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Kinetic Studies on Electron Transfer and Interaction between Nitrogenase Components from *Azotobacter vinelandii*†

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ABSTRACT: Kinetic properties of electron transfer by nitrogenase of *Azotobacter vinelandii* are dependent on the concentration of the two components of nitrogenase. An excess of the MoFe protein inhibits electron transfer in a distinctive manner, and the inhibition is reversed by increasing levels of reductant. The saturation curve for Fe protein is hyperbolic, indicating that only one Fe protein molecule per MoFe protein is required for full activity in ATP hydrolysis and electron transfer. These results can be interpreted on the basis of a complex between the Fe protein and the MoFe protein that dissociates rapidly during turnover. Both 2:1 and 1:1 complexes (Fe-MoFe) are active. Dithionite appears to be a relatively poor reductant for nitrogenase from *Azotobacter vinelandii,* whereas azotobacter flavodoxin is much better. In the presence of the flavodoxin it is possible to increase the specific activity of the Fe protein more than 50% relative to its activity with dithionite alone as a reductant; specific activities greater than 3000 nmol of C_2H_4 formed min^{-1} (mg of Fe protein)$^{-1}$ have been observed.

Nitrogenase consists of two proteins, the larger containing molybdenum and iron (the MoFe protein) and the smaller containing iron (the Fe protein). These are readily separated and purified independently (Winter and Burris, 1976). The easy separation and recombination of the two proteins without irreversible loss of activity allow one to vary the ratio of the nitrogenase components in reaction mixtures. The nitrogenase of *Azotobacter vinelandii* also can be isolated as a particulate complex that is relatively resistant to oxygen denaturation and contains a set ratio (for a given preparation) of the two nitrogenase components (Winter and Burris, 1976). A thorough understanding of the nature of the interactions between the two proteins would eliminate many of the uncertainties inherent in studying a two-protein component system.

The interaction between the two proteins has been studied extensively (Shah et al., 1975; Emerich and Burris, 1976; Davis et al., 1975; Thorneley et al., 1975; Thorneley, 1975; Bui and Mortenson, 1969; Ljones and Burris, 1972a; Bergersen and Turner, 1973; Silverstein and Bulen, 1970) by steady-state kinetics, rapid-reaction kinetics, sedimentation velocity, and gel-filtration experiments. The dilution effect (decreasing the specific activity when the concentration of the nitrogenase is decreased at a constant ratio of the two proteins) serves as a useful probe (Thorneley et al., 1975; Silverstein and Bulen, 1970). Although this approach can provide an estimate of the association constant between the two proteins (Thorneley et al., 1975), it is a relatively insensitive measure of the stoichiometry of the complex. Other experiments have involved the titration of one component with the other (Emerich and Burris, 1976; Bergersen and Turner, 1973). These results, however, must be interpreted with caution, because when the MoFe protein is present in sufficient excess it causes inhibition. The inhibition by excess MoFe protein has been used as evidence of the nature of the active complex (Ljones and Burris, 1972a). Sedimentation velocity studies have indicated that a 1:1 complex (Fe-MoFe) is formed (Thorneley et al., 1975), whereas both kinetic and gel-filtration experiments with tight-binding but inactive heterologous crosses (the MoFe protein from one organism plus the Fe protein from another) of the nitrogenase proteins have indicated that a 2:1 complex

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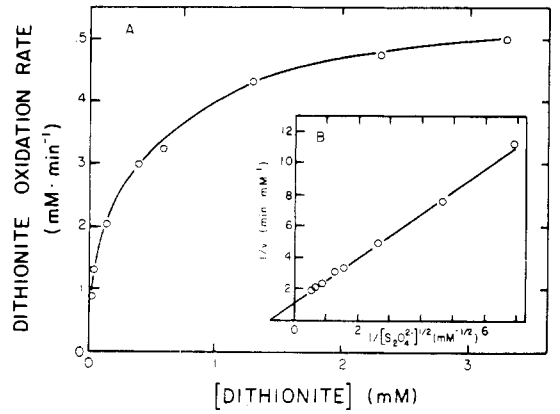

FIGURE 1: Dithionite oxidation velocity as a function of dithionite concentration. The system was under an argon gas phase as described under Materials and Methods. The assay mixture contained 1.55 μM MoFe protein and 9.03 μM Fe protein. Double-reciprocal plot of $1/v$ vs. $1/[S_2O_4^{2-}]^{1/2}$ to determine kinetic constants as described in the text.

can be formed (Emerich and Burris, 1976). Other kinetic studies have indicated that a 2:1 complex is active (Bergersen and Turner, 1973).

Many of the studies on the active complex have used reduction of N_2 or C_2H_2 as a measure of nitrogenase activity. However, because there may be effects of the Fe protein on reduction of these substrates that are not exhibited for H_2 production, it seems advisable to evaluate the activity of the complex by measuring total electron flux and ATP hydrolysis as well. Titrations of the MoFe protein with the Fe protein have yielded both hyperbolic (Ljones and Burris, 1972a; Tso et al., 1972) and sigmoidal (Bergersen and Turner, 1973) saturation curves for enzymatic activity. Sigmoidal saturation curves most commonly have been observed for N_2 or C_2H_2 reduction but not for H_2 evolution.

Stopped-flow kinetics (Thorneley, 1975) and rapid-reaction EPR studies (Zumft et al., 1974; Smith et al., 1973) have indicated that the association rate between the two components is rapid, $\tau < 10$ ms, but it is not known what the dissociation rate of the complex is. At present the stoichiometry of the active complex is in question, and the lifetime of the complex is unknown. Specifically, it is not known whether the complex dissociates during every turnover or whether it remains associated for many turnovers.

Materials and Methods

Nitrogenase components were purified from *A. vinelandii* OP by a modification of the method of Shah and Brill (1973). *Azotobacter* flavodoxin (AzFld) was purified by a modification of the method of Benemann et al. (1969). ATP, creatine kinase (EC 2.7.3.2), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma Chemical Co., creatine phosphate was from Pierce Chemical Co., and all other chemicals were the highest commercial grade. Cylinder gases were purified by passage over hot (150 °C) BASF catalyst R3-11 purchased from Chemical Dynamics Corp. Acetylene was generated by adding calcium carbide to water. Ethylene, formed from acetylene, was measured by gas chromatography (Burris, 1972); H_2 evolved also was estimated by gas chromatography (Eady et al., 1972). Ammonia formed was measured after microdiffusion by the indophenol method of Chaykin (1969). ATP hydrolysis was estimated by the release of creatine as measured by the method of Eggleton et al. (1943). Protein concentration was determined by the microbiuret method of Goa (1953) with bovine serum albumin as the standard. The concentrations of the MoFe protein and the Fe protein were estimated by accepting molecular weights of 242 000 and 60 500, respectively (Swisher et al., 1975). Flavodoxin concentration was estimated with an extinction coefficient of 10 600 M^{-1} cm^{-1} at 450 nm for the oxidized form (Hinkson and Bulen, 1967). Nitrogenase components had specific activities of at least 1900 nmol of C_2H_2 formed min^{-1} (mg of Fe protein)$^{-1}$ and 2200 nmol of C_2H_2 formed min^{-1} (mg of MoFe protein)$^{-1}$ when measured under standard assay conditions with 20 mM dithionite, an argon gas phase with 10% C_2H_2, and optimal levels of the complementary protein.

Unless otherwise specified, all assay mixtures contained in 1.0 mL, 5 μmol of ATP, 25 μmol of creatine phosphate, 0.1 mg of creatine kinase, 10 μmol of magnesium acetate, 50 μmol of Hepes–Tris at a pH of 7.45. Argon was the usual gas phase in 22-mL vaccine bottles. Dithionite, flavodoxin, and nitrogenase were added as indicated. Assay mixtures containing creatine phosphate, creatine kinase, 5 μmol of magnesium acetate, buffer, and flavodoxin were made anaerobic by evacuating and filling with argon three times. Dithionite and acetylene then were added, followed by the nitrogenase components added with gas-tight syringes. After preincubating at 30 °C, reactions were started by the addition of an anaerobic solution of MgATP (pH 7.5). No differences were noted if the assays were started by the addition of either nitrogenase component. After incubating for the desired interval, the reaction was stopped by the addition of either 0.1 mL of 25% trichloroacetic acid or 1.0 mL of saturated K_2CO_3, and the gas phase was analyzed for the C_2H_4 and/or H_2. Assays stopped with K_2CO_3 were analyzed for creatine release, whereas assays stopped with trichloroacetic acid were analyzed for ammonia after microdiffusion initiated by the addition of K_2CO_3. Dithionite oxidation assays (Ljones and Burris, 1972b) were performed in 1.0-mm light-path cuvettes, and an extinction coefficient of 1300 M^{-1} cm^{-1} at 350 nm was used (Davis et al., 1975). Velocities were taken from the tangents to a single progress curve, and dithionite concentration was measured by allowing the reaction to exhaust all the dithionite to establish a blank reference value.

Results

Dithionite Oxidation Kinetics. Figure 1 shows a typical plot of velocity of dithionite oxidation vs. dithionite concentration in the presence of excess Fe protein. In agreement with Watt and Burns (1977), the reaction is dependent on the half-power of the dithionite concentration. However, we find that the reaction does saturate at high dithionite concentrations. This saturation agrees with the H_2 evolution data of Watt and Burns (1977) but not with their dithionite oxidation data. The reason for this discrepancy is unknown, as we have observed saturation even at ratios of 2:1 Fe–MoFe which approximates the component ratio in the particulate nitrogenase they employed. A rather high level of dithionite is required to achieve saturation at this ratio; e.g., $K_{0.5} \doteq 14$ mM. This high level for saturation agrees with some previous evidence for *A. vinelandii* (Hwang and Burris, 1972), but is remarkably different from the micromolar levels of dithionite required to saturate *Clostridium pasteurianum* nitrogenase (Ljones and Burris, 1972).

The kinetics observed fit an equation of the form

$$v = \frac{V_M[S_2O_4^{2-}]^{1/2}}{K^{1/2} + [S_2O_4^{2-}]^{1/2}}$$

where V_M and $K^{1/2}$ are functions of the concentration of the nitrogenase proteins. As suggested by Thorneley et al. (1976), this is consistent with $SO_2^{-}\cdot$ being the active reductant.

Figure 2 shows the dependence of $K^{1/2}/V_M$ and $1/V_M$ on

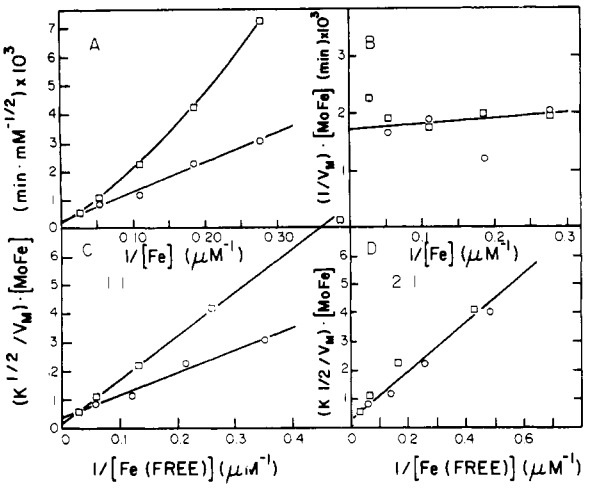

FIGURE 2: Plots of kinetic parameters for dithionite oxidation as functions of Fe protein concentration. The assays were performed as described under Materials and Methods, and data were analyzed as in Figure 1: (○ - ○) 0.77 μM MoFe protein; (□ - □) 1.55 μM MoFe protein. (A) $K^{1/2}/V_M$[MoFe] vs. total Fe protein concentration. (B) $1/V_M$[MoFe] vs. total Fe protein concentration. (C) $K^{1/2}/V_M$[MoFe] vs. free Fe protein calculated by assuming one Fe protein is bound to each MoFe protein molecule and subtracting this amount from the total Fe protein concentration to obtain the free Fe protein concentration. (D) $K^{1/2}/V_M$[MoFe] vs. free Fe protein calculated assuming two Fe protein molecules are bound to each MoFe protein molecule.

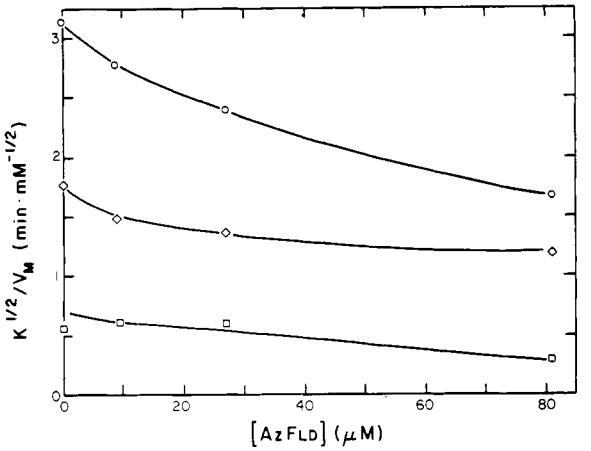

FIGURE 3: Kinetic parameters for dithionite as a function of AzFld concentration and Fe protein concentration. Dithionite oxidation assays were performed as described under Materials and Methods with 1.15 μM MoFe protein: (○ - ○) 4.71 μM Fe protein; (◇ - ◇) 9.41 μM Fe protein; (□ - □) 23.5 μM Fe protein.

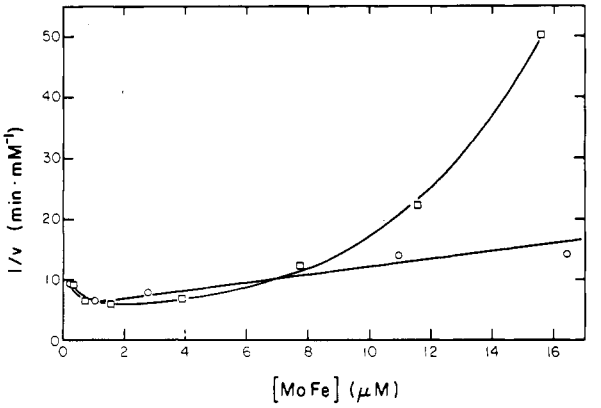

FIGURE 4: H_2 evolution and C_2H_2 reduction assays were carried out as described under Materials and Methods. 1.8 μM Fe protein, 10 mM dithionite, and MoFe protein were present as indicated: (○ - ○) H_2 evolution; (□ - □) C_2H_2 reduction.

the concentration of the Fe protein. Since V_M is relatively insensitive to the Fe protein concentration under these conditions, it is obvious that the apparent $K^{1/2}$ for dithionite is dependent on the Fe protein concentration and increases dramatically at low levels of Fe protein. Figures 2C and 2D show the effect on $K^{1/2}/V_M$, assuming that either one (Figure 2C) or two (Figure 2D) Fe protein molecules are bound to each MoFe protein molecule. $K^{1/2}/V_M$ is plotted vs. the concentration of the Fe protein that is in excess of the assumed stoichiometry. The assumption that two Fe protein molecules are bound yields a result that is independent of the MoFe protein level (Figure 2D), whereas the assumption that only one is bound (Figure 2C) or that binding effects can be ignored (Figure 2A) gives a result that is dependent on the MoFe protein concentration. Although this assumption of tight binding is an oversimplification, it does indicate that there may be sites on the MoFe protein for binding two Fe protein molecules. The assumption of tight binding is justified if the dissociation constant of the complex is 0.1 μM as estimated by Emerich and Burris (1976). They also found evidence for formation of a 2:1 complex, although the relative activities of the 1:1 and the 2:1 complexes could not be estimated. The invariance of V_M with the changing Fe protein level (Figure 2B) also indicates that the complex between the two proteins is relatively tight and that the concentration of the complex does not change as the Fe protein concentration increases.

The dependence of $K^{1/2}/V_M$ on the Fe protein level while V_M remains invariant is important in interpreting the mechanism of electron transfer, because this indicates that the total pool of the Fe protein is involved in electron transfer to the complex. This action could occur either by exchange of free Fe protein for Fe protein bound to the complex or by direct reduction of the complex by free reduced Fe protein.

The dependence of $K^{1/2}/V_M$ on the level of flavodoxin supports the role of free Fe protein in electron transfer. Figure 3 shows how increasing the level of either AzFld or the Fe protein lowers the $K^{1/2}/V_M$ for dithionite. Again V_M was relatively insensitive to the variation in Fe protein or AzFld concentration. Thus, the pool of Fe protein that is not bound to the MoFe protein seems to act as an electron carrier to the complex of Fe protein with MoFe protein. This could occur directly if dithionite, flavodoxin, or free Fe protein could each reduce a complex that is not readily dissociated. Alternatively, oxidized Fe protein could dissociate from the MoFe protein and then be slowly reduced in solution. Increasing the Fe protein concentration would dilute out the oxidized Fe protein and make it more likely for the MoFe protein to bind reduced Fe protein rather than rebinding oxidized Fe protein. Thus, if the total Fe protein concentration were increased, a lower concentration of dithionite could maintain the same proportion of reduced to oxidized Fe protein, and a lower concentration of dithionite would be required for saturation.

MoFe Inhibition. When the MoFe protein is present in excess, electron transfer is inhibited (Tso et al., 1972; Vandecasteele and Burris, 1970). Figure 4 shows the inhibition for H_2 evolution and acetylene reduction. H_2 evolution is seen to be linearly inhibited by increasing MoFe protein levels. Acetylene reduction is inhibited more strongly and in a nonlinear manner. Data of Davis et al. (1975) also indicate that acetylene reduction, nitrogen reduction, and hydrogen evolution are not all equivalent measures of nitrogenase activity when component ratios are varied.

Figure 2 shows the increase in $K^{1/2}/V_M$ for dithionite with

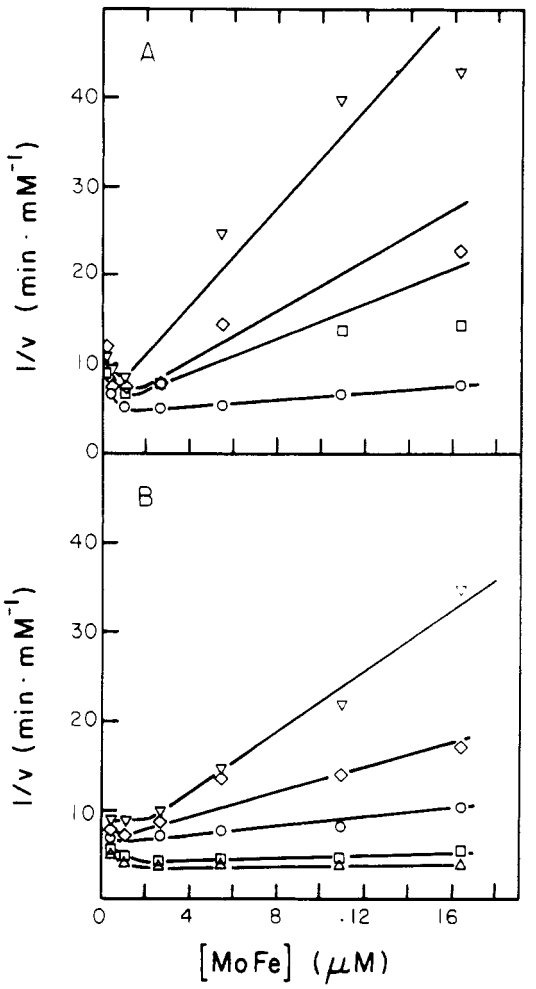

FIGURE 5: Reversal by dithionite and AzFld of the MoFe protein inhibition. Assays were performed under argon as described under Materials and Methods with MoFe protein, dithionite, and AzFld present as indicated. (A) Reversal by dithionite: (▽ - ▽) 2 mM dithionite; (◇ - ◇) 5 mM dithionite; (□ - □) 10 mM dithionite; (○ - ○) 20 mM dithionite. (B) Reversal by AzFld in the presence of 5 mM dithionite: (▽ - ▽) no AzFld; (◇ - ◇) 5 μM AzFld; (○ - ○) 15 μM AzFld; (□ - □) 50 μM AzFld; (△ - △) 100 μM AzFld. 1.79 μM Fe protein was used in each assay.

TABLE I: Fe Protein Specific Activity.

MoFe protein (μM)	nmol of C_2H_4 produced min^{-1} (mg of Fe Protein)$^{-1}$ [a]	
	20 mM dithionite	5 mM dithionite, 150 μM AzFld
0.54	690	
1.09	1968	3195
2.73	1968	3387
5.45	1744	3131
10.91	978	1981
16.36		2172

[a] 2.59 μM Fe protein was present.

decreasing Fe protein concentration. It seemed possible that this trend in the K_M for dithionite would continue when excess MoFe protein was present. If so, then inhibition by MoFe protein would merely reflect the rising K_M for dithionite in assays run at a constant level of dithionite, and it should be possible to reverse the inhibition by increasing the concentration of dithionite or by using AzFld to lower the K_M for dithionite. Figure 5 shows that inhibition by the MoFe protein is reversed with dithionite and AzFld. At all levels of AzFld the inhibition by increasing MoFe protein is linear, and the flavodoxin is approximately competitive with the MoFe protein. Dithionite at concentrations higher than 5 mM inhibits electron flow at noninhibitory levels of the MoFe protein, while it stimulates at higher levels of the MoFe protein. This inhibition is apparently due to ionic-strength effects such as have been observed before by Shah et al. (1972). Salts such as sodium sulfate also produce the inhibition. Ionic strength also affects both V_M and $K^{1/2}/V_M$ for dithionite when measured by dithionite oxidation in the presence of excess Fe protein (data not shown).

An interesting observation made during the preceding experiments was that AzFld supported higher activities than dithionite alone. This increase has also been noted by Schering et al. (1977). This suggested that the reversal of MoFe inhibition might lead to an increase in the specific activity of the Fe protein. Standard assays, either with 20 mM dithionite or with 150 μM AzFld plus 5 mM dithionite as the reductant, showed (Table I) that the maximal activity in the presence of the flavodoxin was over 50% higher than with dithionite alone. This 50% increase is consistent with our other experiments that were not designed to measure optimal Fe protein activity. The peak activity occurs at a higher MoFe protein concentration when AzFld is present; hence, under commonly used assay conditions without flavodoxin, significant inhibition by MoFe protein occurs even with optimal levels of the MoFe protein.

In determining the nature of the active nitrogenase complex, it is of interest to establish whether the inhibition by MoFe protein is partial or complete. As this involves measurements with very high concentrations of the MoFe protein, it presents problems related to the solubility of the MoFe protein. Modifications of the assay conditions required to increase the solubility of the MoFe protein are indicated in Figure 6. The low level of dithionite used increased the inhibition by the excess MoFe protein. The figure shows that inhibition by excess MoFe protein is partial both for H_2 evolution and ATP hydrolysis. Inhibition of ATP hydrolysis has not been reported by others, but we observed it consistently when high levels of the MoFe protein were used. The partial inhibition of H_2 evolution was clearly demonstrable, as no problems were apparent with solubility in this experiment. However, the partial inhibition of ATP hydrolysis became apparent at lower levels of MoFe protein. H_2 evolution decreased with increasing concentration of MoFe protein to about 5% of the best rate observed in this experiment or to about 1.5% of the maximal rate for this Fe protein. ATP hydrolysis decreased to about 25% of its highest rate in this experiment or to about 10% of its maximal rate. These results indicate that dithionite can reduce the complex of oxidized Fe protein plus MoFe protein, although at a much slower rate than the maximal rate of electron transfer operating through uncomplexed Fe protein. Extrapolation of the H_2-evolution curve to infinite MoFe protein indicates a rate of approximately 3 μM min^{-1}, whereas 2 μM MoFe protein gives a rate of 25 μM H_2 evolved min^{-1}. At a concentration of 2 μM MoFe protein, only 3% of the total Fe protein is in the unbound state, but it is capable of supporting a rate of H_2 evolution eightfold the rate at infinite MoFe protein concentration, a condition that leaves essentially no free Fe protein. Thus, the major reduction of Fe protein must occur when it is not bound to the MoFe protein. The data in Figure 3b of Thorneley et al. (1975) support this view; they showed that inhibition by excess MoFe protein occurred at relatively high total protein concentrations, whereas the same

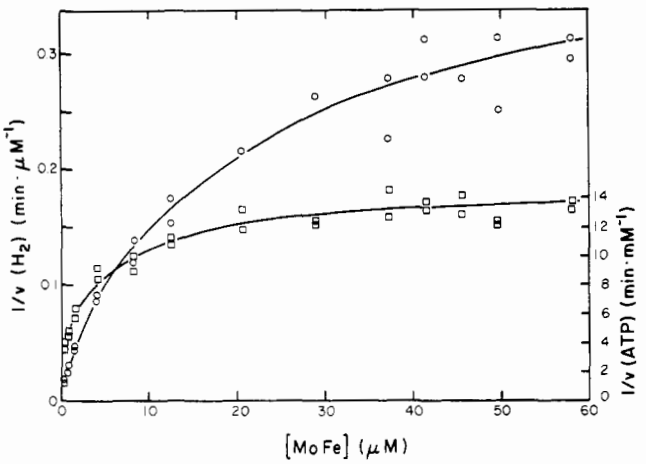

FIGURE 6: Inhibition of H_2 evolution and ATP hydrolysis by excess MoFe protein. Assay mixtures contained 25 mM Hepes-KOH (pH 7.45), 10 mM creatine phosphate, 0.1 mg/mL creatine phosphokinase, 5 mM ATP, 7 mM $MgOAc_2$, 1.04 μM Fe protein, and 2 mM dithionite. MoFe protein was added as indicated: (○ - ○) H_2 evolution, left axis; (□ - □) ATP hydrolysis, right axis.

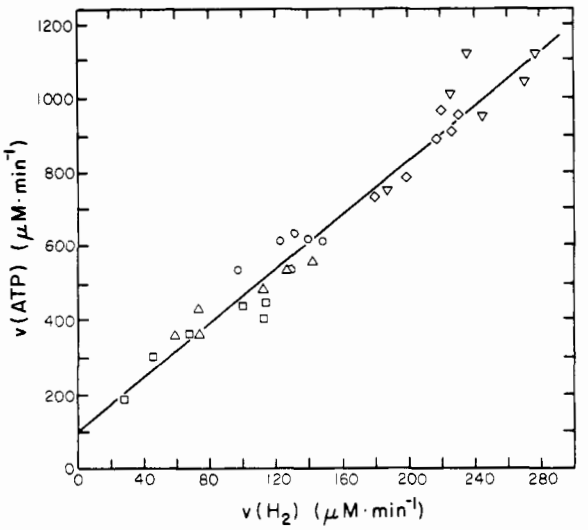

FIGURE 7: ATP hydrolysis as a function of hydrogen evolution. Standard assay conditions were used with 1.79 μM Fe protein and 0.55 to 16.4 μM MoFe protein: (□) 0 μM AzFld; (△) 5 μM AzFld; (○) 15 μM AzFld; (◇) 50 μM AzFld: (▽) 100 μM AzFld. The correlation line is from a linear least-squares fit of the data.

ratio of components at lower protein concentrations did not cause inhibition. The inhibition seems to result from the decrease in the ratio of free to bound Fe protein expected on the basis of mass action.

One of the intriguing problems concerning the functioning of nitrogenase is the coupling between ATP hydrolysis and electron transfer. Reported values for the ratio of ATP/$2e^-$ transferred range from a minimum of 4 up to 20 or more (Silverstein and Bulen, 1970; Ljones and Burris, 1972; Watt et al., 1975; Winter and Burris, 1976). In addition, there is the observation that in the absence of reductant ATP still is hydrolyzed at a rate about 10% of the highest rate in the presence of reductant (Winter and Burris, 1976). As mentioned, we have consistently observed inhibition of ATP hydrolysis in the presence of levels of the MoFe protein higher than those usually used in previous work. Tests of *Clostridium pasteurianum* preparations with similar levels of the MoFe protein have not indicated inhibition of ATP hydrolysis (Walker, 1974). Figure 7 shows the results of an experiment in which AzFld and the MoFe protein concentrations were varied to change the degree of inhibition effected by excess MoFe protein. It is clear that inhibition of ATP hydrolysis and inhibition of H_2 evolution are correlated, and that there is a finite level of ATP hydrolysis at zero H_2 evolution. Although there does not seem to be a significant deviation from this correlation line at any given AzFld concentration, it is possible that there is a trend hidden in the scatter of the data. The rate of ATP hydrolysis at zero H_2 evolution corresponds closely to the rate expected if reductant-independent ATP hydrolysis had been measured under similar conditions. The slope of the correlation line is somewhat less than 4, although ATP/H_2 is about 4 for the highest rates of evolution. The line can be fitted to an equation of the form:

$$v_{ATP} = Av_{H_2} + B$$

where $A = 3.67$ and $B = 95.7$ nmol min^{-1}. If the coupled ratio (ATP/$2e^-$) for ATP hydrolysis is assumed to be 4 [based on two ATP binding sites per Fe protein (Tso and Burris, 1973) and one electron carried per Fe protein molecule (Winter and Burris, 1976)], the above equation can be rearranged to give the more informative equation:

$$v_{ATP} = 4v_{H_2} + D\left(1 - \frac{v_{H_2}}{C}\right)$$

where 4 is the assumed rate of coupled ATP hydrolysis, C is the maximal velocity of H_2 evolution, and D is the maximal rate of uncoupled ATP hydrolysis. In this case, $D = 95.7$ nmol min^{-1}. Similar results are obtained if dithionite and MoFe protein concentrations are varied to change the degree of inhibition. In each case, as the rate of coupled ATP hydrolysis increases, the rate of the uncoupled ATP hydrolysis decreases.

Fe Protein Saturation Curve. Both sigmoidal (Bergersen and Turner, 1973) and hyperbolic (Ljones and Burris, 1972a; Tso et al., 1972; Vandecasteele and Burris, 1970) saturation curves have been reported for the titration of the MoFe protein with the Fe protein. Such studies are complicated by the possibility of inhibition by MoFe protein affecting the results, and, in addition, when acetylene or N_2 reduction rather than H_2 evolution is measured, it is possible that the Fe protein may influence the process in ways other than simply by affecting electron transfer (Bergersen and Turner, 1973; Davis et al., 1975). For example, our Figure 2 and the work of Davis et al. (1975) show that acetylene reduction and N_2 reduction can respond differently than total electron flow. Figure 8 shows the Fe protein saturation curve for H_2 evolution. The experimental conditions were such (high level of AzFld and a relatively low level of the MoFe protein) that there was minimal inhibition by MoFe protein. A Hill plot of the H_2-evolution titration data shows excellent linearity over a wide range of velocities. The Hill coefficient of 1.25 indicates that, although there may be some interaction between the two sites for the Fe protein on the MoFe protein, only one Fe protein molecule is needed per MoFe protein molecule for the complex to express essentially full activity. Clearly, only one Fe protein per MoFe protein is required for H_2 evolution and ATP hydrolysis.

Electron Partitioning. Figure 9 shows the distribution of electrons to alternative substrates and the total electron flux when the concentration of MoFe protein is varied under conditions designed to minimize inhibition by the MoFe protein. As the concentration of the MoFe protein is increased, progressively fewer electrons are allocated to nitrogen. With increasing MoFe protein there is an increase in the percentage of electrons going to acetylene, but with further increases in MoFe protein concentration most of the electrons support H_2

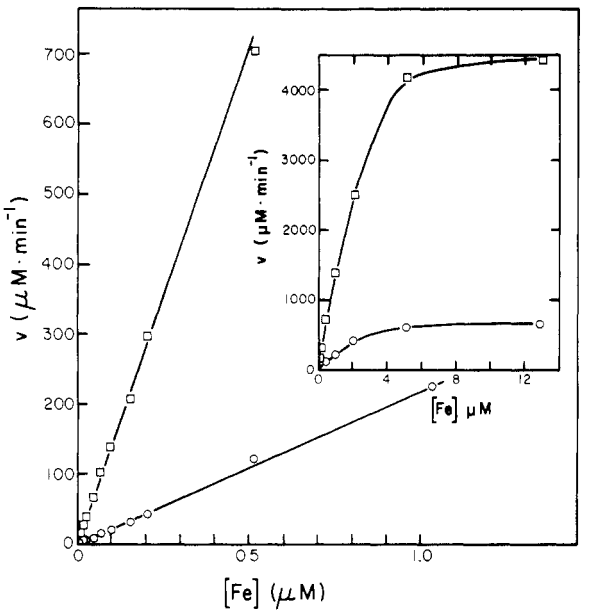

FIGURE 8: Saturation curve for Fe protein; the standard assay conditions, as described under Materials and Methods, were used, with 5 mM dithionite, 150 μM AzFld, and 0.91 μM MoFe protein: (○ - ○) H_2 evolution; (□ - □) ATP hydrolysis. Inset, shows the saturation curve extended to 13 μM Fe protein.

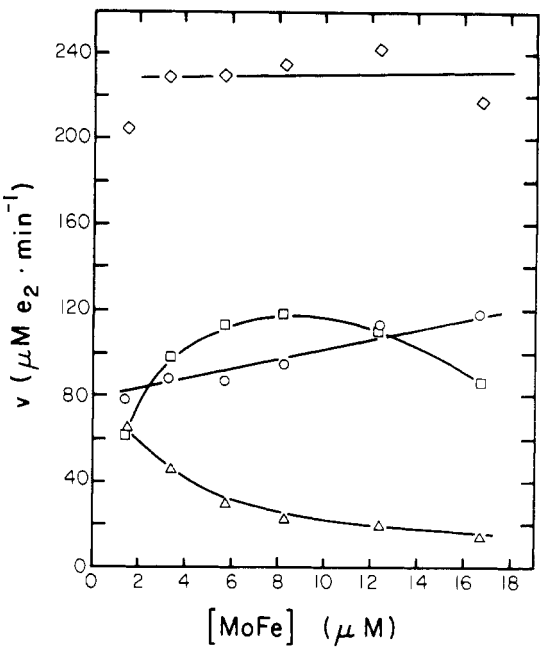

FIGURE 9: Allocation of electrons with changing concentrations of MoFe protein. Standard assay conditions were used, with 0.5% acetylene in nitrogen as a gas phase, 5 mM dithionite, 150 μM AzFld, and 2.16 μM Fe protein: (○ - ○) electron pairs to H_2 (μM min^{-1}); (□ - □) electron pairs to ethylene; (△ - △) electron pairs to ammonia; (◇ - ◇) total electron pairs transferred.

evolution. The important point is that the allocation pattern changes with changing levels of the MoFe protein at a constant electron flux per Fe protein. Apparently, the Fe protein and the MoFe protein do not remain bound as an active complex for many turnovers. The electron flux through a stable complex would remain the same with increasing MoFe protein concentration, and there would be no change in the complex to affect the electron allocation. If the complex dissociates on a time scale comparable to the turnover time, then the electron flux through any given MoFe protein molecule would change with varying MoFe protein concentration and this could affect allocation of electrons. Others have seen this change in electron allocation, but earlier experiments were complicated by inhibition of the total electron flux through the Fe protein by excess MoFe protein.

Discussion

Inhibition of nitrogenase activity by excess MoFe protein has been observed by several groups (Eady, 1973; Ljones and Burris, 1972a; Moustafa, 1970; Tso et al., 1972; Vandecasteele and Burris, 1970); however, no satisfactory explanation for this effect has been provided. The suggestion that the 1:1 complex of Fe-MoFe is inactive contradicts the evidence from the hyperbolic saturation curve for Fe protein. Our data indicate that inhibition by MoFe protein represents an extension of the trend toward an increasing K_M for dithionite with increasing MoFe protein concentrations at noninhibitory levels. Thus, inhibition by the MoFe protein reflects the nonsaturation with reductant when assays are performed at a constant level of reductant. This explanation is supported by the observation that added AzFld virtually eliminates MoFe inhibition. With added AzFld, the system is more nearly saturated with reductant, and this increases the specific activity of the Fe protein from *A. vinelandii* to a level comparable to that of the Fe protein from *C. pasteurianum*.

Other data in this paper suggest an explanation why the K_M for dithionite rises with an increase in the concentration of MoFe protein. Knowing that there are two binding sites for the Fe protein on the MoFe protein that produce a catalytically active complex (Emerich and Burris, 1976), one can visualize four possibilities for the interactions of the two proteins during electron transfer and ATP hydrolysis. First, it must be established whether both the 1:1 and the 2:1 complexes are essentially fully active or whether only the 2:1 complex can transfer electrons effectively. Second, it must be shown whether the active complex stays associated for many turnovers, i.e., it forms a stable complex, or whether it is dissociated with each electron transfer, i.e., it forms a transient complex. For a stable complex to operate, reductants must be able to reduce the complex formed between oxidized Fe protein and MoFe protein, and to explain the decreasing K_M for dithionite with increasing Fe protein concentration it must be postulated that free, reduced Fe protein also can reduce the complex. If the transient complex is the active species, then the Fe protein is reduced free in solution, although possibly it also can be reduced when bound in the complex.

The mechanism for inhibition by excess MoFe protein differs for the different models. If the 2:1 complex is the only active complex, then decreasing the proportion of the 2:1 and increasing the proportion of the 1:1 complex will cause inhibition; this will occur as the concentration of the MoFe protein is increased. The stable complex will be inhibited if dithionite is a much poorer reductant for the complex than is free Fe protein. Inhibition of the transient complexes will be caused by the rapid binding of oxidized Fe protein by the large pool of MoFe protein before reduction of the Fe protein occurs. The observed electron-partitioning effects require that the nature of the active complex change with increasing levels of the MoFe protein under conditions of constant total electron flow.

The observed kinetic effects are as follows: (1) inhibition by excess MoFe protein is linear, partial, and reversed by reductant; (2) the saturation curve for the Fe protein is hyperbolic; (3) partitioning of electrons among substrates varies with changing MoFe protein levels. If a stable 2:1 complex is the only active species, then complete inhibition by excess MoFe

TABLE II: Experimental Results and Predicted Properties for Various Models of Nitrogenase.[a]

property	exptl result	model predictions: stable complexes of Fe-MoFe, 1:1 and 2:1 active	stable complexes of Fe-MoFe, only 2:1 active	transient complexes of Fe-MoFe, 1:1 and 2:1 active	transient complexes of Fe-MoFe, only 2:1 active
inhibit. by excess MoFe					
completeness	appears partial	partial	*complete*	might be partial	*complete*
form	linear	linear	linear *parabolic*	linear	linear *parabolic*
reversal by reduct	yes	no slope effect	? yes	yes	? yes
saturat of Fe protein	hyperbolic	hyperbolic	*sigmoidal*	hyperbolic	*sigmoidal*
e^- partitioning effects	yes	*not after major form is 1:1*	*no*	yes	maybe

[a] Italicized predictions are in disagreement with experimental results.

protein is expected. This inhibition could be either parabolic or linear, depending on whether or not free, reduced Fe protein is a significant factor in reducing the complex. If the inhibition is linear (the case if the small amount of free Fe protein is not significant in reducing the complex), then the reversal by reductant has no ready explanation. If free Fe protein is a significant reductant for the stable 2:1 complex, the inhibition will be parabolic, and increasing levels of reductant will be expected to convert the parabolic into linear inhibition. The stable 2:1 complex also implies sigmoidal saturation kinetics for the Fe protein. If a stable 2:1 complex is the only active species, then a given molecule of the MoFe protein is either transferring electrons or not at a constant electron flow through the Fe protein. There would be only short periods when the MoFe protein molecule is changing states relative to its time in a given state. Thus, if the MoFe protein concentration is increased while maintaining constant electron flux, the excess MoFe protein would not be involved in electron flow and, hence, would not affect electron partitioning. The stable 2:1 complex model contradicts the experimental observations in several respects. The transient 2:1 complex as the only active species also can be ruled out for most of the above reasons. However, the model is compatible with the electron allocation, as this could change with increasing concentration of the MoFe protein. At a constant electron flux through the Fe protein, the flux through any given MoFe protein molecule would be changing if the complex could dissociate after every electron transfer, and this in turn could affect allocation of electrons to substrates.

The functioning of both 1:1 and 2:1 stable, active complexes predicts that inhibition by excess MoFe protein be partial, as dithionite must be able to reduce the complex. Thus, the degree of inhibition indicates how good a reductant dithionite is compared to free, reduced Fe protein. Figure 6 shows clearly that free Fe protein is the major site of reduction by dithionite. Thus, free, reduced Fe protein must be a significant reductant of the stable complex. Inhibition by excess MoFe protein then would be linear, with reductant serving only to modify the final degree of inhibition but not the slope of the inhibition curve. Titration of the MoFe protein with the Fe protein would yield a hyperbolic saturation curve if both the 1:1 and 2:1 complexes were active. Partitioning effects possibly could be observed during the transition from predominantly 2:1 to predominantly 1:1 complexes if both were active, but after titration yielded the 1:1 complex as the major component present no further effects on electron allocation would be observed if the complex did not dissociate during turnover.

If transient complexes of both 1:1 and 2:1 ratios are active, then inhibition by the MoFe protein would be linear as the lifetime of the free, oxidized Fe protein decreased. Reversal by reductants would be competitive with the excess MoFe protein. Inhibition could be partial if dithionite could reduce the complex of oxidized Fe protein plus MoFe protein. The degree of inhibition would reflect the rate at which dithionite could reduce the complex compared to the rate of reduction by dithionite of free oxidized Fe protein. The much slower velocity at infinite MoFe protein compared to the rate at a finite level of MoFe protein, but zero free Fe protein, indicates that the lifetime of the free, oxidized Fe protein is important in the reduction. This indicates that the major route of electron transfer is via free, oxidized Fe protein. The saturation curve would again be hyperbolic if the 1:1 complex were active. Effects on electron allocation would be expected as the electron flux through a given molecule of the MoFe protein decreased with increasing MoFe protein concentration.

The preceding considerations are summarized in Table II; it shows that the experimental data rule out all models except the transient complex with both 1:1 and 2:1 complexes being active. This view does not contradict the available evidence for the dissociation rate constant for the complex. If an association rate constant 10^8 M^{-1} s^{-1} is assumed (Thorneley, 1975) and the dissociation constant of 10^{-7} M is used (Thorneley et al., 1975), then the dissociation rate constant is calculated to be 10 s^{-1}. This is approximately equal to the turnover rate per electron for Fe protein of specific activity 3000 nmol min^{-1} (mg of Fe protein)$^{-1}$ which can be calculated to be 6.4 s^{-1}.

The knowledge of how the two-component proteins of nitrogenase interact should be very helpful in designing and interpreting experiments in which the ratio of the two proteins is varied. The use of *Azotobacter* flavodoxin to eliminate inhibition by excess MoFe protein has been of great importance in simplifying and clarifying the experimental work.

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Subsites and Catalytic Mechanism of Ribonuclease T_1: Kinetic Studies Using GpA, GpC, GpG, and GpU as Substrates†

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ABSTRACT: Steady-state kinetic studies of the ribonuclease T_1 catalyzed transesterification of GpA and GpG in 0.2 M buffer and GpC and GpU in 0.02 M buffer were performed at 25 °C in the pH range 2.5-9 and the results were analyzed together with those for GpC and GpU in 0.2 M buffer (Zabinski, M., & Walz, F. G. (1976) *Arch. Biochem. Biophys. 175,* 558). The pH dependences of k_{cat}/K_m for the four dinucleoside monophosphates in 0.2 M buffer were similar and suggested the involvement of two unprotonated groups on the free enzyme having apparent p*K* values of 3.4 and 4.3 and two protonated groups having apparent p*K* values of 7.5 and 8.1. These apparent p*K*s agree with the intrinsic p*K*s found for Glu-58 (Walz, F. G. (1977) *Biochemistry 16,* 4568), His-40 and His-92 (Arata, K., et al. (1976) *Biochem. Biophys. Res. Commun. 37,* 247) and it is concluded that these residues, and an unidentified carboxyl group, take part in binding and/or catalysis at the active site. The effect of the lower ionic strength buffer on the pH dependence of k_{cat}/K_m supports the involvement of Glu-58 and suggests that both active site carboxyl groups are adjacent to cationic species. The pH independent values of k_{cat}/K_m characterizing the four substrates are virtually identical, while the individual values for k_{cat} and K_m range within an order of magnitude of each other and follow the sequence: GpC > GpA > GpG > GpU. A common mechanism of catalysis for these substrates is proposed in which Glu-58, interacting with His-92, acts as a general base in proton transfer from the Guo-2′-hydroxyl group, and His-40, possibly associated with the unidentified carboxyl residue, serves as a general acid in protonating the 5′ oxygen of the leaving nucleoside group. The different effects of the substrate leaving nucleoside groups on the pH independent values of k_{cat} and K_m are discussed in terms of this mechanism where the intrinsic free energies of binding (Jencks, W. P. (1975) *Adv. Enzymol. 43,* 219) for all substrates are considered to be identical and are differently partitioned into binding or catalytic modes by virtue of coupled interactions of the enzyme with the substrate leaving nucleoside and Guo-2′-hydroxyl groups.

The mechanism of action for RNase[1] T_1 (EC 2.7.7.26) has been studied by a variety of methods; yet the chemical role of essential groups at the active site is still uncertain. Early chemical modification studies clearly indicated the importance of Glu-58 for activity (Takahashi et al., 1967) and led to the proposal of a concerted catalytic mechanism in which Glu-58 and a single histidine residue act as proton transfer agents (Takahashi, 1970). Subsequent pH dependent kinetic studies of the enzyme using GpC and GpU as substrates suggested the participation of at least one carboxylate and two imidazolium groups in binding and/or catalysis (Zabinski & Walz, 1976). This view was supported by more recent chemical modification

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[1] Abbreviations used: RNase, ribonuclease; CM-RNase T_1, γ-carboxymethyl-Glu-58-ribonuclease T_1; NMR, nuclear magnetic resonance; Tris, 2-amino-2-hydroxy-1,3-propanediol; nucleotide designations follow the recommendations of the IUPAC-IUB commission as reported ((1970) *Biochemistry 9,* 4025).

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