Evidence for a Two-Electron Transfer Using the All-Ferrous Fe Protein during Nitrogenase Catalysis*

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The nitrogenase-catalyzed $H_2$ evolution and acetylene-reduction reactions using Ti(III) and dithionite (DT) as reductants were examined and compared under a variety of conditions. Ti(III) is known to make the all-ferrous Fe protein ($[\text{Fe}_4\text{S}_4]^{0}$) and lowers the amount of ATP hydrolyzed during nitrogenase catalysis by approximately 2-fold. Here we further investigate this behavior and present results consistent with the Fe protein in the $[\text{Fe}_4\text{S}_4]^{0}$ redox state transferring two electrons ($[\text{Fe}_4\text{S}_4]^{2+}/[\text{Fe}_4\text{S}_4]^{0}$) per MoFe protein interaction using Ti(III) but transferring only one electron ($[\text{Fe}_4\text{S}_4]^{1+}/[\text{Fe}_4\text{S}_4]^{0}$) using DT. MoFe protein specific activity was a function of Fe:MoFe protein ratio for both a one- and a two-electron transfer reaction, and nearly identical curves were obtained. However, Fe protein specific activity curves as a function of MoFeFe protein ratio showed two distinct reactivity patterns. With DT as reductant, typical MoFe inhibition curves were obtained for operation of the $[\text{Fe}_4\text{S}_4]^{2+}/[\text{Fe}_4\text{S}_4]^{1+}$ redox couple, but with Ti(III) as reductant the $[\text{Fe}_4\text{S}_4]^{2+}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple was functional and MoFe inhibition was not observed at high MoFeFe protein ratios. With Ti(III) as reductant, nitrogenase catalyzed the formation of acetylene, yielding a $V_{\text{max}}$ for the Fe protein specific activity of about 3200 nmol of $H_2$ min$^{-1}$ mg$^{-1}$ Fe protein, significantly higher than for reactions conducted with DT as reductant. Lag phase experiments (Hageman, R. V., and Burris, R. H. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2699–2702) were carried out at MoFeFe protein ratios of 100 and 300 using both DT and Ti(III). A lag phase was observed for DT but, with Ti(III) product formation, began immediately and remained linear for over 30 min. Activity measurements using Av-Cp heterologous crosses were examined using both DT and Ti(III) as reductants to compare the reactivity of the $[\text{Fe}_4\text{S}_4]^{2+}/[\text{Fe}_4\text{S}_4]^{1+}$ and $[\text{Fe}_4\text{S}_4]^{2+}/[\text{Fe}_4\text{S}_4]^{0}$ redox couples and both were inactive. The results are discussed in terms of the two-electron transfer reactions per MoFe protein encounter using the $[\text{Fe}_4\text{S}_4]^{2+}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple with Ti(III) as reductant.

Nitrogenase is a two-component metalloprotein system that carries out the reduction of dinitrogen to ammonia under mild conditions (1 atm, 30 °C) and can also catalyze the reduction of a number of other reducible substrates such as $H^+$, acetylene, etc. (1). The generally accepted view of nitrogenase catalysis with dithionite (DT) as reductant is that the Fe protein ($\gamma$$_{2}$, $M_r$ ~ 63,000) transfers one electron to the MoFe protein ($\alpha_2\beta_2$, $M_r$ ~ 230,000) using the $[\text{Fe}_4\text{S}_4]^{2+}/[\text{Fe}_4\text{S}_4]^{1+}$ redox couple with concomitant hydrolysis of two ATPs per Fe-MoFe protein interaction (1–4). Using this redox couple, four to five ATPs are hydrolyzed for each pair of electrons transferred to the MoFe protein (5, 6). When sufficient electrons have accumulated on the MoFe protein, the bound substrate ($N_2$, $H_2$, acetylene, etc.) is reduced to product at the FeMoco cofactor center located within the $\alpha$ subunit of the MoFe protein. Eight electrons are typically required to reduce dinitrogen and two protons to ammonia and $H_2$ ($N_2 + 8H^+ + 8e^- = 2NH_3 + H_2$) accompanied by the hydrolysis of 16–20 ATPs. Nitrogenase also reduces a variety of other non-physiological substrates using DT as reductant with similar ATP requirements.

With the discovery and characterization (7–11) of the all-ferrous Fe protein ($[\text{Fe}_4\text{S}_4]^{0}$) and identification of conditions that allow operation of the $[\text{Fe}_4\text{S}_4]^{2+}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple to function during catalysis (10, 11), new nitrogenase catalytic reactivity has been observed. For example, using 5–7 mm Ti(III) as reductant, nitrogenase is active and the ATP utilization per electron pair transferred (ATP/2e$^-$) decreases from 4–5, typically observed with DT, to values near 2 (10, 11). This behavior was proposed to arise from operation of the $[\text{Fe}_4\text{S}_4]^{2+}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple with transfer of two electrons to the MoFe protein accompanied by hydrolysis of only two ATPs. Utilization of about one-half as much ATP per Fe protein is the impetus for studying how $[\text{Fe}_4\text{S}_4]^{0}$ interacts with and transfers two electrons to the MoFe protein yet requires hydrolysis of only two molecules of ATP per Fe protein cycle. To better understand this two-electron transfer process and gain insights into the role of the MoFe protein in accommodating both one- and two-electron transfer reactions and coupling them to substrate reduction, we examined several aspects of nitrogenase catalysis involving use of both the $[\text{Fe}_4\text{S}_4]^{2+}/[\text{Fe}_4\text{S}_4]^{1+}$ and the $[\text{Fe}_4\text{S}_4]^{2+}/[\text{Fe}_4\text{S}_4]^{0}$ redox couples.

Such characteristic reactions as component protein titrations (6, 12), MoFe inhibition (6, 12), the dilution effect (13–16), the delay in product formation (the lag phase) at high MoFe:Fe

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‡ The abbreviations used are: DT, sodium dithionite; MoFe protein, molybdenum iron protein; Fe protein, iron protein; $[\text{Fe}_4\text{S}_4]^{2+}$, oxidized Fe protein; $[\text{Fe}_4\text{S}_4]^{0}$, singly reduced Fe protein; $[\text{Fe}_4\text{S}_4]^{1+}$, all-ferrous Fe protein; Ti(III), titanium (III) citrate; Av1, A. vinelandii MoFe protein; Av2, A. vinelandii Fe protein; Cp1, Clostridium pasteurianum MoFe protein; Cp2, C. pasteurianum Fe protein; Ac, A. chroococcum; Kp, K. pneumoniae; ATP, MgATP.

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protein ratios (17, 18), and inhibition due to Av-Cp heterologous cross reactions (19–21) have been studied. Each of these reactions is established and has been important in developing an understanding of nitrogenase catalysis using DT as reductant, where only the $[\text{Fe}_4\text{S}_4]^{2-}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple is functional. With the discovery of the $[\text{Fe}_4\text{S}_4]^{0}$ redox state of the Fe protein (7) and the demonstration that the $[\text{Fe}_4\text{S}_4]^{2-}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple functions during catalysis (10), it is of interest to evaluate these characteristic nitrogenase reactions during nitrogenase catalysis using the $[\text{Fe}_4\text{S}_4]^{2-}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple capable of transferring two electrons from the Fe protein to the MoFe protein. Here we describe several fundamental nitrogenase reactions using Ti(III) as reductant under conditions where the $[\text{Fe}_4\text{S}_4]^{2-}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple operates and compare the results with those obtained using DT as reductant, where only the $[\text{Fe}_4\text{S}_4]^{2-}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple can operate.

MATERIALS AND METHODS

All reactions involving the storage and handling of air-sensitive solids or solutions were conducted in a Vacuum Atmospheres glove box under $N_2$ ($O_2 < 0.1$ ppm). TiCl$_3$ was purchased from Aldrich as a 10% TiCl$_3$ solution in concentrated HCl. Water and HCl were removed to provide solid anhydrous TiCl$_3$ (evap) by allowing the solution to stand in a vacuum desiccator over anhydrous CaCl$_2$ and NaOH while inside a vacuum atmospheres glove box (11). Stock 83 mM Ti(III) in 0.20 mM citrate and 0.30 mM Tris, pH 8, solutions were prepared in the glove box, standardized optically ($e_{280} = 730$ M$^{-1}$ cm$^{-1}$), and used for the experiments described below (11).

Azotobacter vinelandii MoFe and Fe proteins with specific activities of 1800–2000 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ component protein, determined from dithionite assays, were prepared and characterized as described previously (22). Rick Baer at Exxon Research and Engineering Corporation prepared Cp component proteins with comparable activities. The Li27Δ mutant was a gift from Lance Seefeldt at Utah State University and was prepared as previously reported (23). Standard nitrogenase assays were conducted using both dithionite and Ti(III) as reductants in 5.0- or 12-ml vials under the conditions outlined below using limiting ATP and/or the ATP regeneration system under argon or 10% acetylene gas. When the headspace gas due to the removal of consecutive 0.2-ml aliquots. Dilution experiments were carried out as described previously using 50 mM DT reactions with increasing Fe:MoFe protein ratios above 1.0–2.0. This result was also observed when low Ti(III) concentrations (1.0–2.0) were used, due to the predominance of the $[\text{Fe}_4\text{S}_4]^{2-}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple (data not shown). At a MoFe:Fe protein ratio of about 1.0–2.0 with DT, the Fe protein specific activity for H$_2$ formation was at a maximum at 1850 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ Fe protein but declined to 20% of this value at a MoFe:Fe protein ratio of 7.0 mM Ti(III) as reductant. At 7.0 mM Ti(III), inhibition by this reductant is minimal, but the $[\text{Fe}_4\text{S}_4]^{2-}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple is fully operative (11). Under both sets of conditions, the MoFe protein activity curves were fitted by non-linear least squares and gave $K_m$ values for MoFe protein activity of 6.0 ± 0.2 and 6.2 ± 1.2 for DT and Ti(III), respectively. The $V_{\text{max}}$ values are quite comparable at 582 ± 37 and 582 ± 37 μmol min$^{-1}$ (2240 ± 40 and 2520 ± 160 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ MoFe protein) for DT and Ti(III), respectively. Although similar $V_{\text{max}}$ values are obtained that suggest nearly identical behavior, the use of DT and Ti(III) produced distinct ATP utilization patterns characterized by ATP2e values near 4 and 2, respectively, consistent with previous results (10, 12).

RESULTS

MoFe Protein Titration with Fe Protein—Fig. 1 shows the MoFe protein activity curves for H$_2$ evolution as a function of the Fe:MoFe protein ratio using either 20 mM DT or 7 mM Ti(III) as reductant. At 7.0 mM Ti(III), inhibition by this reductant is minimal, but the $[\text{Fe}_4\text{S}_4]^{2-}/[\text{Fe}_4\text{S}_4]^{0}$ redox couple is fully operative (11). Under both sets of conditions, the MoFe protein activity curves were fitted by non-linear least squares and gave $K_m$ values for MoFe protein activity of 6.0 ± 0.2 and 6.2 ± 1.2 for DT and Ti(III), respectively. The $V_{\text{max}}$ values are quite comparable at 582 ± 37 and 582 ± 37 μmol min$^{-1}$ (2240 ± 40 and 2520 ± 160 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ MoFe protein) for DT and Ti(III), respectively. Although similar $V_{\text{max}}$ values are obtained that suggest nearly identical behavior, the use of DT and Ti(III) produced distinct ATP utilization patterns characterized by ATP2e values near 4 and 2, respectively, consistent with previous results (10, 12). Nearly identical behavior was seen for acetylene reduction (data not shown). For the acetylene reduction reaction using either DT or Ti(III), about 10% of the total electron flow is directed toward H$_2$ evolution even though the amount of ATP hydrolyzed is 2-fold higher using DT.

Fe Protein Titration with MoFe Protein—Fig. 2 compares the Fe protein specific activity as a function of the MoFe:Fe protein ratio for H$_2$ formation using DT and Ti(III) and shows that the Fe protein specific activity varied with DT and Ti(III) produced distinct ATP utilization patterns characterized by ATP2e values near 4 and 2, respectively, consistent with previous results (10, 12). Near identical behavior was seen for acetylene reduction (data not shown). For the acetylene reduction reaction using either DT or Ti(III), about 10% of the total electron flow is directed toward H$_2$ evolution even though the amount of ATP hydrolyzed is 2-fold higher using DT.

FIG. 1. The MoFe protein specific activity (H$_2$ evolution) as a function of Fe:MoFe protein ratio using Ti(III) and DT. The data (○) are for 20 mM DT reactions with increasing Fe:MoFe protein ratios. The MoFe protein concentration is 1.13 μM and the $K_m$ and $V_{\text{max}}$ values are 6.0 ± 0.2 and 568 ± 10 μmol min$^{-1}$ (2240 ± 40 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ MoFe protein), respectively. The data (■) are for 7 mM Ti(III) reactions with increasing Fe:MoFe protein ratio. The MoFe protein concentration is 0.98 μM and the $K_m$ and $V_{\text{max}}$ values are 6.2 ± 1.2 and 582 ± 37 μmol min$^{-1}$ (2520 ± 160 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ MoFe protein), respectively.
The rate of H$_2$ production is shown over a 25-min interval, and Ti(III) conducted with a MoFe:Fe protein ratio of 300:1. This rate is constant, even beyond the 25-min period of examination. The small positive y intercept is seen in all of the experiments we have conducted. Control reactions containing Ti(III) and the nitrogenase proteins but no ATP regeneration system were used to correct for background H$_2$ produced by Ti(III) during the course of the catalytic reaction, but it appears that the amount of H$_2$ produced during nitrogenase turnover may be slightly larger than these controls. ATP hydrolysis also begins immediately upon initiating the reaction and quickly reaches a steady state. Division of the ATP hydrolysis rate by the rate of product formation gives ATP/2e values of near 2. The Fe protein specific activity using Ti(III) at a 300:1 MoFe:Fe protein ratio was 4100 ± 50 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ Fe protein, which is consistent with that found in Fig. 2.

Also shown in Fig. 3 is a comparison reaction under the same conditions with DT as reductant. The lag phase of 1.3 ± 0.1 min is much shorter than the 4.3-min lag phase of Hageman and Burris (17) at a ratio of 100:1 MoFe:Fe protein ratio. To further investigate the reason for this behavior, an identical lag phase experiment was carried out under identical conditions to duplicate the original experiment reported by Hageman and Burris (17). Using a 100:1 MoFe:Fe protein ratio, we were not able to duplicate the 4.3-min lag phase but instead measured only a 0.4-min lag (data not shown) at the same Fe protein, ATP, and DT concentrations reported by Hageman and Burris (17). This difference could be attributable to the low Fe protein specific activity of about 130 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ Fe protein obtained from their slope, whereas the Fe protein activity using DT and 300:1 and 100:1 MoFe:Fe protein ratios was about 2000 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ Fe protein. The greater the Fe protein specific activity, the faster electron transfer occurs to the MoFe protein, resulting in a shorter lag phase. Because the activity of our protein was significantly greater than that used by Hageman and Burris, it was necessary to increase the ratio to 300:1 MoFe:Fe protein to reliably measure a lag phase of 1.3 min with DT as reductant.

To more closely replicate the results of Hageman and Burris, Fe protein with an activity of about 300 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ Fe protein, determined by assays using DT and the ATP regeneration system, was used. Fig. 4 compares the results obtained using this partially active Fe protein with both DT and Ti(III) as reductants. A lag phase of 4.1 ± 0.7 min was obtained using DT at a MoFe:Fe protein ratio of 100:1, a value comparable to that reported by Hageman and Burris. In contrast, no lag phase was observed under the same conditions using Ti(III), indicating that apparently the active portion of partially active
Fe protein concentration at a fixed Fe:MoFe protein ratio that gave results comparable to both Kp and Ac (25). The dilution effect was also recently examined with both Av and Cp formation between the nitrogenase component proteins. The and proposed that low protein concentrations prevent complex formation previously reported using DT as reductant (13, 19–21).

Results obtained with DT were identical to those previously reported (19, 21). showing that the Av1-Cp2 and Cp1-Av2 combinations are totally inactive at 1:1 ratios. When these same combinations react using Ti(III), both combinations were inactive as well.

To determine if the Av and Cp Fe proteins were interacting in the same manner regardless of their redox state, duplicate heterologous combinations at 1:1 protein mixtures were examined. One reaction contained Ti(III) and the other DT under normal assay conditions. After the reaction was initiated, the proteins were removed for native gel electrophoresis. Control reactions containing Av1-Av2 and Cp1-Cp2 were also prepared, and samples were removed for native electrophoretic analysis under identical conditions. Using a 1:1 Fe:MoFe protein ratio assured that, if a complex formed, the Fe protein band would disappear and the MoFe protein band would be shifted. Both the Ti(III) and DT assays gave a shifted MoFe protein band near 300 kDa and the Fe protein band disappeared. In contrast, no complex formation was observed using either the Ti(III) or the DT with a Cp1-Av2 mixture, which is consistent with previous results (19, 21).

In addition to these heterologous cross reactions, reaction between the L127Δ Fe protein (which mimics the ATP hydrolysis transition state) (23) and Av1 was investigated. Although not a true heterologous cross, this combination forms an inactive, tight complex similar to the heterologous crosses above. No product was measured when the L127Δ Fe protein was mixed at a 1:1 ratio with native Av1 in the presence of 7.0 mM Ti(III). Native Electrophoretic analysis of this mixture showed that the L127Δ Fe protein band disappeared and an Av1 protein band was once again shifted up to about 300 kDa, indicative of complex formation. Similar results were obtained for DT.

**DISCUSSION**

Characteristic nitrogenase reactions were conducted to determine if differences in reactivity of the Fe protein occur depending on whether DT or Ti(III) is used as reductant during nitrogenase catalyzed H2 evolution and acetylene reduction. Under all conditions, the Fe protein could only be reduced by one electron using DT and consequently only the [Fe3S4]2+/[Fe3S4]3+ redox couple operated, transferring a single electron to the Mo protein. However, using Ti(III) as reductant under proper conditions, the Fe protein was reduced to the [Fe3S4]1+ state and the [Fe3S4]2+/[Fe3S4]3+ redox couple operated, transferring two electrons per MoFe-Fe interaction. Comparison of the results obtained using these two reductants provides information about how the different Fe protein redox couples operate.
ate during nitrogenase catalysis and how they influence electron transfer and ATP hydrolysis during the interaction of the two component proteins.

The titration of the MoFe protein with Fe protein (Fig. 1) was conducted under conditions where either the $[\text{Fe}_4\text{S}_4]^{2+/1+}$ (DT) or the $[\text{Fe}_4\text{S}_4]^{2+/0}$ (Ti(III)) redox couple functions, and similar $V_{\text{max}}$ values near 580 μmol of H$_2$ min$^{-1}$ were observed. At these high Fe:MoFe protein ratios, product formation occurs at the same maximal rate and is independent of which redox state of the Fe protein is functioning. This is understandable, because in either case the Fe protein saturates the MoFe protein and the rate of product formation is limited by the rate at which the MoFe protein processes electrons to product. At high Fe protein concentrations, the $[\text{Fe}_4\text{S}_4]^{2+/1+}$ redox couple transfers two electrons via two separate Fe:MoFe interactions at a rate comparable to a single two-electron transfer using the $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple. However, it is important to recognize that, although the maximum rate of reduced product formation is constant and independent of the operating Fe protein redox couple, operation of the $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple causes a 2-fold decrease in ATP utilization.

It is of interest now to consider the behavior produced by these two different reductants shown in Fig. 1 as the Fe:MoFe protein ratio decreases. At Fe:MoFe protein ratios below complete saturation of the MoFe protein, the rate at which the MoFe protein generates product is essentially identical and independent of the redox couple that is operating. Stated differently, the transfer of two electrons via two, single-electron steps using the $[\text{Fe}_4\text{S}_4]^{2+/1+}$ redox couple occurs at the same rate as the transfer of two electrons via one two-electron step using the $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple. However, at all ratios studied, the ATP requirement was decreased by 2-fold using the $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple. The results of Fig. 1 suggest that the electron flux through the MoFe protein cannot be increased using the two electron $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple, because the MoFe protein controls the rate of electron transfer, but the ATP hydrolysis can be decreased by 2-fold because in this case the Fe protein redox state controls the amount of ATP required in the reaction.

The results in Fig. 5 are consistent with that just discussed by showing that the affinity of the Fe protein for the MoFe protein in either of its reduced states ($[\text{Fe}_4\text{S}_4]^{1+}$ or $[\text{Fe}_4\text{S}_4]^0$) is invariant with concentration over the range investigated. Combining the results of Figs. 1 and 5 shows that at a 1:1 Fe:MoFe protein ratio near 0.25 μM, the rate of electron transfer through the MoFe protein is constant and independent of whether a one- or a two-electron transfer reaction occurs from the Fe protein. Additionally, the dilution data show that the rate of product formation at a 1:1 Fe:MoFe protein ratio or any other protein ratio is comparable for the two redox couples over a wide range of concentrations.

In contrast to the results just discussed, a much different behavior is observed when the Fe protein is titrated with the MoFe protein. The Fe protein specific activity curve using DT as reductant has a peak activity of about 2000 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ Fe protein at a MoFe:Fe protein ratio of about 1:1 to 2:1. At MoFe:Fe protein ratios above 2:1, the activity decreases due to MoFe inhibition. MoFe inhibition is a characteristic nitrogenase reaction common to all nitrogenases thus far examined and is manifest as decreasing product formation with increasing MoFe protein concentration at constant Fe protein concentration. A proper explanation has remained elusive, although such reactivity has been used to suggest possible stoichiometric relationships for nitrogenase complex formation during catalysis and to assess the specific activity of the Fe protein (29, 30). MoFe inhibition was proposed by Johnson et al. (26) to be due to two Fe proteins interacting cooperatively with one active site of the MoFe protein. If this is the case, significantly different behavior would be expected using Ti(III) as is verified in Fig. 2, which shows that, using Ti(III) as the reductant, MoFe inhibition is eliminated and the peak activity is near 3100 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ Fe protein. In other experiments, activities as high as 4200 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ Fe protein were observed. The elimination of MoFe inhibition suggests that, when the $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple is operating, two electrons instead of one are transferred from the Fe protein to the same catalytic site of the MoFe protein, and product formation occurs with each interaction. With the elimination of MoFe inhibition using the $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple, nitrogenase catalysis follows a simple hyperbolic curve suggesting a simple Michaelis-Menten behavior for the Fe:MoFe protein interaction. It is interesting that under these conditions the Fe protein increases in activity to greater than 3100 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ Fe protein only when the $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple is operating. This is because the rate-limiting step with a large excess of MoFe protein is the rate with which the Fe protein can transfer electrons to the MoFe protein. Consequently, transfer of two electrons by the $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple, in the presence of excess MoFe protein, can generate product at almost twice the rate of the $[\text{Fe}_4\text{S}_4]^{2+/1+}$ redox couple under identical conditions.

Another characteristic nitrogenase reaction studied here is the delay in product formation occurring at high MoFe:Fe protein ratios as initially reported by Hageman and Burris (17). In the original report, Hageman and Burris found that it took approximately 8 min for the product formation to reach steady state. When the linear portion of the curve was extrapolated backward in time, it fitted to approximately a 4.3-min lag phase. This experiment was interpreted as a random transfer of electrons from the Fe protein to the MoFe protein with product formation occurring only when two electrons accumulate in the same catalytic center of the MoFe protein. The difference in reactivity between a completely random one-electron electron transfer reaction using the $[\text{Fe}_4\text{S}_4]^{2+/0}$ and a two-electron electron transfer reaction using the $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple would be quite different. With the random $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple, a significant delay in product formation would be expected before a steady-state rate of product formation is observed. However, if a concerted two-electron transfer occurs at the same catalytic site on the MoFe protein, then the delay in product formation should be eliminated and product formation would be observed upon initiation of the reaction. Figs. 3 and 4 demonstrate this difference between the $[\text{Fe}_4\text{S}_4]^{2+/0}$ and $[\text{Fe}_4\text{S}_4]^{1+}$ redox couples and suggest that the latter operates by transferring two electrons at a time to the MoFe protein center. The difference between our results and those of Hageman and Burris is the protein used here was significantly more active, and this is the reason our reactions were performed at 300:1 MoFe:Fe protein ratio rather than 100:1.

To demonstrate that protein activity influenced the length of the lag phase for the $[\text{Fe}_4\text{S}_4]^{2+/0}$ redox couple, we examined a partially inactive Fe protein (~300 nmol of H$_2$ min$^{-1}$ mg$^{-1}$ Fe protein). By doing this we were able to more closely replicate the results of Hageman and Burris. Fig. 4 shows the difference between DT and Ti(III) assays run with the 100:1 MoFe:Fe protein ratio and the low activity Fe protein. The DT assay yielded a lag phase of 4.1 ± 0.7 min, whereas the Ti(III) assay generated product linearly from time zero. Because little has been reported regarding what happens to an Fe protein when it is inactivated, it is difficult to speculate as to
why the Ti(III) reaction were 4-fold more active than the DT reaction.

Heterologous crosses have been well studied and in general Fe-MoFe crosses from all diazotrophs are active except for crosses with Cp. We reported previously (10) that Cp2 could form the [Fe₄S₄]⁰ and that a 5:1 Cp2-Cp1 mixture under Ti(III) decreases the ATP/2e by 2-fold (10). Here we report that whether DT or Ti(III) is used as the reductant, the CpAv crosses are inactive. The Av1-Cp2 cross yielded a stable complex in the presence of DT but generated no measurable product, because it could not transfer more than one electron per MoFe protein interaction. The Cp1-Av2 heterologous cross did not form a stable complex nor was it active using DT. The Ti(III) assays performed under similar conditions were inactive even though Ti(III) was used as reductant (one turnover may have occurred, but this amount of product was below our detection limit). To determine if the Av1-Cp2 complex forms in the presence of Ti(III), we ran native gels and found that a component protein complex formed and appeared to be the same as that formed with DT. Additionally, when L127ΔFe protein was assayed with native Av1 and analyzed by native gel electrophoresis, a stable complex formed in both cases independent of the reductant used. It appears that the [Fe₄S₄]⁰ is similar to [Fe₄S₄]¹⁺ in its ability to interact with the MoFe protein and form a complex. The [Fe₄S₄]¹²⁺ does not cause a more favorable interaction in the case of a heterologous cross.

The questions that accompanied the discovery of the all-ferrous Fe protein were: 1) Is it physiologically relevant; and 2) Does it behave differently from the DT reduced Fe protein. The results presented here specifically address the second question and show that distinct differences and similarities are observed between the [Fe₄S₄]¹²⁺/[Fe₄S₄]⁰ and the [Fe₄S₄]¹⁺/[Fe₄S₄]¹⁻ redox couples. We suggest that their differences arose from a two-electron transfer reaction as opposed to the traditionally observed single-electron transfer. With regard to physiological relevance, previous data (7, 10, 11), along with the data presented here specifically address the second question and show that distinct differences and similarities are observed between the [Fe₄S₄]¹²⁺/[Fe₄S₄]⁰ and the [Fe₄S₄]¹⁺/[Fe₄S₄]¹⁻ redox couples. We suggest that their differences arose from a two-electron transfer reaction as opposed to the traditionally observed single-electron transfer. With regard to physiological relevance, previous data (7, 10, 11), along with the data presented here, suggest that the [Fe₄S₄]¹²⁺/[Fe₄S₄]⁰ redox couple is operative at physiologically attainable redox potentials and can in fact transfer two electrons with each FeMoFe interaction. The ATP/2e ratio is a sensitive measure of this reactivity and indicates an increased efficiency of the nitrogenase-catalyzed reduction of substrates when this redox couple is functioning. Both the [Fe₄S₄]¹⁺/[Fe₄S₄]⁰ and [Fe₄S₄]¹⁺/[Fe₄S₄]¹⁻ redox couples can be functional during in vitro nitrogenase catalysis. Are these in vitro results relevant to in vivo nitrogenase catalysis? ATP/2e values of 2.5 for Cp (30), 3.75 for Kp (32), and 0.63 for Ac (33) were reported for ATP utilization in whole cells. Although these in vitro results are not in close agreement, they are all significantly lower than values near 5.0 obtained using DT. The in vivo results are closer to the ATP/2e values near 2.0 reported previously and here. Reactions conducted with Av flavoprotein (a possible physiological reductant) also eliminate MoFe inhibition and give ATP/2e values near 2 (10). Taken together, the results suggest the [Fe₄S₄]¹²⁺/[Fe₄S₄]⁰ redox couple is physiologically relevant.

REFERENCES

1. Burgess, B. K., and Lowe, D. J. (1996) Chem. Rev. 96, 2983–3011
2. Kim, J., and Rees, D. C. (1994) Biochemistry 33, 389–397
3. Smith, B. E., and Eady, R. R. (1992) Eur. J. Biochem. 205, 1–15
4. Zumbi, W. G., and Mortenson, L. E. (1975) Biochim. Biophys. Acta 416, 1–52
5. Watt, G. D., Wang, Z.-C., and Knotts, R. R. (1986) Biochemistry 25, 8156–8162
6. Thorneley, R. N. F., and Lowe, D. J. (1985) in Molybdenum Enzymes (Spiro, E., ed) pp. 221–284, Wiley-Interscience, New York
7. Watt, G. D., and Reddy, K. R. N. (1994) J. Inorg. Biochem. 53, 281–294
8. Angove, H. C., Yoo, S. J., Burgess, B. K., and Munch, E. (1997) J. Am. Chem. Soc. 119, 8730–8731
9. Angove, H. C., Yoo, S. J., Munch, E., and Burgess, B. K. (1998) J. Biol. Chem. 273, 26330–26337
10. Erickson, J. A., Nyborg, A. C., Johnson, J. L., Truscott, S. M., Gunn, A., Nordmeyer, F. R., and Watt, G. D. (1999) Biochemistry 38, 14279–14285
11. Nyborg, A. C., Erickson, J. A., Johnson, J. L., Gunn, A., Truscott, S. M., and Watt, G. D. (2000) J. Inorg. Biochem.
12. Johnson, L., Tolley, A. M., Erickson, J. A., and Watt, G. D. (1996) Biochemistry 35, 11326–11342
13. Thorneley, R. N. F., Eady, R. R., and Yates, M. G. (1975) Biochim. Biophys. Acta 403, 269–284
14. Sorger, G. J. (1971) Biochem. J. 122, 305–309
15. Shah, V. K., Davis, L. C., and Brill, W. J. (1972) Biochim. Biophys. Acta 256, 498–511
16. Silverstein, R., and Bulen, W. A. (1970) Biochemistry 9, 3809–3815
17. Hageman, R. V., and Burris, R. H. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2699–2702
18. Hageman, R. V., and Burris, R. H. (1979) J. Biol. Chem. 254, 11189–11192
19. Detroy, R. W., Witz, D. F., Parejko, R. A., and Wilson, P. W. (1968) Proc. Natl. Acad. Sci. U. S. A. 61, 557
20. Emerich, D. W., and Burris, R. H. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4369–4373
21. Orme-Johnson, W. H., Davis, L. C., Henzl, M. T., Averill, B. A., Orme-Johnson, N. R., Munck, E., and Zimmerman, R. (1977) in Recent Developments in Nitrogen Fixation (Newton, W. E., Postgate, J. R., and Rodriguez-Barrueco, C., eds) pp. 131–178, Academic Press, London
22. Burgess, B. K., Jacobs, D. B., and Stiefel, E. I. (1980) Biochim. Biophys. Acta 614, 196–209
23. Lanzilotta, W. N., Fisher, K., and Seeffeldt, L. C. (1996) Biochemistry 35, 7188–7196
24. Fisk, C. H., and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–380
25. Johnson, J. N., Nyborg, A., Wilson, P. E., Tolley, A. M., Nordmeyer, F. R., and Watt, G. D. (2000) Biochim. Biophys. Acta 1543, 24–35
26. Johnson, J. N., Nyborg, A., Wilson, P. E., Tolley, A. M., Nordmeyer, F. R., and Watt, G. D. (2000) Biochim. Biophys. Acta 1543, 36–46
27. Hageman, R. V., Orme-Johnson, W. H., and Burris, R. H. (1980) Biochemistry 19, 2333–2342
28. Thorneley, R. N. F., and Lowe, D. J. (1984) Biochem. J. 224, 903–909
29. Orme-Johnson, W. H., and Davis, L. C. (1977) in Iron-Sulfur Proteins: Structure and Metabolic Mechanisms (Lovenberg, W., ed) Vol. III, pp. 15–60, Academic Press, New York
30. Mortenson, L. E., Walker, M. N., and Walker, G. A. (1976) in Proceedings of the 1st International Symposium on Nitrogen Fixation (Newton, W. E., and Nyman, C. J., eds) Vol. 1, pp. 117–149, Washington State University Press, Pullman, WA
31. Daesch, G., and Mortenson, L. E. (1968) J. Bacteriol. 96, 346–351
32. Postgate, J. R. (1971) The Chemistry and Biochemistry of Nitrogen Fixation, Plenum Press, London
33. Dalton, H., and Postgate, J. R. (1969) J. Gen. Microbiol. 56, 307–311