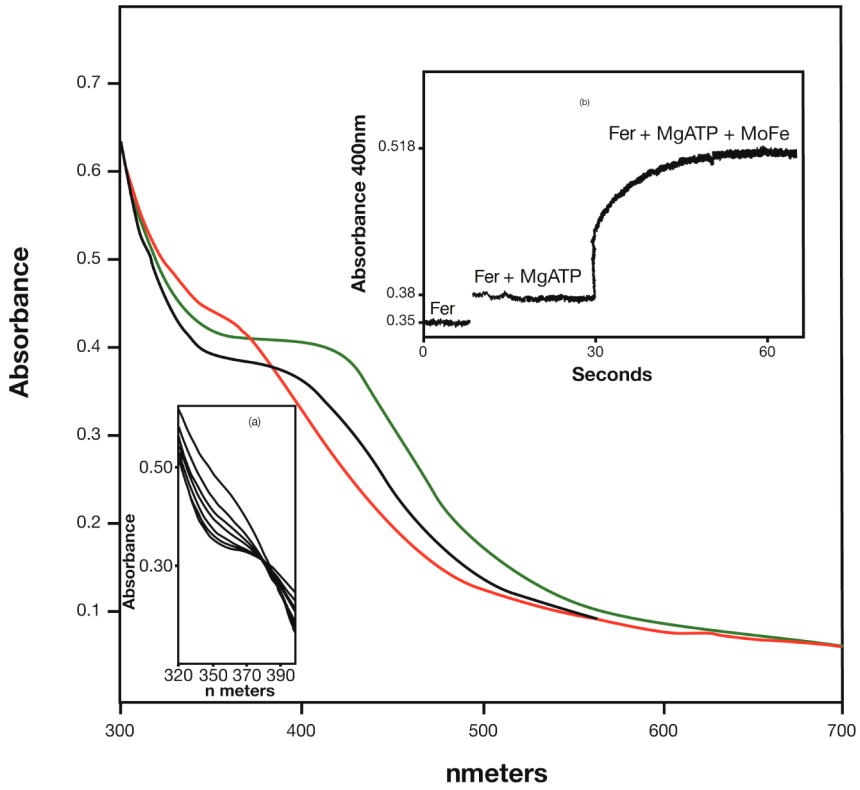

Figure 3. Main figure shows the optical spectrum of reduced but DT-free Fe protein (red). MgATP was then sequentially added as shown by the optical titration from 320 to 400 nm in Inset (a), which attained a final concentration of 5.0 mM giving the black spectrum. Inset (b) shows the reaction of reduced Fer in 5.0 mM MgATP, which has undergone Reaction 3, with 0.37 μM MoFe, and the resulting Fer(MgATP)$_2$ is oxidized according to Reaction 4 giving the green spectrum.

mechanistically viable Ferr(MgATP)$_2$ that would transfer two electrons to the MoFe and hydrolyze only two ATPs during catalysis. The overall ATP/2e value would be 4.0, resulting from the hydrolysis of two ATPs via reaction 2 and the subsequent hydrolysis of two ATPs during the two-electron transfer from Ferr(ATP)$_2$ to the MoFe protein. While of potential interest because Ferr(MgATP)$_2$ forms upon addition of MgATP, this reaction is not mechanistically viable because addition of ATP to Fer does not form ADP as confirmed by HPLC and by the absence of the optical spectrum of Feo(ADP)$_2$.

Reaction 3. This reaction represents the MgATP assisted disproportionation of Fer into Feo(ATP)$_2$ and Ferr. However, when the free energy change for reaction 3 was initially calculated,[9] the binding of MgATP to Ferr was not established and the free energy calculation did not include MgATP binding to Ferr. The results now show that MgATP binds to Ferr, making this reaction even more spontaneous ($K = 1.5 \times 10^{12}$ M^{-4}) than originally determined.

Figure 3 shows the optical spectrum of Fer (red) and the resulting spectrum (black) after MgATP is added. The original spectrum of Fer undergoes a decrease in absorbance from 300 to 390 nm and an increase from 390 to 550. The use of highly purified Fe protein free of MoFe protein was essential because the presence of ~nanomolar quantities MoFe causes immediate enzymatic turnover with formation of H_2 and Feo(ADP)$_2$ as shown by Reaction 4 and the top (green) spectrum in Figure 3.

$$2\text{Fer} + 4\text{MgATP} = 2\text{Feo(MgADP)}_2 + \text{H}_2 + 4\text{Pi} \qquad (4)$$

The green spectrum and coulometry show that catalytic oxidation of Fer by Reaction 4 did not occur but that Fer has undergone Reaction 3.

Close inspection of the ATP-modified spectrum of Fer (black) in Figure 3 shows similarities to Feo(ATP)$_2$ shown as the upper spectrum but is of a lower intensity. Using the spectrum of Ferr in Figure 1 and the spectrum of Feo(ATP)$_2$ previously published,[22] the black, modified spectrum of Fer in the presence of MgATP was resolved into a 1:1 mixture of Ferr(MgATP)$_2$ and Feo(ATP)$_2$, in accordance with Reaction 3. Coulometry gave a value of 0.47 $\pm$ 0.05 e/Fer, a value consistent with Reaction 3, suggesting an ATP-assisted disproportionation reaction with formation of Feo(MgATP)$_2$ and Ferr(MgATP)$_2$. The absence of an absorption band at 520 nm demonstrates the absence of the $S = 4$ spin state and the exclusive formation of Ferr ($S = 0$).

Figure 3(a) is an optical titration of Fer with MgATP and shows the stepwise conversion of Fer into Feo(MgATP)$_2$ and Ferr(MgATP)$_2$. The optical titration results are shown in Figure 4 along with corresponding EPR results from which an overall equilibrium constant of 1.5×10^{12} M^{-4} was calculated. The following set of reactions and previous results[9] for Reactions 5 and 1 combine to yield Reaction 6 giving an MgATP binding constant of 6.6×10^6 M^{-2}. This value represents modest MgATP binding and is slightly larger than that of 4.5×10^5 M^{-2} for ATP binding to Fer calculated previously[22] and known to be a functional species during nitrogenase catalysis.

dx.doi.org/10.1021/bi301547b | *Biochemistry* 2013, 52, 4791−4799

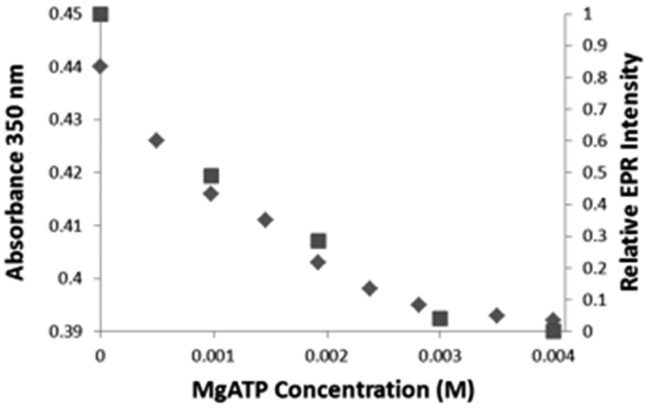

Figure 4. Plot of the optical titration results in Figure 3(a) monitored at 350 nm (♦). EPR amplitude at $g = 1.94$ (right axis) taken during the optical titration (■). An equilibrium constant of 1.5×10^{12} M^{-4} was determined.

$$2\text{Fer} + 4\text{MgATP} = \text{Feo}(\text{MgATP})_2 + \text{Ferr}(\text{MgATP})_2$$
$$K = 1.5 \times 10^{12}\text{M}^{-4} \quad (3)$$

$$\text{Feo}(\text{MgATP})_2 = \text{Feo} + 2\text{MgATP}$$
$$K = 1.5 \times 10^{-8}\text{M}^{2} \quad (5)$$

$$\text{Feo} + \text{Ferr} = 2\text{Fer} \qquad K = 2.9 \times 10^{2} \quad (1)$$

$$\text{Ferr} + 2\text{MgATP} = \text{Ferr}(\text{MgATP})_2$$
$$K = 6.6 \times 10^{6}\text{M}^{-1} \quad (6)$$

Addition of MgADP to Fer produced similar optical changes but they differ slightly from those with MgATP, indicating that MgADP causes a related reaction but perturbs the cluster differently than MgATP, consistent with the observed optical spectrum of $\text{Feo}(\text{ADP})_2$. The reaction required a higher concentration of MgADP than that shown in Figure 3a, suggesting that MgADP is less effective than MgATP in promoting the MgADP equivalent of Reaction 3. The results indicate that MgADP binding to Ferr is weaker than to Feo and Fer.

Reaction 3 shown as inset (a) in Figure 3 might suggest a mechanism where Fer disproportionation first occurs forming $\text{Ferr}(\text{MgATP})_2$, which then transfers two electrons to MoFe to initiate catalysis as shown as Inset (b) in Figure 3 with hydrolysis of two MgATPs. However, two factors argue against this possibility. The first is that Reaction 3 occurs over several seconds and this non-instantaneous rate could limit Reaction 3 as a viable step in nitrogenase catalysis unless it is catalyzed by its interaction with the MoFe protein.

The more serious problem is illustrated by Reactions 3−7, which show that Fer could produce the mechanistically viable $\text{Ferr}(\text{MgATP})_2$ by Reaction 3 and produce H_2 and hydrolyze two MgATPs. Reaction 4 requires the presence of MoFe protein to complete the catalytic step (Figure 3b) and $\text{Feo}(\text{MgATP})_2$ formed in Reaction 3 can be subsequently

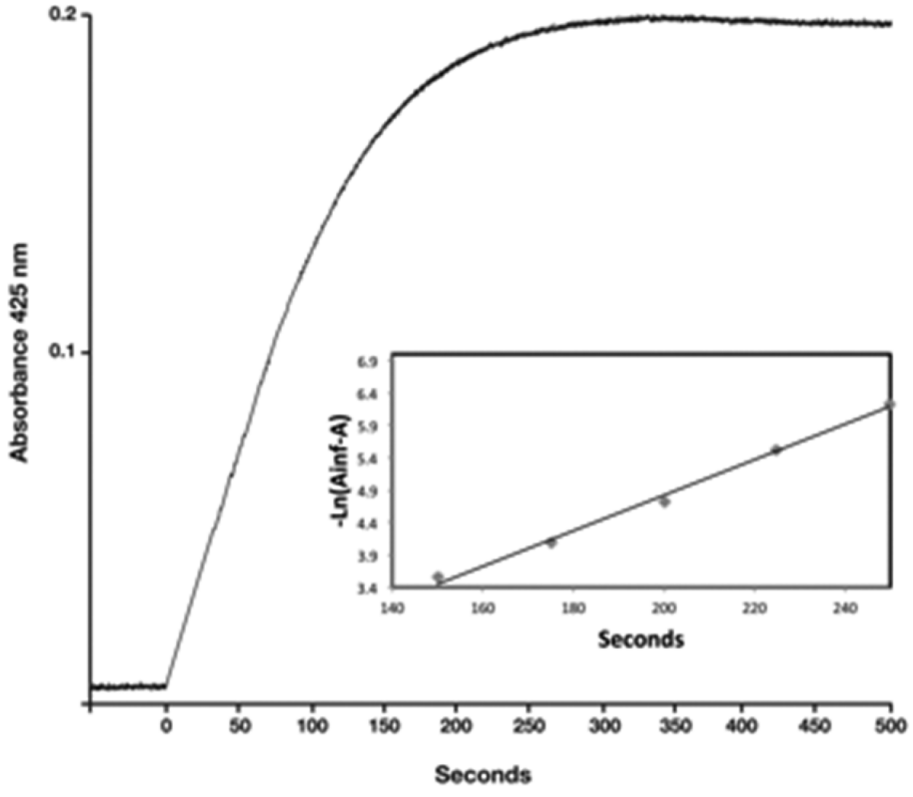

Figure 5. Reaction of Fer (no DT) with MoFe in the presence of 5.0 mM MgATP regeneration system monitored at 425 nm. 30.3 μM Fer and 1.12 nM MoFe in 0.05 M Tes pH 7.4 were equilibrated at 30 C and then MgATP regeneration system was added to 5.0 mM MgATP. A zero order reaction occurred to ~100 s from which a specific activity of 2514 nmol H_2/min-mg was calculated. A first order reaction from ~150 to 300 s occurred (see inset) as Fer was completely oxidized giving a specific activity of 2733 nmol H_2/min-mg. Ln(Ainf-A) is the ln of the absorbance after the reaction is completed minus the absorbance at a given time plotted as a function of time.

dx.doi.org/10.1021/bi301547b | *Biochemistry* 2013, 52, 4791−4799

reduced to Fer(MgATP)$_2$ and disproportionate again to form Feo(MgATP)$_2$ + Ferr(MgATP)$_2$

$$2\text{Fer} + 4\text{MgATP} = \text{Feo(MgATP)}_2 + \text{Ferr(MgATP)}_2 \quad (3)$$

$$\text{Ferr(MgATP)}_2 + 2\text{H}^+ = \text{Feo(MgADP)}_2 + \text{H}_2 + 2\text{MgADP}_2 + 2\text{Pi} \quad (4)$$

$$2\text{Fer} + 4\text{MgATP} = \text{Feo(MgATP)}_2 + \text{Feo(MgADP)}_2 + \text{H}_2 + 2\text{Pi} \quad (7)$$

Inspection of Reaction 7 shows the important result that the MgATP in the product Feo(MgATP)$_2$ has not been hydrolyzed, so the net utilization of MgATP is only 2MgATPs per H_2. This gives an ATP/2e value of 2.0 and not 4.0−5.0 as is observed from operation of the Fer protein cycle. This result combined with the marginal rate of Reaction 3 demonstrates that Reaction 3 cannot be a component reaction of nitrogenase catalysis using Fer. We conclude that even with the various interconversions of Fer outlined above that could produce Ferr(MgATP)$_2$ as a viable intermediate, none are feasible. Therefore, only single electron transfers are possible using Fer, which result in ATP/2e values of 4−5.

Having concluded that Reactions 1−3 are not mechanistically viable, we evaluated the relative catalytic rates of nitrogenase catalysis using Fer and Ferr to provide further insights into the reactivity of Fer and Ferr in the $S = 4$ and $S = 0$ spin states.

Fer as Electron Donor. Figure 5 shows the rate of Fer oxidation when reductant-free Fer and MoFe protein are first combined followed by addition of MgATP to initiate the reaction. This sequence of addition eliminates Reaction 3 from first occurring and assures that Fer is the initial reductant and not Ferr. The resulting rate profile consists of two regions: a linear zero order reaction and a first order reaction beginning about halfway through the reaction as Fer becomes limiting. From both kinetic regions, specific activities of 2514 and 2733 nmol H_2/mgMoFe-min were determined, respectively, compared to 2100 nmol H_2/mg.MoFe-min determined by assay procedures using DT. The ∼25% increase in specific activity could be due to a combined consequence of Feo reduction by DT, which is not instantaneous,[23,24] and nucleotide exchange. The rate of Fer oxidation varies linearly with a 15-fold variation of MoFe protein, indicating first order behavior in both proteins. Reaction 4 occurs at a MoFe protein concentration <1 nM and yields a dissociation constant of $<10^{-7}$ M^{-1}, a similar value reported from protein dilution studies.[25,26] Removal of such small amounts of MoFe protein requires rigorous purification of the Fe protein prepared by standard isolation procedures.

ATP/2e values of 4−5 were determined, demonstrating that the Fe protein transfers a single electron to MoFe protein with hydrolysis of two MgATPs. The reaction in Figure 5 consists of two cooperative binding and two dissociation steps of Fer to deliver two electrons to the MoFe protein and serves as a reference for reactions discussed next.

Ferr as Electron Donor. Figure 6a shows the rate of reaction of Ferr ($S = 4$) with MoFe protein initially in ∼4.0 mM Ti(III) during which Ti(III) was totally consumed. At 4.0 mM Ti(III), Ferr(MgATP)$_2$ is the functional Fe protein species but as Ti(III) is consumed, the reaction transitions into the

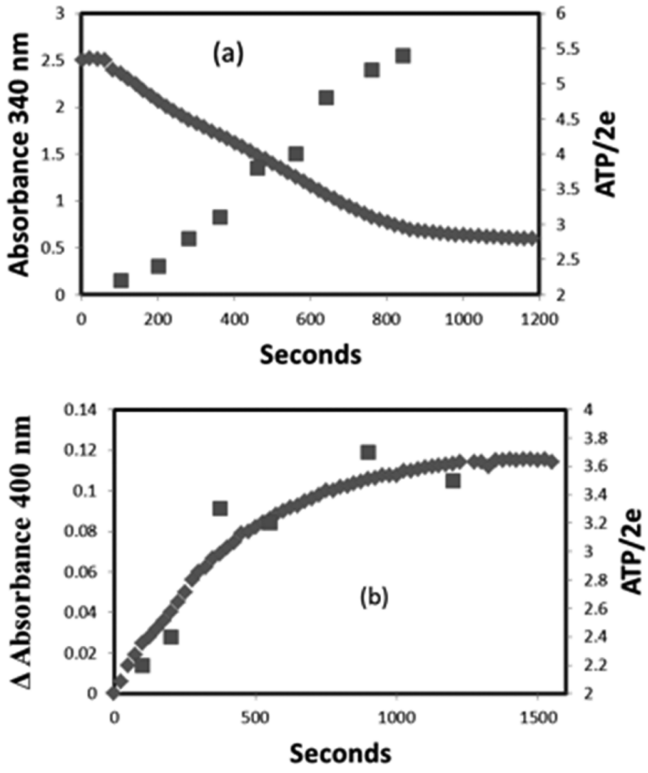

Figure 6. Oxidation of Ferr ($S = 4$) and Ferr ($S = 0$) by MoFe protein. (a) A 5/1protein ratio of Fe/MoFe in 0.050 M Mops pH 7.4 containing ∼4.0 mM Ti(III) was equilibrated at 30 °C and a concentrated MgATP regeneration system was added to initiate the reaction. The Ti(III) concentration was followed at 340 nm as the nitrogenase catalyzed reaction transferred electrons from Ti(III) through the Fe protein to form H_2. ATP hydrolysis was monitored by HPLC and shown as the ATP/2e ratio on the right axis. (b) 15 μM Ferr ($S = 0$) prepared by Reaction 3 was equilibrated at 30 °C with 3.2 nM MoFe in 0.05 M MOPS buffer pH 7.4 and MgATP was added to 5.0 mM to initiate the reaction. MgADP was measured by HPLC in separate but identical reactions at the indicated times and converted into ATP/2e values (right axis).

reaction dominated by Fer(MgATP)$_2$, with intermediate reaction simultaneously operating with both Ferr(MgATP)$_2$ and Fer(MgATP)$_2$. The rate of Ti(III) utilization is essentially constant demonstrating a constant rate of electron transfer from Ferr(MgATP)$_2$, Fer(MgATP)$_2$, or a mixture of both to the MoFe protein. However, ATP/2e values indicate that the rate of MgATP hydrolysis increases ∼2.5-fold under the same conditions. The ATP/2e values were determined in separate experiments with the reactions initiated at the indicated initial Ti(III) concentrations.[10] The results are consistent with a constant rate of electron transfer to the MoFe protein independent of the redox state of the Fe protein but with a ∼2.5-fold increase of MgATP hydrolysis during the reaction sequence.

Close inspection of Figure 6a reveals that near 500 s a small deviation in rate is observed. Analysis of the linear portion from 82 to 262 s and that from 442 to 722 s shows the former rate is ∼1.10 times faster than the latter. This result has been observed consistently in five separate experiments and suggests that Ferr(MgATP)$_2$ reacts slightly faster than Fer(MgATP)$_2$. This behavior is not seen in Figure 6b, which utilizes the $S = 0$ form of Ferr and suggests that the $S = 4$ state may react slightly faster than the $S = 0$ or Fer forms of the Fe protein.

dx.doi.org/10.1021/bi301547b | Biochemistry 2013, 52, 4791−4799

Figure 6b shows the kinetic profile for oxidation of Ferr(MgATP)$_2$ (S = 0), formed by Reaction 3, to form H_2 and hydrolyze MgATP (right axis). During this reaction, Ferr(MgATP)$_2$ is initially oxidized to Feo(MgADP)$_2$ with a measured ATP/2e value of ∼2.0. However, as the reaction proceeds, the concentration of Feo(MgADP)$_2$ increases and, with nucleotide exchange, immediately reacts with the remaining Ferr(MgATP)$_2$ to form 2Fer(MgATP)$_2$ (the reverse of Reaction 1a). Fer(MgATP)$_2$ then reacts according to Figure 5, producing an ATP/2e value of ∼5.0. The limiting ATP/2e values reported in Figure 6b are consistent with Ferr(MgATP)$_2$ transforming into Fer(MgATP)$_2$, with overall ATP/2e values increasing from ∼2.0 to ∼5.0 and giving an average cumulative ATP/2e value near 3.8.

The rate of electron transfer remains constant at 2.8 × 10^{-8} M/s up to ∼480 s but during this time interval the species change from Ferr(MgATP)$_2$ to a mixture of Ferr(MgATP)$_2$ and Fer(MgATP)$_2$ and finally to Fer(MgATP)$_2$ as the dominant species at ∼300 s. The rate of electron transfer is constant and independent of whether Ferr(MgATP)$_2$ or Fer(MgATP)$_2$ or both is the electron donor, but the rate of MgATP hydrolysis increases by a factor of ∼2.5 as the species distribution changes. The results are consistent with a single, two-electron step when Ferr(MgATP)$_2$ reacts but with two, single-electron steps for Fer(MgATP)$_2$ interacting with the MoFe protein with both reactions occurring at the same rate.

The results presented here and previously[10,12] show that the rate of nitrogenase catalysis is identical when Ferr (S = 4 or 0) or Fer (S = 1/2, 3/2) is the electron donor to the MoFe protein, even though the number of electrons transferred with each MoFe encounter differs by a factor of 2. Dissociation of the Fe protein *cannot* be the rate-limiting step because the rates should differ by a factor of 2 instead of being invariant. We conclude that the rate-limiting step occurs after the accumulation of two electrons within the MoFe protein as observed previously for nitrogenase turnover.[15−18]

Further information regarding the interaction of Fer and both forms of Ferr with the MoFe protein was obtained by measuring their rates of reactions at a MoFe concentration of 0.92 nM. Under these conditions, Fer is oxidized rapidly similar to Figure 5, but when either form of Ferr reacted under similar conditions, no Ferr oxidation occurred. The results actually showed that the $[Fe_4S_4]^0$ cluster was slowly removed, presumably by chelation of Fe^{2+} by the phosphate species present in the MgATP regeneration mixture. As the MoFe protein concentration is increased, cluster destruction is minimized and a low level of reactivity similar to Figure 6b is observed. Only when the MoFe protein concentration is >5-fold higher than the concentration where Fer rapidly reacts does the Ferr (S = 0) cluster react at a rate comparable to that of Fer. The Ferr (S = 4) species requires a slightly higher MoFe protein concentration to react, suggesting it has an even lower affinity for the MoFe protein than the Ferr (S = 0) cluster state.

DISCUSSION

Figure 1 shows that reduction of Fer by FSA to form Ferr is a simple method for forming Ferr with S = 0. FSA also reduces Feo first to Fer and then Fer →Ferr, with the former step being slightly faster. The value of using FSA is that it can be obtained in a pure state (>98%), the dry solid is not oxidized by air, and it does not have optical or EPR spectral properties that interfere with observing the various redox states of the Fe protein. Aqueous solutions are very slowly oxidized by O_2 and require anaerobic conditions.

FSA reacts at a similar low potential[27−29] as DT (SO_2^-) and raises the question, "why doesn't DT form the all-ferrous state?" The likely explanation is that FSA can be obtained in a pure state, whereas DT has significant amounts (>15%) of impurities.[21] If DT forms the all-ferrous state, the impurities oxidize it to Fer as previously proposed.[9] Formation of the all ferrous cluster using Ti(III) does not decompose DT and suggests that the S = 4 spin state cannot interact with DT or its impurities. However, the S = 0 form readily reacts with and destroys DT (Figure 2b). This result predicts that pure DT should readily form Ferr with S = 0.

The first report[9] of the all-ferrous cluster in the nitrogenase Fe protein used MV as reductant and consequently formed Ferr with S = 0. The S = 0 spin state also readily forms by reduction with the natural flavoprotein reductant.[12] Other studies used Ti(III) and formed Ferr with S = 4.[20] While it is important to clarify which form of the Ferr is being used in nitrogenase experiments, the results reported here show that all reduced forms of the Fe protein react with the MoFe protein at identical rates during catalysis. Nevertheless, a proper description of the redox state of the Fe protein used for catalysis is important in clarifying other aspects of nitrogenase reactivity. From the results reported previously[9,12] and confirmed and extended here, it is possible to form the all-ferrous Fe protein (S = 0) with reduced methyl viologen,[9] flavoprotein,[12] and now FSA or by Reaction 3.

The results reported here demonstrate an important aspect of nitrogenase catalysis. Electron transfer to the DT-reduced P-clusters (S = 0) of the MoFe protein from Fer with a spin state (S = 1/2 and 3/2) mixture,[30,31] Ferr with S = 4, or Ferr with S = 0 occurs at identical rates even though large spin state changes are normally associated with slow rates of reaction (see Figure 2). Oxidized P-clusters[32,33] also have spin state mixtures (S = 3/2, 5/2, etc.) as do reduced P-clusters.[34] The magnetic state flexibility of the P-clusters is likely responsible for accommodating the various spin states of the Fe protein to eliminate these kinetic barriers.

The prediction was previously made that interconversion from 2Fer → Feo + Ferr (Reaction 1) was unfavorable.[9] However, other results (Reactions 2 and 3) and Reaction 1b suggest that such interconversions to Ferr(MgATP)$_2$ are allowed and might be relevant to nitrogenase catalysis. These predictions have now been examined and indicate that, while the cluster state of Fer can be manipulated to form Ferr by addition of nucleotides, these interconversions do not take place prior to catalysis and are not relevant to nitrogenase catalysis when Fer is the reductant. The results show that Fer only functions as a single electron reductant during nitrogenase catalysis but two, cooperative, single electron interactions with the MoFe protein are required in a rate-determining step.[18,26]

The S = 0 all-ferrous cluster is readily formed by FSA (Figure 1) as well as by the natural nitrogenase reductant flavoprotein and is likely of physiologically importance. The facile interconversion of Fer occurs and forms Ferr(MgATP)$_2$, which catalytically transfers two electrons to MoFe during each catalytic step (Figures 3b and 6b). The current view of nitrogenase catalysis is that the Fe protein cycle delivers multiple electrons to the resting state of the MoFe protein by *identical* one-electron steps and that Feo(MgADP)$_2$ dissociation is the rate limiting step.[7,8] This view cannot be correct. Steady state kinetic measurements previously demonstrated that

dx.doi.org/10.1021/bi301547b | *Biochemistry* 2013, 52, 4791−4799

cooperative and therefore nonidentical reactions of the Fe protein occurred with the MoFe protein.[18,26] Fe protein cooperatively explained the behavior known as MoFe protein inhibition at high MoFe/Fe protein ratios.[18,26] Figure 6 and previous results[10,12] clearly show that the rate of nitrogenase catalysis is independent of whether the Fe protein functions to deliver two electrons to the MoFe protein in two one-electron steps or in one two-electron step. The significance of this result is that if dissociation of the "spent" $Feo(ADP)_2$ is rate limiting, then the rates of these two processes should differ by a factor of 2. Because they do not, we conclude that Fe protein dissociation is not the rate-limiting step. Previous nitrogenase turnover results show that the rate-limiting step occurs as an accumulation of two electrons and establish that nitrogenase turnover requires a two-electron step.[15−18] The present results suggest that accumulation of two electrons or their internal electron transfer within the MoFe protein is responsible for the rate-limiting step during nitrogenase catalysis. This conclusion is consistent with a gating process occurring within the MoFe protein that is triggered by the Fe−MoFe protein interaction prior to electron transfer[13,14] to reducible substrate, but the results presented here suggest it is a two-electron gating process. The redox state of the reduced Fe protein therefore controls the amount of ATP consumed during electron transfer but does not control the rate of catalysis. The rate of catalysis remains constant and is determined by the MoFe protein and independent of the redox state of the Fe protein.

AUTHOR INFORMATION

Corresponding Author

*E-mail: gdwatt@chem.byu.edu. Tel: 801-422-4561.

Funding Statement

This research was supported by Grant 82-CRCR-1−1122 from the USDA/SEA Competitive Grants Program to G. D. W. and partially by the College of Mathematics and Physical Sciences at Brigham Young University.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

Feo, Fer, and Ferr the $[Fe_4S_4]^{2+}$, $[Fe_4S_4]^{1+}$, and $[Fe_4S_4]^0$ cluster states of the Fe protein from *Azotobacter vinelandii*, respectively; MV methyl viologen; DT sodium dithionite; MOPS 3-(*N*-morpholino)propanesulfonic acid; Ti(III) Ti(III)-citrate solution

REFERENCES

(1) Burgess, B. K., and Lowe, D. J. (1996) Mechanism of molybdenum nitrogenase. *Chem. Rev. 96*, 2983−3011.

(2) Seefeldt, L. C., Hoffman, B. M., and Dean, D. R. (2009) Mechanism of Mo-dependent nitrogenase. *Annu. Rev. Biochem. 78*, 701−729.

(3) Georgiadis, M. M., Komiya, H., Chakrabarti, Woo, P. D., Kornuc, J. J, and Rees, D. C. (1992) Crystallographic structure of the nitrogenase iron protein from Azotobacter vinelandii. *Science 257*, 1653−1659.

(4) Schlessman, J. L., Woo, D., Joshua-Tor, L., Howard, J. B., and Rees, D. C. (1998) Crystallograpic structure of the nitrogenase iron protein from *Azotobacter vinelandii*. *J. Mol. Biol. 280*, 669−685.

(5) Kim, J., and Rees, D. C. (1992) Crystallographic structure and functional implications of the nitrogenase molybdenum-iron protein from *Azotobacter vinelandii*. *Nature 360*, 553−560.

(6) Peters, J. W., Stowell, M. H. B, Soltis, S. M., Finnegan, M. B., Johnson, M. K., and Rees, D. C. (1997) Redox-dependent structural changes in the nitrogenase P-cluster during turnover. *Biochemistry 36*, 1181−1187.

(7) Thorneley, R. N. F, and Lowe, D. J. (1983) Nitrogenase of Klebsiella pneumonia, Kinetics of the dissociation of oxidized iron protein from molybdenum-iron protein: identification of the rate-limiting step for substrate reduction. *Biochem. J. 393*, 403−409.

(8) Thorneley, R. N. F., and Lowe, D. J. (1984) *Molybdenum Enzymes* (Spiro, T., Ed.) p 221, Vol *1*, Wiley-Interscience, New York.

(9) Watt, G. D, and Reddy, K. R. N. (1994) Formation of an all ferrous Fe4S4 Cluster in the Iron Protein Component of *Azotobacter vinelandii* Nitrogenase. *J. Inorg.Biochem. 53*, 281−294.

(10) Nyborg, A. C., Erickson, J. A., Johnson, J. L., Gunn, A., Truscott, S. M., and Watt, G. D. (2000) Reactions of *Azotobacter vinelandii* nitrogenase using Ti(III) as reductant. *J. Inorg. Biochem. 78*, 371−381.

(11) Erickson, J. A., Nyborg, A. C., Johnson, L. L., Truscott, S. M., Gunn, A., Nordmeyer, F. R., and Watt, G. D. (1999) Analysis of steady state Fe and MoFe protein interactions during nitrogenase catalysis. *Biochemistry 38*, 14279−14285.

(12) Lowery, T. J., Wilson, P. E., Zhang, B., Bunker, J., Harrison, R. G., Nyborg, A. C., Thiriot, D., and Watt, G. D. (2006) Flavodoxin hydroquinone reduces Azotobacter vinelandii Fe protein to the all-ferrous redox state with a S = 0 spin state. *Proc. Natl. Acad. Sci. U.S.A. 106*, 17131−17136.

(13) Danyal, K., Mayweather, D., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2010) Conformational gating of electron transfer from the nitrogenase Fe protein to MoFe protein. *J. Am. Chem. Soc. 26*, 6894−6895.

(14) Danyal, K., Dean, D. R., Hoffman, B. M., and Seefeld, L. C. (2011) Electron transfer within nitrogenase: evidence for a deficit-spending mechanism. *Biochemistry 50*, 9255−9363.

(15) Evans, M. C. W., and Albrecht, S. L. (1974) Determination of the applied oxidation-reduction potential required for substrate reduction by chromatium nitrogenase. *Biochem. Biophys. Res. Commun. 61*, 1187−1192.

(16) Lough, S., Burns, A., and Watt, G. D. (1983) Redox reactions of the nitrogenase complex from *Azotobacter vinelandii*. *Biochemistry 22*, 4062−4000.

(17) Hallenbeck, P. C. (1983) Nitrogenase reduction by electron carriers-influence of redox potential on activity and the ATP/2e ratio. *Arch. Biochem. Biohys. 220*, 657−662.

(18) Johnson, J. L., Nyborg, A. C., Wilson, P. E., Tolley, A. M., Nordmeyer, F. R., and Watt, G. D. (2000) Analysis of steady state Fe and MoFe protein interactions during nitrogenase catalysis. *Biochem. Biophys. Acta. 1543*, 24−35.

(19) Johnson, J. L., Tolley, A. M., Erickson, J. A., and Wat, G. D. (1996) Steady-state kinetic studies of dithionite utilization, component protein interaction, and the formation of an oxidized iron protein intermediate during Azotobacter vinelandii nitrogenase catalysis. *Biochemistry 35*, 11336−11342.

(20) Angove, H. C., Yoo, S. J., Burgess, B. K., and Munck, E. (1997) Mossbuer and EPR evidence for an all-ferrous Fe4S4 cluster with S=4 in the Fe protein of nitrogenase. *J. Am. Chem. Soc. 119*, 8730−8731.

(21) McKenna, C. E., Gutheil, W. G., and Song, W. (1991) A method for preparing analytically pure sodium dithionite. Dithionite quality and observed nitrogenase-specific activities. *Biochim. Biophys. Acta 1075*, 109−117.

(22) Watt, G. D., Wang, Z.-C., and Knotts, R. R. (1986) Redox reactions of and nucleotide binding to the iron protein of *Azotobacter vinelandii*. *Biochemistry 25*, 8156−8162.

(23) Thorneley, R. N. F., Yates, M. G., and Lowe, D. J. (1976) Nitrogenase of *Azotobacter chroococcum* kinetics of the reduction of oxidized iron protein by sodium dithionite. *Biochem. J. 155*, 137−142.

(24) Wilson, P. E., Bunker, J., Lowery, T. J., and Watt, G. D. (2004) Reduction of nitrogenase Fe protein from *Azotobacter vinelandii* by dithionite: quantitative and qualitative effects of nucleotides, temperature, pH and reaction buffer. *Biophys. Chem. 109*, 305−324.

(25) Thorneley, R. N. F., Eady, R. R., and Yates, M. G. (1975) Nitrogenases of *Klebsiella pneumonia* and *Azotobacter chroococum*. Complex formation between the component proteins. *Biochim. Biophys. Acta 403*, 269−284.
(26) Johnson, J. L., Nyborg, A. C., Wilson, P. E., Tolley, A. M., Nordmeyer, F. R., and Watt, G. D. (2000) Mechanistic interpretation of the dilution effect for *Azotobacter vinelandii* and *Clostridium pasteurianum* nitrogenase catalysis. *Biochem.Biophys. Acta. 1543*, 36−46.
(27) Shashoua, V. E. (1964) Formadine sulfinic acid as a biochemical reducing agent. *Biochemistry 3*, 1719−1720.
(28) Mayhew, S. G. (1978) The redox potential of dithionite and SO_2^- from equilibrium reactions with flavodoxins, methyl viologen and hydrogen plus hydrogenase. *Eur. J. Biochem. 85*, 535−547.
(29) Watt, G. D., and Burns, A. (1975) The thermochemical characterization of sodium dithionite, flavin mononucleotide, flavin-adenine dinucleotide and methyl and benzyl viologens as low-potential reductants for biological systems. *Biochem. J. 152*, 33−37.
(30) Lindahl, P. A., Orme-Johnson, W. H., Kent, T. A., Day, E. P., and Munck, E. (1985) Mossbauer, Electron-Paramagnetic Resonance and Magnetization studies of the Azotobacter vinelandii Fe Protein. Evidence for a $[4Fe\text{-}4S]^{1+}$ cluster with spin S = 3/2. *J. Biol. Chem. 260*, 1160−1173.
(31) Watt, G. D., and McDonald, J. W. (1985) Electron paramagnetic resonance spectrum of the iron protein of nitrogenase: existence of a g = 4 spectral component and its effect on spin quantization. *Biochemistry 24*, 7226−7231.
(32) Pierik, A. J., Wassink, H., Haaker, H., and Hagen, W. R. (1993) Redox properties and EPR spectroscopy of the P clusters of Azotobacter vinelandii MoFe protein. *Eur. J. Biochem. 212*, 51−61.
(33) Tittsworth, R. C., and Hales, B. J. (1993) Detection of EPR signals assigned to the 1-equiv-oxidized P-clusters of the nitrogenase MoFe-protein from Azotobacter vinelandii. *J. Am. Chem. Soc. 115*, 9763−9767.
(34) Chan, J. M., Christiansen, J., Dean, D. R., and Seefeldt, L. C. (1999) Spectroscopic evidence for changes in the redox state of the nitrogenase P-cluster during turnover. *Biochemistry 38*, 5779−5785.