Enhanced Efficiency of ATP Hydrolysis during Nitrogenase Catalysis Utilizing Reductants That Form the All-Ferrous Redox State of the Fe Protein†

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ABSTRACT: The amount of MgATP hydrolyzed per pair of electrons transferred (ATP/2e) during nitrogenase catalysis (1.0 atm N_2, 30 °C) using titanium(III) citrate (Ti(III)) as reductant was measured and compared to the same reaction using dithionite (DT). ATP/2e values near 2.0 for Ti(III) and 5.0 for DT indicate that nitrogenase has a much lower ATP requirement using Ti(III) as reductant. Using reduced *Azotobacter vinelandii* flavoprotein ($AvFlpH_2$), a possible in vivo nitrogenase reductant, ATP/2e values near 2.0 were also observed. When the reaction was conducted using Ti(III) under N_2, 5% CO in N_2, Ar, 5% CO in Ar, or acetylene, ATP/2e values near 2.0 were also observed. With Ti(III) as reductant, ATP/2e values near 2.0 were measured as a function of temperature, Fe:MoFe protein ratio, and MoFe:Fe protein ratio, in contrast to measured values of 4.0−25 when DT is used under the same conditions. Both Ti(III) and $AvFlpH_2$ are capable of forming the $[Fe_4S_4]^0$ cluster state of the Fe protein whereas DT is not, suggesting that ATP/2e values near 2.0 arise from operation of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple with hydrolysis of only 2 ATPs per pair of electrons transferred. Additional experiments showed that ATP/2e values near 2.0 correlated with slower rates of product formation and that faster rates of product formation produced ATP/2e values near 5.0. ATP/2e values of 5.0 are consistent with the operation of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple while ATP/2e values of 2.0 could arise from operation of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple. These results suggest that two distinct Fe protein redox couples may be functional during nitrogenase catalysis and that the efficiency of ATP utilization depends on which of these redox couples is dominant.

Nitrogenase, a complex enzyme system composed of two dissimilar metalloprotein components, is responsible for biological nitrogen fixation ($N_2 + 6e^- + 6H^+ = 2NH_3$) (*1*−*5*). The structures of both the Fe[1] and MoFe proteins are known (*6*−*9*). The MoFe protein ($\alpha_2\beta_2$, $M_r \sim 230\,000$) contains two sets of two distinct redox-active metal clusters: The P-cluster centers that consist of two $[Fe_4S_4]$ cubanes connected by two bridging cysteine thiol ligands (α88 and β95) bound at the αβ-subunit interface and the FeMoco ($MoFe_7S_8$) that resides primarily within the α-subunit where substrate binding, activation, and reduction are believed to occur (*5*). The Fe protein (α_2, $M_r \sim 63\,000$) contains a single redox-active $[Fe_4S_4]$ cluster which bridges the two identical subunits (*8*). The Fe protein binds two ATPs that induce a negative shift in the redox potential of the $[Fe_4S_4]$ cluster (*8*).

During nitrogenase catalysis using DT, the Fe protein exclusively utilizes the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple to transfer a single electron to the MoFe protein. Repeated, but identical, one-electron transfers from the Fe protein to the MoFe protein provide the eight electrons required for N_2 reduction to NH_3 and obligatory H_2 evolution (*10*, *11*). The role of the MoFe protein in the catalytic cycle is to store incoming electrons provided by the Fe protein cycle until a sufficient number have accumulated to carry out substrate reduction. During nitrogenase catalysis at 30 °C with DT as reductant, the amount of ATP hydrolyzed per electron pair transferred (ATP/2e) is constant near 5.0, a result consistent with the hydrolysis of two ATPs for each electron transferred by the Fe protein.

The report that the Fe protein can exist as $[Fe_4S_4]^0$ and that this redox state is functional in producing H_2 and reducing acetylene raises the possibility that an alternate Fe protein cycle utilizes the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple (*12*). Here we explore the reactivity of $[Fe_4S_4]^0$ and examine the hypothesis that if two electrons are transferred to the MoFe protein and only two ATPs are hydrolyzed during each Fe

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[1] Abbreviations: FeMoco, the molybdenum−iron-containing cofactor; Av, *Azotobacter vinelandii*; Cp, *Clostridium pasteurianum*; Ac, *Azotobacter chroococcum*; Kp, *Klebsiella pneumoniae*; MoFe protein, molybdenum−iron protein; Fe protein, iron protein; Av1 and Cp1, Av and Cp MoFe proteins, respectively; Av2 and Cp2, Av and Cp Fe proteins, respectively; $AvFlpH_2$, reduced Av flavoprotein; $AvFlp^{\bullet}$, Av flavoprotein semiquinone; AvFlp, oxidized Av flavoprotein; Ti(III), titanium (III) citrate; MV, methyl viologen; DT, sodium dithionite; ATP, MgATP; $[Fe_4S_4]^{2+}$, oxidized Fe protein; $[Fe_4S_4]^{1+}$, singly reduced Fe protein; $[Fe_4S_4]^0$, doubly reduced or all-ferrous Fe protein.

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protein cycle, then ATP/2e values could be as low as 2.0 instead of 4.0−5.0 as observed with DT. Initial studies showed that reduced MV, Ti(III), and $AvFlpH_2$ were capable of forming the $[Fe_4S_4]^0$ state in the Fe protein (*12*, *13*; Watt, unpublished results) and consequently use of these reductants in place of DT during nitrogenase catalysis might be expected to produce ATP/2e values near 2.0 if the above considerations are correct.

MATERIALS AND METHODS

Nitrogenase component proteins were prepared anaerobically from *Azotobacter vinelandii* OP cells as described (*14*). Several Fe and MoFe protein preparations were used with specific activities of 1700−2100 and 1800−2000 nmol of H_2 mg^{-1} min^{-1}, respectively. Component proteins from *Clostridium pasteurianum* were a gift from Richard Baer of Exxon (Annandale, NJ) with Fe and MoFe protein activities of 1800−2000 and 1800−2000 nmol of H_2 mg^{-1} min^{-1}, respectively. AvFlp was collected during nitrogenase component protein isolation, purified separately, and concentrated to 2−5 mM using an Amicon concentrator.

$TiCl_3$ was obtained from Aldrich and 83 mM stock Ti(III) solutions were prepared as described (*15*). Concentrated AvFlp was reduced in a Vacuum Atmospheres glovebox (<1.0 ppm O_2) with a 10-fold excess of Ti(III) or DT, allowed to react for 5−10 min, and then passed through an anaerobic Sephadex G-50 column to remove excess reductant and obtain the pale yellow hydroquinone form ($AvFlpH_2$) at 1−2 mM.

Nitrogenase activity was determined by two separate methods. The first was the standard nitrogenase assay (*14*) using DT (10−20 mM) or Ti(III) (1−20 mM) conducted in 5.0- or 10.0-mL vials to quantify H_2, ethylene, or NH_3. H_2 and ethylene formation were determined by gas chromatography, and ammonia formation was determined by either the Nessler or indophenol methods following microdiffusion. All assays were performed at least in triplicate and averaged, and the uncertainty represents the standard deviation of all measurements. The ammonia analyses on all Ti(III) solutions investigated gave variable backgrounds depending upon the Ti(III) source; therefore, controls consisting of all reaction components excluding ATP or enzyme were conducted to correct for this background. The second method measured reductant utilization (*15*) in a thermostated optical cell under 1.0 atm nitrogen in a Vacuum Atmospheres glovebox using a Milton Roy Spectronic 401 spectrophotometer interfaced with an IBM personal computer for data collection.

Both standard and optical assays using Ti(III) were carried out using substrate levels of ATP and the ATP regeneration system. Ti(III) solutions slowly produce H_2 so controls consisting of all assay components minus enzyme were conducted to correct for background H_2. The background correction typically accounted for less than 5% of the total product formed. For reactions using substrate level ATP, nucleotide concentrations were determined using a Rainin Rabbit HPLC equipped with a Rainin Microsorb MV 100-Å, 5-μm, C18 reversed-phase column (4.6 mm i.d. × 15 cm) (*16*). The mobile phase consisted of solution A (0.02 M KH_2PO_4, 0.01 M tributylamine, pH 2.63) and solution B (methanol). A Macintosh computer controlled the HPLC and a Rainin Dynamax HPLC Method Manager was used for collecting and analyzing the resulting chromatograms. The background corrections for the HPLC method accounted for less than 3% of the ATP hydrolyzed. For reactions utilizing the ATP-regenerating system, ATP hydrolysis was assessed as inorganic phosphate determined by the Fiske and Subbarow method (*17*) with appropriate controls that account for less than 10% of the phosphate measured.

Optical Determination of ATP/2e Values. To a thermostated 1.0-cm optical cell was added buffer, ∼1.0 mM Ti(III), and the nitrogenase component proteins at various ratios after which the spectrophotometer was zeroed. A Ti(III) stock solution (80−100 mM) was then added to produce the desired final concentration in a volume of 1.0 mL. The mixture was then incubated until absorbance at 340 nm stabilized. Typically, 5 μL of 0.1 M MgATP was then added to start the nitrogenase reaction and the change in absorbance of the Ti(III) was monitored at 340 nm ($\epsilon = 0.73$ $mM^{-1}cm^{-1}$) to determine the reducing equivalents converted to product. At regular intervals, 50-μL samples were removed from the optical cell for nucleotide analysis. Both the unreacted ATP and the ADP formed during nitrogenase catalysis were used in calculating ATP/2e values.

The same optical procedure was used with $AvFlpH_2$ at initial concentrations near 1.0 mM by following the amount of $AvFlp^{\bullet}$ formed ($\epsilon = 5765$ M^{-1} cm^{-1} at 580 nm) for the nitrogenase-catalyzed reaction. The presence of the flavoprotein interfered with the HPLC determination of ATP; therefore, ATP hydrolysis was determined by measuring ADP relative to an ADP standard.

The reaction order and rate constant for Ti(III) or $AvFlpH_2$ utilization during nitrogenase catalysis were determined from the rate of change of reductant concentration as described above and fit using an Applied Photophysics Kinetics Global Analysis program or Microsoft Excel. Rate constants were measured as a function of temperature and used to calculate the activation energy of Ti(III) utilization.

$[Fe_4S_4]^{1+}$ and $[Fe_4S_4]^{2+}$ Reduction Kinetics. $[Fe_4S_4]^{1+}$ was prepared by passing DT-reduced Fe protein through an anaerobic 1 × 10 cm Sephadex G-25 column equilibrated with 0.05 M Tris, 0.05 M NaCl, pH 7.5, in a Vacuum Atmospheres glovebox. $[Fe_4S_4]^{2+}$ was prepared by anaerobic oxidation with brilliant alizarin blue and was separated as outlined above. Fe protein (15 μM) was loaded into an anaerobic 1.0-mL, 1.0-cm path length optical cell to equilibrate at 30 °C and the spectrum recorded on a Hewlett-Packard 8453 diode array spectrophotometer in the kinetics mode. Ti(III) (0.5, 1.0 or 5.0 mM) was added and absorbance versus time measurements were recorded every 10 s during the reduction of either $[Fe_4S_4]^{1+}$ or $[Fe_4S_4]^{2+}$. The absorbance change at 340 and 654 nm were fitted to first-order equations using Microsoft Excel.

RESULTS

Two distinct experimental approaches were used to assess nitrogenase activity in the presence of different reductants and under a variety of conditions. The first measured reductant disappearance (Ti(III), $AvFlpH_2$, or DT) by an optical method and the second measured product formation (H_2, ethylene, and ammonia) using standard nitrogenase assay procedures. In both cases, experiments were conducted with substrate levels of ATP and with the ATP regeneration system.

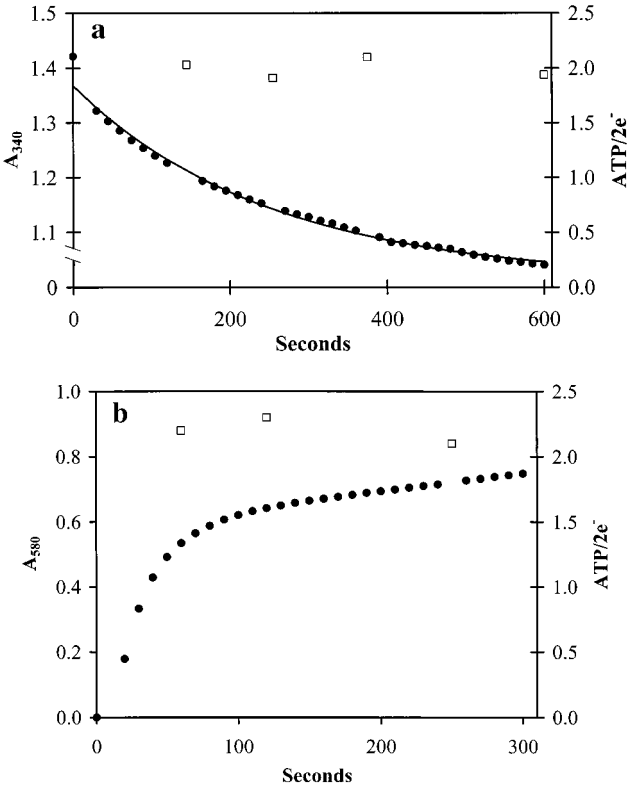

FIGURE 1: Reductant utilization and ATP/2e measurements for Av nitrogenase catalysis under 1 atm N_2 using Ti(III) or $AvFlpH_2$ as reductant. (a) The absorbance change at 340 nm (●, left axis) due to the oxidation of Ti(III) during catalysis. The solid line is the nonlinear least-squares fit under pseudo-first-order conditions with $k = 4.0 \times 10^{-3}$ s^{-1}. This corresponds to an Av1 specific activity of 435 nmol of H_2 min^{-1} mg^{-1}. (b) The absorbance change at 580 nm (●, left axis) due to the formation of $AvFlp^{\bullet}$ during catalysis. From the initial rate, the Av1 specific activity is 390 nmol of H_2 min^{-1} mg^{-1}. For both (a) and (b), the ATP/2e values measured at various times are shown (□, right axis). The gaps in the absorbance data result when samples were removed for nucleotide analysis by HPLC. The reactions contained 0.6 μM Av1 at an Av2:Av1 ratio of 5.2 and 0.5 mM ATP with no ATP regenerating system.

Optical Assays. Figure 1a shows the rate of disappearance of Ti(III) measured optically during nitrogenase catalysis under 1 atm N_2 at 30 °C in a Vacuum Atmospheres glovebox held below 0.10 ppm O_2. The reaction was initiated by adding ATP to a final concentration of 0.5−1.0 mM (no regenerating system) followed by periodically removing samples (gaps in the data of Figure 1) for nucleotide analysis until 60−90% of the ATP was hydrolyzed to ADP. The number of electrons transferred from Ti(III) to product was determined from the absorbance change. The upper data points show that ATP hydrolyzed per pair of electrons catalytically transferred (ATP/2e) is constant near 2.0. For this ATP-limiting reaction, ADP builds up during the course of the reaction, but the low ADP concentrations produced in this experiment, if inhibitory, had no effect on the ATP/2e values. Figure 1b confirms that ATP/2e values near 2.0 are also obtained by measuring the $AvFlp^{\bullet}$ produced by $AvFlpH_2$ oxidation under conditions similar to those in Figure 1a. Because $AvFlpH_2$ is difficult to obtain in high concentrations, the $AvFlpH_2$ assay (Figure 1b) was not conducted under pseudo-first-order conditions and the specific activity was calculated from the initial rate.

Table 1: ATP/2e Values Measured under Various Conditions Using the ATP Regeneration System[a,b]

Ti(III) (mM)[c−e,h,k]	1.0	2.0	3.0	5.0	7.0
ATP/2e	5.5 ± 0.5	4.1 ± 0.3	3.3 ± 0.3	2.3 ± 0.3	2.1 ± 0.7
MoFe (μM)[c,d,h,i]	2.5	1.2	0.61	0.31	0.15
ATP/2e	4.7 ± 0.2	3.2 ± 0.2	2.7 ± 0.3	2.8 ± 0.2	2.1 ± 0.4
ATP (mM)[d,f,j]	2.5	2.0	1.5	1.0	0.50
ATP/2e	4.5 ± 0.6	3.4 ± 0.3	3.0 ± 0.3	2.5 ± 0.3	2.1 ± 0.4
Fe:MoFe[c,g−i]	1	2	5	20	
ATP/2e	2.1 ± 0.2	2.2 ± 0.2	2.5 ± 0.3	2.0 ± 0.2	

[a] Assayed under 10% acetylene. [b] Assayed under Ar. [c] [ATP] = 2.5 mM. [d] Fe:MoFe protein ratio = 5.0. [e] [MoFe] = 0.52 μM. [f] [MoFe] = 0.27 μM. [g] [MoFe] = 0.60 μM. [h] Verified using substrate level ATP. [i] [Ti(III)] = 5.0 mM. [j] [Ti(III)] = 2.0 mM. [k] Assayed under N_2.

ATP/2e values of 5.3 ± 0.4 were measured with DT as reductant using the same optical method under similar conditions. This value markedly contrasts with the values of 2.1 ± 0.2 obtained using DT compared to Ti(III) and $AvFlpH_2$ as reductants. These results suggest that nitrogenase catalysis is mechanistically different using Ti(III) and $AvFlpH_2$ (an artificial and a physiological nitrogenase reductant, respectively), which results in decreased ATP utilization as manifest by the lower ATP/2e values.

Standard Assays. The results in Figure 1a were confirmed using standard nitrogenase assays conducted under identical conditions (1 atm N_2) with a [Ti(III)] between 1 and 20 mM. The number of electrons transferred during nitrogenase catalysis was measured by quantifying H_2 and NH_3 instead of reductant disappearance. The results using this independent method confirm the ATP/2e values near 2.0 reported in Figure 1a and also provide an opportunity to examine product distribution during nitrogenase catalysis with other reducible substrates using Ti(III) as reductant.

The measured product distribution of 57% NH_3 and 43% H_2 with Ti(III) suggests that obligatory H_2 evolution also occurs during nitrogenase catalysis even under conditions where the ATP/2e value is near 2.0. When this experiment is repeated with 5% CO in N_2, H_2 is the only product formed, and the ATP/2e value remains constant near 2.0. Additional studies showed that identical rates of H_2 production were observed under argon or 5% CO in argon, and in each case the ATP/2e values were near 2.0. Finally, with 10% acetylene in argon, 7−14% H_2 evolution accompanies ethylene formation and gives ATP/2e values near 2.0. Comparison of these results with corresponding reactions using DT as reductant shows that nearly identical product distributions are observed, but with DT the ATP/2e values were near 5.0 instead of 2.0.

Ti(III) as Reductant. Table 1 summarizes ATP/2e values obtained under four separate sets of conditions: (1) variation of Ti(III) concentration; (2) variation of enzyme concentration; (3) variation of ATP concentration; and (4) variation of Fe:MoFe protein ratio. These conditions were chosen because each influences the rate of product formation (and reductant disappearance) and should provide information about how ATP utilization is linked to reductant reactivity during nitrogenase catalysis.

The top row of Table 1 shows that, at fixed ATP and nitrogenase concentrations, the ATP/2e values decrease uniformly from 5.5 to 2.1 as the Ti(III) concentration increases from 1.0 to 7.0 mM. Above 7 mM Ti(III), ATP/

2e values remain constant near 2.0; however, under these conditions nitrogenase becomes increasingly inhibited and the uncertainty in the measurements increases because less product is formed.[2] These results show that variation of the Ti(III) concentration produces two different reactions: one producing ATP/2e values near 5.0 at low Ti(III) concentrations and another producing ATP/2e values near 2.0 at high Ti(III) concentrations. Intermediate ATP/2e values result from combinations of these two reactions.

The second row in Table 1 shows that, under specified ATP and Ti(III) concentrations, the ATP/2e values depend on the concentration of nitrogenase proteins used during catalysis. At high protein concentrations, ATP/2e values near 5.0 are observed, while at low protein concentrations, ATP/2e values near 2.0 are observed. Intermediate protein concentrations give ATP/2e values between 5.0 and 2.0. Because the rates of reductant usage and product formation are directly related to protein concentration, the results suggest that ATP/2e values near 5.0 may arise from rapid nitrogenase turnover, while values near 2.0 may be a consequence of slower enzyme turnover.

At fixed reductant and enzyme concentrations, the third row shows that the variation of ATP concentration (with the ATP regeneration system) produces the same behavior reported above. At high ATP concentrations, reductant disappearance and product formation are rapid giving ATP/2e values near 5.0, while low ATP concentrations cause a slower rate of reductant utilization and product formation giving ATP/2e values near 2.0. In assays containing less than 0.5 mM ATP, the amount of product formed was small, resulting in a less precise measurement of ATP hydrolysis.

The fourth row compares ATP/2e values as a function of Fe:MoFe protein ratio under high Ti(III) and controlled ATP concentrations. The results show that the ATP/2e value is constant near 2.0 over the range of Fe:MoFe protein ratios covered. Although an accelerated rate of product formation is observed at 20:1 compared to 1:1 Fe:MoFe protein ratios, the ATP/2e value does not change, indicating that in this case the increase in the rate of enzymatic catalysis does not change the ATP utilization behavior.

Reduction of $[Fe_4S_4]^{2+}$ and $[Fe_4S_4]^{1+}$ with Ti(III) and $AvFlpH_2$. ATP/2e values near 5.0 and 2.0 are observed using Ti(III), but only values near 5.0 are observed with DT. To better understand nitrogenase reactivity using Ti(III), we

$$[Fe_4S_4]^{2+} + 2Ti(III) = [Fe_4S_4]^{0} + 2Ti(IV) \quad (1)$$

$$[Fe_4S_4]^{1+} + Ti(III) = [Fe_4S_4]^{0} + Ti(IV) \quad (2)$$

$$[Fe_4S_4]^{2+} + Ti(III) = [Fe_4S_4]^{1+} + Ti(IV) \quad (3)$$

investigated the rate of Fe protein reduction by Ti(III). The reduction of 15 μM $[Fe_4S_4]^{1+}$ according to reaction 2 using 0.5−5.0 mM Ti(III) is first order in both $[Fe_4S_4]^{1+}$ and [Ti(III)] with an overall second-order rate constant of ~66 M^{-1} s^{-1} at 30 °C. In comparison, 0.5 mM $AvFlpH_2$ reduces more than 95% of the $[Fe_4S_4]^{1+}$ to $[Fe_4S_4]^{0}$ before the first measurement at 10 s. Therefore the second-order rate constant for the $AvFlpH_2$ reduction of Fe protein is $k > 1000$ M^{-1} s^{-1} at 30 °C.

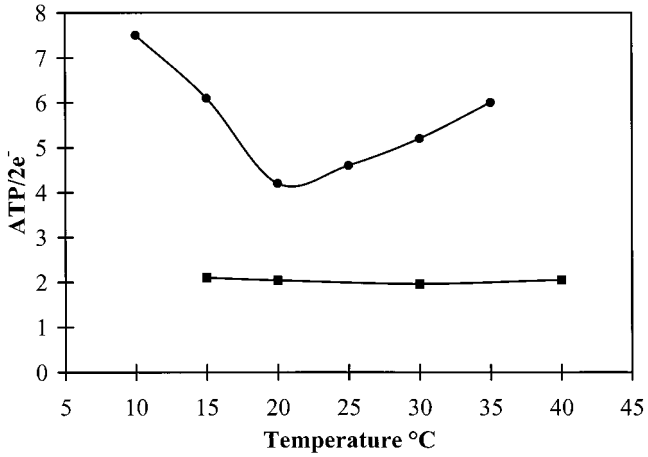

FIGURE 2: Variation in ATP/2e values with temperature during Av nitrogenase catalysis with DT and Ti(III) as reductants. The data in the upper curve (●) for DT as reductant were taken from Watt et al. (*18*). The data for the lower curve (■) were determined under the same conditions with Ti(III) as reductant at an Fe:MoFe protein ratio of 5.2.

The reduction of $[Fe_4S_4]^{2+}$ to $[Fe_4S_4]^{0}$ using 0.5 mM Ti(III) also has a second-order rate constant of ~66 M^{-1} s^{-1} at 30 °C even though two electrons are being transferred instead of one. Identical rates of reduction for reactions 1 and 2 arise because reaction 3 is much faster than reaction 2. This important observation shows that the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple will be active at all Ti(III) concentrations during nitrogenase catalysis. Because reaction 2 is much slower, the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{0}$ redox couple will be inoperative at low Ti(III) concentrations but will make an increasing contribution to catalysis as the Ti(III) concentration increases. In contrast, the rate of reduction of $[Fe_4S_4]^{1+}$ by $AvFlpH_2$ is at least 15 times faster than with Ti(III), making it likely that the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{0}$ redox couple is viable even at low $AvFlpH_2$ concentrations. Because both the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{0}$ and $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couples function during nitrogenase catalysis, we examined nitrogenase reactivity using Ti(III) and $AvFlpH_2$ under other conditions.

ATP/2e Variation with Temperature. Figure 2 compares ATP/2e values obtained as a function of temperature, under conditions comparable to Figure 1a, using Ti(III) or DT as reductant. ATP/2e values near 2.0 using Ti(III) are independent of temperature while ATP/2e values using DT are temperature dependent, varying from 4.0 to >7.5 (*18*). The latter, more complex behavior suggests that more than one reaction occurs in the presence of DT (*18*−*20*), while only one reaction occurs when Ti(III) is used. From the variation of the pseudo-first-order rate constant for Ti(III) oxidation during catalysis as a function of temperature, a single activation energy of 67.1 kJ/mol (16.0 kcal/mol) is calculated. However, two distinct activation energies of 51.5 (12.3 kcal/mol) and 146 kJ/mol (35 kcal/mol) are observed with DT (*18*, *21*), a result consistent with two separate reactions.

ATP/2e Variation with MoFe:Fe Protein Ratio. Table 2 summarizes the variation of ATP/2e for Cp (*22*, *23*), Av (*10*), and Kp (*24*) as a function of MoFe:Fe protein ratio. With

[2] Inhibition by Ti(III) is sensitive to reaction conditions, the type of Ti(III) salt, and how the Ti(III) solutions are prepared. Our results show that the onset of inhibition can occur at concentrations as low as 3.0 mM or as high as 10 mM. The value of 5 mM quoted here is a concentration of Ti(III) typical of uninhibited nitrogenase behavior. Details of this inhibition behavior and other characteristics of Ti(III) reactivity will be reported separately.

Table 2: Comparison of ATP/2e Values at 30 °C at Various MoFe:Fe Protein Ratios of Av, Kp, and Cp Nitrogenases Using Ti(III) or DT as Reductant

	ATP/2e				
MoFe:Fe	Av (Ti(III))	Cp (Ti(III))	Av (DT)[a]	Kp (DT)[b]	Cp (DT)[c]
0.2	2.1 ± 0.3 (2.1 ± 0.3)[d]		4.5	4.2	4−5
0.8	1.9 ± 0.3	2.3 ± 0.3	4−5[a,e]	4.2	4−5
2.0	2.2 ± 0.3		10.2	4.8	11.2
2.5			11.5	5.4	16
5.0	2.1 ± 0.3	1.9 ± 0.3	12.8	8.8	>16
7.5			14.2	>15	>16

[a] From Figure 6 of Hageman and Burris (*10*). At MoFe:Fe protein ratios greater than about 50, the ATP/2e values for Av level off at 25. [b] From Figure 1 of Eady and Postgate (*24*). [c] From Figure 3 of Ljones and Burris (*22*). From Figure 3, values could be evaluated only-to-a ratio of 2.5. The trend in the ATP/2e values with increasing MoFe:Fe protein ratio continued upward but values could not be estimated from the figure. Similar ATP/2e values are reported in Mortenson et al. (*23*). [d] This value was determined using $AvFlpH_2$ as reductant. [e] From Figure 2 of Watt et al. (*18*).

DT, the ATP/2e value increases rapidly with increasing MoFe:Fe protein ratio for the Cp, Kp, or Av nitrogenases and reaches values greater than 25 at high ratios. However, the ATP/2e values for both Av and Cp nitrogenases remain invariant near 2.0 under the same conditions using either Ti(III) or $AvFlpH_2$ as reductant. These results show that, under identical conditions, the reductant used influences the mechanistic behavior of nitrogenase and determines whether the measured ATP/2e values remain constant near 2.0 or vary as a function of increasing MoFe:Fe protein ratio.

DISCUSSION

Biological nitrogen reduction is a complex biocatalytic process involving interactions of two dissimilar metalloproteins, a reducible substrate, a low-potential reductant, ATP, and protons. The coupling of ATP hydrolysis to electron transfer is of central importance in understanding the mechanism of nitrogenase catalysis, but it is a complex and poorly understood process (*5*). Numerous studies have been conducted using DT, and a detailed model of nitrogenase catalysis using the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox cycle has been proposed (*25*). The effect of alternate reductants on nitrogenase catalysis has received limited study because $[Fe_4S_4]^{1+}$ was thought to be the only possible reduced state of the Fe protein. The report that the Fe protein forms a stable $[Fe_4S_4]^0$ redox state at physiologically relevant redox potentials (*12*) suggests that nitrogenase reactivity different from that produced by DT may occur using the Fe protein in the $[Fe_4S_4]^0$ redox state.

The initial report describing the formation of the $[Fe_4S_4]^0$ cluster of the Fe protein showed that $[Fe_4S_4]^0$ reacts with ATP and the MoFe protein and transfers two electrons to form H_2 and reduce acetylene to ethylene (*12*). From these and other results, it was proposed that the two-electron $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ Fe protein cycle was responsible for this reactivity. Operation of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ cycle could significantly decrease ATP utilization during nitrogenase catalysis if both electrons are transferred to the MoFe protein with hydrolysis of only two ATPs per Fe protein cycle. We have extensively examined this possibility with experiments that have evaluated: (1) both reductant disappearance and product formation to assess electron transfer; (2) three different reductants; (3) various reducible substrates; (4) reactions using both substrate level ATP and the ATP regeneration system; (5) inhibitory conditions with CO; and (6) variation of temperature and MoFe:Fe protein ratio to assess nitrogenase reactivity.

$[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ Redox Couple. It is commonly accepted that two ATPs are hydrolyzed per Fe−MoFe interaction using the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple (*5*). Our results clearly show that, during nitrogenase catalysis using Ti(III) or $AvFlpH_2$, two electrons are transferred with the hydrolysis of only two ATPs. These results suggest that there is only one tight Fe−MoFe interaction because only two ATPs are hydrolyzed per cycle. We considered a number of possible explanations for the decreased ATP requirement: (1) Two electrons are being transferred during a single Fe−MoFe encounter using the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple; (2) the Fe protein is being reduced while complexed to the MoFe protein; (3) an Fe protein is transferring an electron to a different site on the MoFe protein that does not require a tight complex nor ATP hydrolysis; or (4) Ti(III) or $AvFlpH_2$ is directly reducing the MoFe protein one electron equivalent further than DT. The last possibility is unlikely because there has been no evidence that Ti(III) or $AvFlpH_2$ can reduce the MoFe protein further than DT. The crystal structure of the Fe−MoFe complex does not show the presence of a second Fe protein binding site, making the third option unlikely (*26*). The fact that the L127Δ and the AlF_4^- complexes are inactive once they are locked together argues against the second possibility (*27*−*29*). Because Ti(III) and $AvFlpH_2$ can form $[Fe_4S_4]^0$ and because the ATP utilization is sensitive to [Ti(III)] (see Table 1, row 1), we hypothesize that the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple is responsible for the observed changes in the efficiency of ATP hydrolysis.

The results in Tables 1 and 2 show that, using Ti(III) and $AvFlpH_2$ as reductants, ATP/2e values can be as low as 2.0 and independent of temperature and MoFe/Fe protein variation. This behavior is mechanistically different from that observed using DT. These two reductants differ from DT in that they form the $[Fe_4S_4]^0$ redox state. Thus, the results are consistent with the hypothesis that ATP/2e values near 2.0 are produced during nitrogenase catalysis by the predominant use of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple with concomitant hydrolysis of only two ATPs per Fe protein cycle.

This conclusion contrasts the presently accepted view that nitrogenase functions using the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple to transfer only one electron per Fe protein cycle while hydrolyzing two ATPs. However, the results presented here indicate that this latter view may be a limiting case of a more general Fe protein reactivity. As previously noted, $[Fe_4S_4]^0$ is unstable in the presence of DT (*12*). Because of this mechanistic anomaly, only the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ couple is functional. Previous nitrogenase studies have primarily used DT as reductant, which has created the impression that nitrogenase catalysis uses this couple exclusively. Our results suggest that using Ti(III) and $AvFlpH_2$, a more extensive redox chemistry of the Fe protein is available. Table 1 shows that intermediate ATP/2e values can arise during catalysis which may be due to the simultaneous operation of both redox couples.

The observations in Table 1 showing that rapid enzyme turnover favors ATP/2e values of 5.0 while slower turnover

conditions favor ATP/2e values of 2.0 suggest that the enzymatic rate-limiting step may change with changes in conditions. Because nitrogenase catalysis is a complex process involving two proteins, ATP hydrolysis, reducible substrates, protons, and different valence states of the Fe and MoFe protein clusters, it is difficult to identify what enzymatic steps are altered from the measurements reported here. Furthermore, if the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ couple is functional, additional complications arise because little is known regarding the affinity of the $[Fe_4S_4]^0$ state for the MoFe protein, how electrons are transferred, and whether the Fe protein dissociates with each cycle or whether it is reduced in situ. These problems prevent a complete understanding of how nitrogenase can alter its ATP usage under the conditions shown in Table 1.

Because electron transfer from the Fe protein to the MoFe protein is central to nitrogenase function and because both the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ and $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ have been implicated in catalysis with Ti(III), we examined the reduction of Fe protein by Ti(III). Our initial measurements show that with Ti(III) reaction 2 is slow compared to reaction 3. Because this reaction is comparable to the rate of enzyme turnover, it may become rate limiting under certain conditions during nitrogenase catalysis and be responsible for the behavior noted in Table 1. For example, slow turnover conditions could effectively maintain a high concentration of $[Fe_4S_4]^0$ by reaction 2, which then reacts with the MoFe protein using the two-electron $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ couple. If two ATPs are hydrolyzed for each pair of electrons transferred, ATP/2e values near 2.0 would result. However, rapid product formation may deplete the $[Fe_4S_4]^0$ concentration faster than it can be replenished by reactions 1−3, making $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ the predominant redox couple used resulting in ATP/2e values near 5.0. For reducing agents capable of forming $[Fe_4S_4]^0$, both redox couples could participate in catalysis as seen in Table 1 with the experimental conditions dictating what proportion of each couple is involved in catalysis. The following calculation based on preliminary Fe protein reduction kinetics supports this possibility. In a typical Ti(III) assay (row 1 of Table 1), the rate of product formation is 1.4×10^{-6} M s^{-1}. Under conditions where ATP/2e values are near 5.0 (1.0 mM Ti(III)), the rate of $[Fe_4S_4]^0$ formation is 2×10^{-7} M s^{-1} which is 7-fold slower than the rate of product formation. Thus, $[Fe_4S_4]^{1+}$ is the predominate reduced species present. On the other hand, under conditions where ATP/2e values are near 2.0 (7.0 mM Ti(III)), the rate of $[Fe_4S_4]^0$ formation is 1.4×10^{-6} M s^{-1}. This corresponds to the rate at which $[Fe_4S_4]^{2+}$ is produced and so $[Fe_4S_4]^0$ becomes a significant catalytic species under these experimental conditions. While this explanation is plausible we note that the rate constant for reduction of the Fe protein was measured in the absence of nucleotide and MoFe protein.

The explanation that reaction 2 is slow and controls the relative concentrations of $[Fe_4S_4]^0$ and $[Fe_4S_4]^{1+}$ that in turn determine the ATP utilization rate during nitrogenase catalysis seems plausible and consistent with the results reported here. Further support comes from a comparison of nitrogenase catalysis using Ti(III) and $AvFlpH_2$ which shows that $AvFlpH_2$ produces ATP/2e values near 2.0 at concentrations below where Ti(III) produces values near 5.0. The differences in reactivity are consistent with the more rapid reduction of $[Fe_4S_4]^{1+}$ to $[Fe_4S_4]^0$ by $AvFlpH_2$ than by Ti(III), resulting in a high steady-state concentration of $[Fe_4S_4]^0$ at lower $AvFlpH_2$ concentrations.

However, nitrogenase catalysis is a complex process and other factors are also likely to influence the concentrations of the two Fe protein species. For example, the original report (*12*) describing $[Fe_4S_4]^0$ predicted from thermodynamic data that reaction 4 occurs if both $[Fe_4S_4]^{2+}$ and $[Fe_4S_4]^0$ are

$$[Fe_4S_4]^{2+} + [Fe_4S_4]^0 = 2[Fe_4S_4]^{1+} \qquad \Delta G = -14.5 \text{ kJ/mol} \quad (4)$$

present. This rapid reaction has been confirmed in our laboratory and independently (*30*). If $[Fe_4S_4]^{2+}$ is formed in the presence of $[Fe_4S_4]^0$, reaction 4 shows that two $[Fe_4S_4]^{1+}$ will form rapidly for each $[Fe_4S_4]^0$ consumed. This reaction represents an alternate pathway for forming $[Fe_4S_4]^{1+}$. Thus, rapid product formation produces $[Fe_4S_4]^{2+}$, which rapidly forms $[Fe_4S_4]^{1+}$ by reactions 3 and 4, and makes $[Fe_4S_4]^{1+}$ the dominant Fe protein species during catalysis, which produces ATP/2e values near 5.0 by predominant operation of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple.

The hypothesis that the results in Table 1 are a consequence of the two distinct limiting types of reactivity provides insights into the results shown in Figure 2. The fact that variation in temperature and MoFe:Fe protein ratio using DT as reductant give rise to substantial changes in ATP utilization (Table 2) suggests that more than one reaction occurs. The possibility of two reactions occurring during operation of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple has been considered previously (*18*, *20*). Since only the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple operates using DT, the presence of two reactions must arise from two different Fe protein interactions with the MoFe protein, each using the $[Fe_4S_4]^{1+}$ redox state. In contrast, only one reaction is observed under a wide range of conditions from use of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple. We are currently exploring what the two distinct $[Fe_4S_4]^{1+}$ reactions might be.

The results in the last row of Table 1 appear anomalous because at high Fe:MoFe protein ratios, the rate of product formation is high and operation of the $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couple would produce ATP/2e values near 5.0. However, at high ratios the MoFe protein is operating in the presence of excess $[Fe_4S_4]^0$ and the concentration of the $[Fe_4S_4]^{1+}$ formed by reactions 3 and 4 is small compared to the excess of the $[Fe_4S_4]^0$, so the latter preferentially reacts. In addition, under these conditions, any $[Fe_4S_4]^{1+}$ that is present has sufficient time to be reduced to $[Fe_4S_4]^0$. Despite the increase in the rate of the enzymatic reaction at high ratios, a high concentration of $[Fe_4S_4]^0$ is maintained and drives the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple.

An excess of reduced MV was the first reductant used to form $[Fe_4S_4]^0$ and, if used as a reductant for nitrogenase catalysis, should also give ATP/2e values near 2.0. But at concentrations of reduced MV (*31*) above 0.4 mM, necessary to conveniently conduct measurements, nitrogenase is severely inhibited (*32*), which prevents a proper test of this prediction. To properly evaluate this predicted effect, additional experiments are being conducted.

Product Distribution. Finally we examined the relationship between ATP utilization and substrate reduction under conditions where ATP/2e values of 2.0 and 5.0 were

obtained. N_2 reduction using 5 mM Ti(III) produced NH_3 (67%) and H_2 (33%) at the indicated percentages of total electron flow and ATP/2e values near 2.0. With DT, 70% NH_3 and 30% H_2 are formed but ATP/2e values near 5.0 are observed. H_2 evolution, acetylene reduction, and reactions conducted in the presence of CO gave identical product distributions independent of ATP/2e values. An important conclusion suggested by these results is that obligatory H_2 evolution for either acetylene or N_2 reduction is completely controlled by the MoFe protein whether electrons are transferred with concomitant hydrolysis of two or five ATPs per cycle. These results also suggest that CO is not reacting with the Fe protein cluster because identical behavior is observed independent of the reductant used. These conclusions support the previous findings that CO interacts with FeMoco as evidenced by distinct EPR signals (*33*, *34*).

If both the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ and $[Fe_4S_4]^{2+}/[Fe_4S_4]^{1+}$ redox couples are functional during in vitro nitrogenase catalysis using Ti(III) and $AvFlpH_2$, this would explain the results reported here. The question that remains is, are the in vitro results relevant to in vivo nitrogenase catalysis? ATP/2e values of 2.5 for Cp (*35*), 3.75 for Kp (*36*), and 0.63 for Ac (*37*) were reported for ATP utilization in whole cells. While these in vivo 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 in Figure 1 and Tables 1 and 2 for reactions that we proposed are catalyzed by the $[Fe_4S_4]^{2+}/[Fe_4S_4]^0$ redox couple. These results suggest that the in vivo electron donor is capable of forming the $[Fe_4S_4]^0$ state of the Fe protein and hydrolyzes only two ATPs per 2e transferred. This process would make the in vivo utilization of ATP a more efficient process than the one-electron-transfer step using DT in vitro. It is also possible that both redox couples operate in vivo depending upon the cellular conditions.

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