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Role of Magnesium Adenosine 5′-Triphosphate in the Hydrogen Evolution Reaction Catalyzed by Nitrogenase from *Azotobacter vinelandii*†

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ABSTRACT: We have investigated the role of MgATP in the reaction catalyzed by nitrogenase from *Azotobacter vinelandii*. There is a rapid burst of ATP hydrolysis in the pre-steady-state reaction that occurs on the same time scale as the electron transfer from dinitrogenase reductase to dinitrogenase. This burst corresponds to two ATP's hydrolyzed per electron transferred between the two proteins. Two MgATP molecules are bound to dinitrogenase reductase with dissociation constants of 430 μM and 220 μM. Investigation of the effect of MgATP concentration on the pre-steady-state kinetics of electron transfer from dinitrogenase reductase to dinitrogenase showed that there are two MgATP's required for this reaction, and the K_m values are 220 μM and 970 μM. These values are similar to the dissociation constants for MgATP from dinitrogenase reductase and indicate that electron transfer between the two proteins is substantially slower than the binding and dissociation of MgATP from dinitrogenase reductase. The K_m values for MgATP in steady-state H_2 evolution were 390 μM and 30 μM. The decrease in the value of the second K_m indicates that a slow, irreversible step occurs after the electron transfer from dinitrogenase reductase to dinitrogenase. It is possible to predict quantitatively the steady-state kinetics from the pre-steady-state kinetics, and this shows that the MgATP dependence of electron transfer is sufficient to account for effects of MgATP concentration on the steady-state H_2 evolution catalyzed by nitrogenase. The hydrolysis of two ATP molecules when an electron is transferred between the two proteins of the nitrogenase system is sufficient to account for all of the ATP hydrolysis occurring in the steady-state reaction. The simplified scheme proposed to account for the MgATP dependency of the nitrogenase reaction indicates that the only role of MgATP is in support of the electron transfer from dinitrogenase reductase to dinitrogenase.

The nitrogenase enzyme system is responsible for the biological reduction of N_2 to NH_3 in an MgATP-requiring reaction. The enzyme system also catalyzes MgATP-dependent H_2 evolution and the MgATP-dependent reduction of acetylene to ethylene. The enzyme system consists of two proteins, dinitrogenase[1] (MoFe protein) and dinitrogenase reductase (Fe protein) (Zumft, 1976). Dinitrogenase carries the substrate reducing site, and dinitrogenase reductase serves as a specific reductase for dinitrogenase (Hageman & Burris, 1978a). The enzymology of nitrogenase has been reviewed frequently (Orme-Johnson et al., 1977; Winter & Burris, 1976; Zumft, 1976), and this paper will concentrate on the role of MgATP [also see a recent review by Ljones (1979)].

Although the nitrogenase reaction of physiological importance is the reduction of N_2, the reduction of protons to H_2 is a more convenient reaction for studies of electron flow through nitrogenase:

$$2H^+ + 2e^- + n\text{MgATP} \rightarrow H_2 + n\text{MgADP} + nP_i$$

The amount of MgATP hydrolyzed in the reaction is variable and ranges from a minimum of 4 ATP per 2 electrons to greater than 20 (Ljones & Burris, 1972), with no evidence for an upper limit. Dinitrogenase reductase contains a single [4Fe–4S] cluster[2] (Orme-Johnson & Davis, 1977), and it donates a single electron per [4Fe–4S] cluster to dinitrogenase in the physiological reaction (Ljones & Burris, 1978a). A molecule of dinitrogenase reductase binds two MgATP molecules (Tso & Burris, 1973), and the binding of both of these MgATP molecules is required to induce a conformational change in the Fe protein (Walker & Mortenson, 1973; Ljones & Burris, 1978b; Zumft et al., 1973). The binding of two MgATP's and the donation of one electron by dinitrogenase reductase agree nicely with the minimum hydrolysis of four ATP per two electrons.

MgATP is required to support electron transfer from dinitrogenase reductase to dinitrogenase (Orme-Johnson et al., 1972; Zumft, 1976). Eady et al. (1978) have shown that MgATP is hydrolyzed with a time course identical with that of the pre-steady-state electron transfer from dinitrogenase reductase to dinitrogenase of *Klebsiella pneumoniae*. They analyzed the stoichiometry of this burst of MgATP hydrolysis

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[1] The nomenclature used in this paper is modified somewhat from that suggested by Hageman & Burris (1978a). The MoFe protein is designated dinitrogenase and the Fe protein is designated dinitrogenase reductase to express the activity of the components of the complete system that is designated nitrogenase.

[2] Abbreviations used: [4Fe–4S], a four-iron- and four-sulfur-containing cluster of the ferredoxin type; BPS, bathophenanthrolinedisulfonate.

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by comparison to the *total* protein concentration and not the *active site* concentration. The active site concentration of the protein must be known to obtain a meaningful relationship in the stoichiometry of a single turnover experiment. Eady et al. (1978) found that two MgATP's were hydrolyzed per molecule of dinitrogenase, and they commented that this is only half the ATP hydrolyzed in the H_2 evolution reaction. Although they quantitated the amount of MgATP hydrolyzed, they did not quantitate the number of electrons transferred in the pre-steady-state burst. The comparison of MgATP hydrolysis to H_2 evolution may be misleading if one does not know that all of the electrons necessary for H_2 production were actually transferred and available. The hydrolysis of 2 MgATP's per dinitrogenase molecule corresponds to ~0.4 ATP per dinitrogenase reductase under their experimental conditions, a stoichiometry not easy to understand. The active-site concentration of the two nitrogenase proteins in the reaction must be known to make a meaningful analysis of the stoichiometry of MgATP hydrolysis in the pre-steady-state burst.

The MgATP dependence of electron transfer from dinitrogenase reductase to dinitrogenase has been analyzed by Thorneley (1975) and Thorneley & Cornish-Bowden (1977). They reported a kinetic requirement for only one MgATP for each electron transferred; however, H_2 evolution by nitrogenase has a kinetic requirement for two MgATP's per electron (Thorneley & Cornish-Bowden, 1977; Watt & Burns, 1977). Thorneley & Cornish-Bowden (1977) suggested that these observations indicated a role for MgATP in addition to the known role in the electron transfer reaction, because their pre-steady-state electron transfer data did not quantitatively predict the kinetics of steady-state H_2 evolution. Watt & Burns (1977) also suggested that the failure to see sigmoidal MgATP kinetic patterns may reflect the failure of investigators to utilize sufficiently low concentrations of MgATP.

The analysis of the pre-steady-state electron transfer by Thorneley (1975) and Thorneley & Cornish-Bowden (1977) applies to only a portion of the MgATP dependency of the pre-steady-state electron transfer. They did not consider that the reaction observed in the stopped-flow experiment is an approach to the equilibrium between the oxidation of dinitrogenase reductase by dinitrogenase and the reduction of oxidized dinitrogenase reductase by dithionite. The reduction reaction must occur as the nitrogenase system completes its catalytic cycle under the experimental conditions used by these authors. Although the back-reaction has little effect at high MgATP concentrations, due to the very high rate of the oxidation reaction, it is very important at low MgATP concentrations, and corrections for the back-reaction lead to parabolic rather than linear double-reciprocal plots. It seems likely to us that a more comprehensive analysis of their data (Thorneley, 1975; Thorneley & Cornish-Bowden, 1977) would lead to a conclusion that two MgATP's are kinetically required for the initial electron transfer in the *K. pneumoniae* nitrogenase system.

The role of MgATP in electron transfer is well documented (Orme-Johnson et al., 1972; Zumft, 1976; Eady et al., 1978), but additional roles for MgATP have been proposed. The support for the proposed second role for MgATP has come from peculiarities in the dependence of substrate reduction on the MgATP concentration and from differential inhibition of MgATP hydrolysis and substrate reduction. Nitrogenase, inhibited by ATP analogues, was reported to give proportionally more H_2 evolution over acetylene reduction than uninhibited nitrogenase, and it was suggested that MgATP had a specific role in determining which substrate reduction was favored by nitrogenase (Eady et al., 1975). Similar interpretations have been based on immunochemical inhibition (Rennie et al., 1978), heterologous crosses of low activity (Smith et al., 1976), and extreme component ratios (Thorneley & Eady, 1977). Silverstein & Bulen (1970) noted that low MgATP concentrations favored H_2 evolution over N_2 reduction by nitrogenase. They suggested that MgATP indirectly controls the distribution of nitrogenase between different states rather than proposing a specific control by MgATP. They postulated a reaction that occurred at a rate dependent on the MgATP concentration (presumably electron transfer between the component proteins) and suggested that the rate of this reaction controlled the distribution of states. This idea was supported by Davis et al. (1975). We since have shown that this control of enzymatic states is not a unique property of MgATP, because any factor that will change the electron flux through dinitrogenase also will alter the distribution of nitrogenase between different substrate-reducing states (Burris & Hageman, 1980).

It appears that no data actually require the existence of a second role for MgATP. In this paper we will show that under some experimental conditions, the effect of MgATP on steady-state H_2 evolution can be predicted on the basis of the pre-steady-state MgATP kinetics. The ability to quantitatively predict the MgATP dependence of the H_2 evolution rate indicates that the only role for MgATP is in the electron transfer reaction between the nitrogenase proteins.

Materials and Methods

General Procedures. The nitrogenase proteins from *Azotobacter vinelandii* were purified by a modification (Hageman, 1979) of the method of Shah & Brill (1973). *A. vinelandii* cell paste (500 g) was dispersed anaerobically in 2.2 L of 20 mM Tris (pH 7.4), 2 mM $Mg(OAc)_2$, 0.5 mM dithiothreitol, and 4 M glycerol. The cells were sedimented, the supernatant was discarded, and the cells were resuspended in 90 mL of the glycerol buffer plus 60 mg of lysozyme, 6 mg of DNase I, and 1 mM $Na_2S_2O_4$. After 15 min the cells were broken by osmotic shock by pouring the suspension into 2.2 L of rapidly stirred anaerobic buffer. The supernatant from centrifugation at 8000*g* for 90 min was placed on an anaerobic 6 × 25 cm column of Whatman DE-52 DEAE-cellulose in equilibrium with 50 mM NaCl. Dinitrogenase was eluted with 200 mM NaCl and dinitrogenase reductase was eluted with 350 mM NaCl in buffer (20 mM pH 7.4 Tris, 2 mM $Mg(OAc)_2$, 1 mM dithiothreitol, and 0.5 mM $Na_2S_2O_4$). The active fractions were precipitated with poly(ethylene glycol) 4000, and then the redissolved dinitrogenase and dinitrogenase reductase fractions were passed individually through DEAE-cellulose columns. Final purification of dinitrogenase reductase was accomplished by preparative polyacrylamide gel electrophoresis, and then it was desalted by passage through Sephadex G-25 fine. The dinitrogenase was crystallized. The preparations were estimated to be over 90% pure and had specific activities of ~2050 and 1750 nmol of C_2H_2 reduced min^{-1} (mg of dinitrogenase)$^{-1}$ for dinitrogenase reductase.

Chemical sources and general assay techniques were the same as described earlier (Hageman & Burris, 1978b). All experiments were performed under an argon atmosphere. The active-site concentration of dinitrogenase was taken to be equal to the Mo concentration, assuming that there are two Mo's per protein molecule (Münck et al., 1975) and one active site per Mo. Dinitrogenase accepts a single electron per Mo (Münck et al., 1975) in the rapid reaction (Smith et al., 1973), leading to the disappearance of the EPR signal of di-

nitrogenase. Mo was measured by the method of Clark & Axley (1955) (Bulen & LeComte, 1966). The active-site concentration of dinitrogenase reductase was taken to be the concentration of [4Fe–4S] clusters in the protein (Orme-Johnson & Davis, 1977; Ljones & Burris, 1978b). Dinitrogenase reductase from *Clostridium pasteurianum* is known to transfer one electron per [4Fe–4S] cluster (Ljones & Burris, 1978a) and dinitrogenase reductase from *A. vinelandii* was assumed to do the same. The [4Fe–4S] cluster content of dinitrogenase reductase from *A. vinelandii* was estimated with bathophenanthrolinedisulfonate (BPS) as described by Ljones & Burris (1978b). The tightly bound iron released by added MgATP was presumed to arise from the [4Fe–4S] clusters of dinitrogenase reductase. It was assumed that all of the iron in the [4Fe–4S] clusters was measured in this treatment. We have found that the concentration of [4Fe–4S] clusters estimated in this manner is proportional to the specific activity of dinitrogenase reductase. When calculating the stoichiometry of MgATP hydrolysis, we assumed that dinitrogenase reductase donated one electron per [4Fe–4S] cluster and that dinitrogenase accepted one electron per Mo.

MgATP binding was estimated by the BPS method described by Ljones & Burris (1978b), who have discussed the reasons for utilizing this technique to study the interactions of MgATP with dinitrogenase reductase. Hydrogen evolution was measured for 5–60 min to give approximately constant amounts of H_2 evolved among experiments and to minimize effects of the hydrogen lag period (Hageman & Burris, 1978a). After the reactions were quenched with trichloroacetic acid, H_2 was estimated by gas chromatography (Eady et al., 1972).

Rapid-Reaction Procedures. Stopped-flow spectrophotometry (Gibson, 1969) was performed in a Gibson-Durrum D-110 spectrometer that had been modified in three significant ways. The syringes utilized glass barrels (Wilmad; 7.13-mm i.d.) and adjustable plungers with o-ring seals, fitted to a demountable Delrin syringe shell from which connections to either the spectrometer or the chemical quench apparatus could be made. The water baths of the D-110 spectrometer were replaced with a single bath enclosing both the syringes and the reaction cuvette and insulated from the mounting frame of the spectrometer. This modification eliminated the refractive index artifact (Miller & Gordon, 1976) associated with operation different from room temperature. The bath was closed with a gasketed Lucite box so that the pO_2 in the vicinity of the syringes and cuvette could be lowered by purging the system with N_2. Absorbance data were accumulated in a Tracor-Northern NS 570 signal averager and were transferred to either magnetic or paper tape for subsequent analysis. The data were visualized immediately on the signal averager, and a permanent record was obtained by transmitting the data to an *X–Y* recorder or to a Univac 110 computer that was used to fit the first-order portion of the reaction profile to an equation of the form

$$A = B + C(1 - e^{-kt}) \quad (1)$$

by a least-squares method (B is the initial absorbance of the mixture and C is the increase in absorbance caused by the oxidation of dinitrogenase reductase).

Chemically quenched reaction mixtures were prepared with the general procedures developed by Bray (1961) and subsequently elaborated by Ballou (1978). A pair of Update Instrument Co. (Madison, WI) syringe rams were connected so that they could be activated in sequence, and syringes as described for the stopped-flow experiments were combined with mixing chambers and delay lines to produce one of the following sequences of events: (1) for delays of 400 ms or longer, enzyme and substrate are rapidly (~1 ms) mixed and passed into a delay line, the ram hesitates while the reaction mixture ages, and then the second ram is activated so that buffer displaces the reaction mixture into a second mixer in which acid is added; (2) for shorter delay times, enzyme and substrate are mixed rapidly and passed through a delay line and thence to the second mixer, the delay time being governed by the length of the delay line and the velocity of the syringe ram. The acid-quenched reaction mixture was collected in a stoppered test tube and stored on ice until analyzed for released phosphate. Most of the protein was removed from the samples by centrifugation, and then phosphate was measured in the supernatant by the method of Penney & Bolger (1978). Corrections were made for variations in the dilution volume of the quenching acid by normalization to the adenine concentration in the quenched reaction mixture as measured by absorption at 259 nm after protein had been precipitated a second time with 5% perchloric acid.

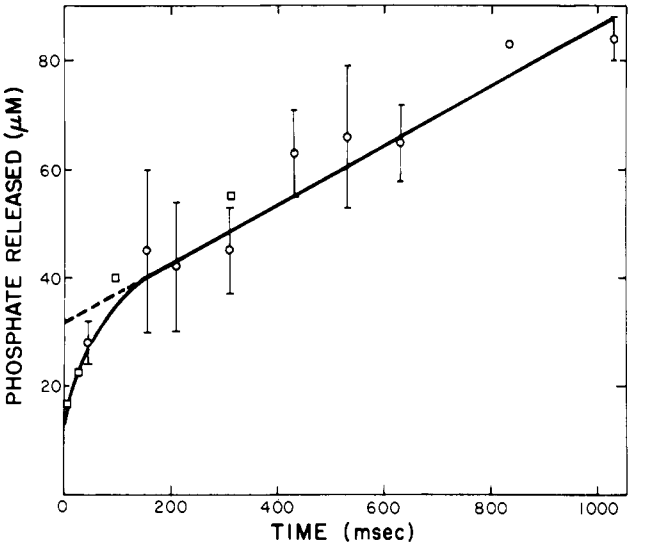


FIGURE 1: Pre-steady-state hydrolysis of ATP by nitrogenase. The chemical quench technique described in the text was used to measure phosphate release during the first second of the nitrogenase-catalyzed reaction. Syringe A contained 25 mM buffer, 5 mM dithionite, and 4 mM ATP. Syringe B contained 25 mM buffer, 5 mM dithionite, 6 mM magnesium acetate, 19.2 μM dinitrogenase (27.8 μM molybdenum), and 47.4 μM dinitrogenase reductase (25.1 μM [4Fe–4S] clusters). Syringe C contained 25% (w/v) perchloric acid. The mixing ratio was 5 of A to 5 of B to initiate the reaction and ~2 of C to stop the reaction. Phosphate released was corrected for the background phosphate in the assay mixture. Error is indicated on the figure as the standard deviation of the replicates at each time point. The linear least-squares fit was calculated from the data at times of 90 ms and longer (the standard error of the fit was 20%, which was used to define the error in the y intercept). Two experiments are shown: (○) represents the data used in the least-squares fit and (□) represents a separate experiment.

Results

Pre-Steady-State ATP Hydrolysis. Eady et al. (1978) reported that a burst of MgATP hydrolysis occurs at the same rate as the initial electron transfer between the two proteins of nitrogenase, and they estimated that two ATP's were hydrolyzed per molecule of dinitrogenase. However, the ratio of nitrogenase proteins that they used may not have supported optimal electron transfer. Figure 1 shows a similar pre-steady-state experiment on MgATP hydrolysis at 30 °C. An initial rapid release of phosphate was followed by a much slower steady-state rate. The initial burst of MgATP hydrolysis in this experiment was too rapid to allow determination of the rate constant, but the results of Eady et al. (1978)

Table 1: Stopped-Flow Measurement of MgATP-Induced Oxidation of Dinitrogenase Reductase[a]

[MgATP] (mM)	k_{app} (s^{-1})	absorbance change (A)	initial velocity ($A\ s^{-1}$)	k_1 (s^{-1})	k_2 (s^{-1})
0.037	4.1 ± 0.9	0.048 ± 0.001	0.20 ± 0.04	1.6 ± 0.2	2.5 ± 0.6
0.050	6.9 ± 0.6	0.066 ± 0.004	0.46 ± 0.05	3.4 ± 0.3	3.3 ± 0.4
0.067	7.6 ± 0.3	0.073 ± 0.003	0.55 ± 0.02	4.2 ± 0.2	3.2 ± 0.3
0.084	12.7 ± 1.0	0.075 ± 0.005	0.92 ± 0.07	7.1 ± 0.2	5.4 ± 0.9
0.100	14.9 ± 0.5	0.083 ± 0.004	1.24 ± 0.02	11.2 ± 0.5	(3.8)
0.167	23.9 ± 0.4	0.099 ± 0.004	2.37 ± 0.10	20.1 ± 0.4	(3.8)
0.200	32.2 ± 2.0	0.108 ± 0.008	3.47 ± 0.46	28.4 ± 2.0	(3.8)
0.500	91.6 ± 3.5	0.119 ± 0.004	10.88 ± 0.18	87.8 ± 3.8	(3.8)
2.500	225.0 ± 20.0	0.129 ± 0.009	29.00 ± 4.40	221.0 ± 20.0	(3.8)

[a] The stopped-flow experiment is described in the text and Figure 3. Variables are described in the text. All values are given with plus or minus standard error.

indicated that the rate constant would be the same as for electron transfer. The equivalence of the observed phosphate release with MgATP hydrolysis is supported by the following two lines of evidence. First, in a separate experiment, the formation of ADP from ATP was measured after an acid quench [ADP was estimated by an enzymatic method (Adam, 1963)], and this experiment also showed a time course of rapid burst followed by a slower steady-state rate. Second, attempts to measure covalently bound adenine or phosphate during turnover have failed [phosphate was measured on an acid-quenched steady-state sample, and the column technique of Penefsky (1977) was used to measure protein-bound [^{14}C]ATP during turnover]. Both experiments indicated that release of phosphate is not accompanied by the formation of a long-lifetime, covalent intermediate with ADP bound to the protein. Such a covalent intermediate would be expected if the phosphate release were not accompanied by an equivalent release of ADP.

The slow steady-state rate of phosphate release (Figure 1) can be extrapolated to time zero to estimate the magnitude of the pre-steady-state burst. The 32 (±6) μM phosphate released (y intercept) corresponds to 1.3 MgATP's hydrolyzed per dinitrogenase reductase molecule. This dinitrogenase reductase had been found to contain 0.53 [4Fe-4S] cluster per molecule by the method of Ljones & Burris (1978b). Thus, the MgATP hydrolysis in the burst phase of Figure 1 approximates 2.5 MgATP's (1.3/0.53 = 2.5) hydrolyzed per [4Fe-4S] cluster in the dinitrogenase reductase or to ~2.5 MgATP's per electron transferred, as dinitrogenase reductase is a one-electron transfer agent.

The MgATP burst also is equivalent to about 3.2 MgATP's hydrolyzed per dinitrogenase molecule. Dinitrogenase from *A. vinelandii* contains 2.0 Mo's per molecule, and it accepts two electrons per molecule (one electron per Mo) in a fast step (Münck et al., 1975; Smith et al., 1973). In Figure 1 the burst of phosphate release represents 2.2 MgATP's hydrolyzed per Mo in dinitrogenase (there were 1.45 Mo's per molecule of dinitrogenase and 3.2/1.45 = 2.2). Thus, the results of this experiment suggest that there are two MgATP's hydrolyzed upon the initial one-electron transfer between the two proteins of nitrogenase from *A. vinelandii*. Such a value accounts for the four ATP's hydrolyzed per two electrons transferred, a figure deduced to be the minimum achievable in the steady state (Ljones & Burris, 1972; Watt et al., 1975).

MgATP Binding. Attempts to measure the binding of MgATP to dinitrogenase reductase from *A. vinelandii* by the method of Tso & Burris (1973) gave erratic results, so the reaction of dinitrogenase reductase with BPS in the presence of MgATP was used to quantitate the binding of MgATP to dinitrogenase reductase (Ljones & Burris, 1978b). The experiments of Ljones & Burris (1978b) yielded a K_d of 85 μM for MgATP from *C. pasteurianum* dinitrogenase reductase,

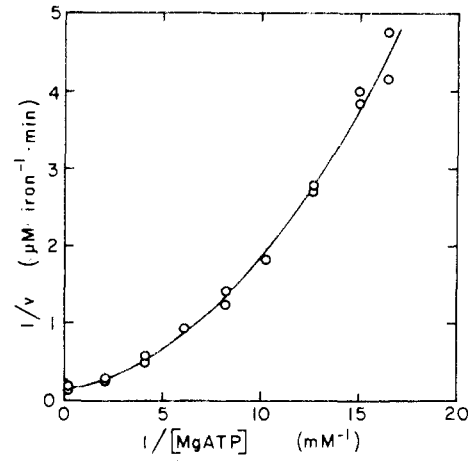


FIGURE 2: A double-reciprocal plot of the rate of the BPS reaction, showing its clearly parabolic dependence on the MgATP concentration. Reaction conditions were 50 mM buffer, 25 mM creatine phosphate, 3 mM magnesium acetate, 5 mM dithionite, 0.1 mg/mL creatine kinase, 0.15 mM BPS, and 9.45 μM dinitrogenase reductase. MgATP was added as indicated to initiate the reaction, and the initial rate of iron release was measured. The rate was measured in μM iron released per min.

and this is in fair agreement with the most recently published direct measurement of K_d for MgATP in the same organism of 50 μM (Emerich et al., 1978). The MgATP-dependent reaction of dinitrogenase reductase with BPS appears to give a reasonable estimate of the MgATP dissociation constant. The dependence of the rate of chelation of Fe from dinitrogenase reductase of *A. vinelandii* is shown in Figure 2. The data were fitted to the equation

$$v = \frac{V_{max}[\text{MgATP}]^2}{K_A K_B + K_B[\text{MgATP}] + [\text{MgATP}]^2} \quad (2)$$

to evaluate the two binding constants (K_A and K_B) for MgATP. This equation is a general equation for a reaction requiring the simultaneous presence of two MgATP molecules and allows the two binding constants for MgATP to vary independently and does not force a relationship between K_A and K_B as does the analysis performed by Ljones & Burris (1978b). The velocity of iron chelation by BPS is not important in this work but the binding constants for MgATP are. The fitted equation gave V_{max} = 6.78 μM min^{-1}, K_AK_B = 0.094 mM^2, K_A = 0.430 mM, and K_B = 0.220 mM with an overall standard error of the fit of 7%. The MgATP dissociation constants measured by this method are much larger in the *A. vinelandii* system than in the *C. pasteurianum* system, and this difference probably accounts for the failure of the Tso & Burris (1973) method to work well with dinitrogenase reductase from *A. vinelandii* (R. V. Hageman and R. H. Burris, unpublished data).

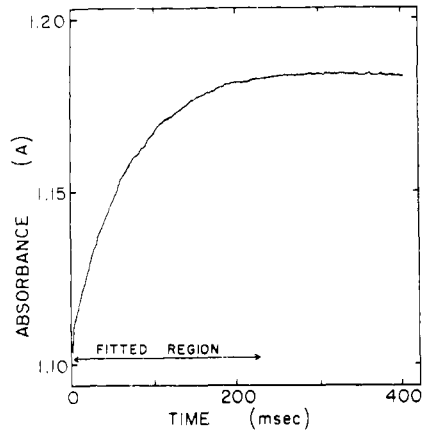


FIGURE 3: Time course of the MgATP-dependent oxidation of dinitrogenase reductase protein by dinitrogenase. The reaction was monitored at 430 nm in a 2.0-cm light path cuvette. Syringe A contained 50 mM buffer, 20 mM creatine phosphate, 2 mM magnesium acetate, 5 mM dithionite, 1.0 mg/mL creatine kinase, and 200 μM MgATP to give the desired final MgATP concentration. Syringe B contained 50 mM buffer, 5 mM dithionite, 19.2 μM dinitrogenase (27.8 μM molybdenum), and 47.4 μM dinitrogenase reductase (25.1 μM [4Fe–4S] clusters). The mixing ratio was 1:1. The initial portion of the reaction is obscured by the dead time of the instrument, and the final portion is complicated by a very slow decay of the absorbance.

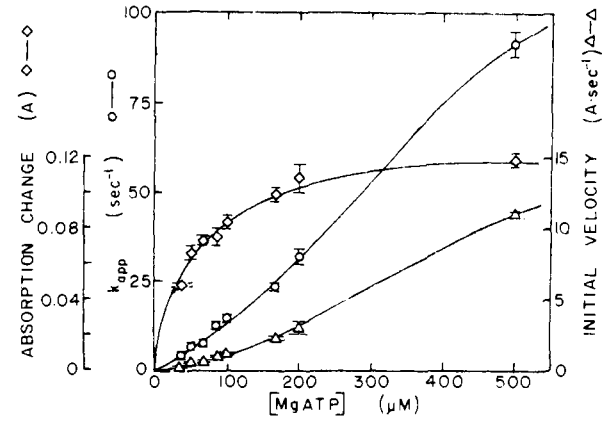


FIGURE 4: Variation in the rate and extent of the MgATP-induced oxidation of dinitrogenase reductase by dinitrogenase. Conditions are as described in Figure 2, with MgATP concentration varied to produce the desired final values. The data at 2500 μM MgATP are not shown but were used in drawing the saturation curves shown. (O) k_{app}, the apparent first-order rate constant for the oxidation; (◇) the extent of the oxidation of dinitrogenase reductase measured by the total absorbance change; (Δ) the initial velocity of the oxidation reaction.

MgATP Dependence of Electron Transfer. The dependence of the electron transfer rate on MgATP was studied at 30 °C with the nitrogenase proteins from *A. vinelandii* in a stopped-flow experiment similar to that described by Thorneley (1975). Our qualitative results were similar to those observed by Thorneley (1975), but there were important quantitative differences. The oxidation of dinitrogenase reductase can be monitored at 430 nm (Ljones, 1973), and a typical time course of the reaction is shown in Figure 3. Thorneley et al. (1976) compared the rate of oxidation measured in a stopped-flow experiment with the electron transfer observed with freeze–quench EPR. Figure 3 shows the rate and extent of oxidation of *A. vinelandii* dinitrogenase reductase by dinitrogenase at one level of MgATP. In other experiments, the MgATP concentration was varied to determine the dependence of the rate and extent of the oxidation on the MgATP concentration. Table I shows the results from the stopped-flow experiments. The apparent first-order rate constant, k_{app}, is obtained by fitting the observed progress curves to eq 1; the absorbance change expresses the extent of oxidation of dinitrogenase reductase as measured by the change in absorbance from reaction time zero to infinity (C in eq 1); the initial velocity is the initial rate of change of absorbance at reaction time zero, and k_1 and k_2 will be defined. The reaction zero time occurs shortly before the flow stops in the stopped-flow apparatus, and values recorded are extrapolations; extrapolation is important only at high MgATP concentrations (Figure 3, for example, requires very little extrapolation). Thorneley's (1975) data also show small changes (~20% of the total with a 10% error) dependent on the MgATP concentration. Table I clearly shows the dependency of the extent of oxidation (absorbance change) on the MgATP concentration.

Figure 4 shows the dependence of the absorbance change, k_{app}, and the initial velocity on the MgATP concentration. The k_{app} dependence is not obviously sigmoidal, and the linear appearance of a double-reciprocal plot indicates hyperbolic saturation behavior. However, the initial velocity dependence is definitely sigmoidal and yields a parabolic reciprocal plot. The initial velocity is directly proportional to the initial rate of electron transfer, whereas k_{app} has a more complicated dependence on the rate of electron transfer and cannot be used directly in evaluating the dependence of the electron transfer on MgATP concentration.

Our interpretation of the stopped-flow experiment in terms of the initial electron transfer differs from that of Thorneley (1975) and Thorneley & Cornish-Bowden (1977). They examined the dependence on MgATP concentration of the apparent rate constant, k_{app}, obtained in their stopped-flow experiment. The observed reaction is not a simple unidirectional electron transfer between the proteins as would be required for k_{app} to be a true first-order rate constant; rather, it represents an approach to the steady state arising from the reduction of the oxidized dinitrogenase reductase by dithionite. This is shown diagrammatically in the equation

$$\text{dinitrogenase reductase}^{red} + \text{dinitrogenase}^{ox} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{dinitrogenase reductase}^{ox} + \text{dinitrogenase}^{red} \quad (3)$$

In this scheme k_1 is the oxidation of dinitrogenase reductase by the dinitrogenase and is MgATP dependent, and k_2 expresses the overall reduction of the oxidized dinitrogenase reductase by dithionite. If both of these reactions are apparent first-order reactions, then

$$k_{app} = k_1 + k_2 \quad (4)$$

(Jencks, 1969), where k_{app} is the observed first-order rate constant, k_1 is the rate constant for the oxidation of dinitrogenase reductase, and k_2 is the rate constant for the reduction of oxidized dinitrogenase reductase. k_2 can have a number of contributory terms, but only the overall rate constant is important assuming k_2 is independent of the oxidation state of dinitrogenase. For evaluation of the MgATP dependence of the oxidation reaction properly, the value of k_1 at each MgATP concentration must be used and not the measured apparent rate constant.

At high MgATP concentrations, k_1 is known to be very large (Thorneley, 1975), and hence the turnover time of dinitrogenase reductase is governed by k_2; this allows an estimate of the magnitude of k_2. Under conditions identical with those of the stopped-flow experiment, a measurement of the rate of H_2 evolution gives a turnover time for dinitrogenase reductase of 3.8 s^{-1} extrapolated to infinite MgATP. As k_1 is ~300 s^{-1}, k_2 is then 3.8 s^{-1} at infinite MgATP (k_2 expressed on a one

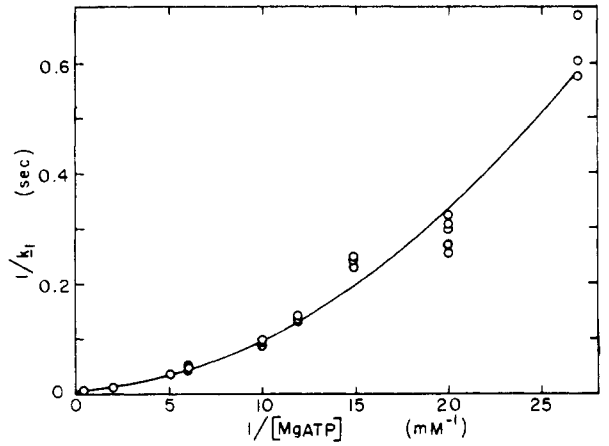


FIGURE 5: A double-reciprocal plot of the rate constant for oxidation of dinitrogenase reductase by dinitrogenase induced by MgATP (k_1). Details of the calculation of k_1 are described in the text. A double-reciprocal plot of the initial velocity of the oxidation rather than the rate constant is very similar.

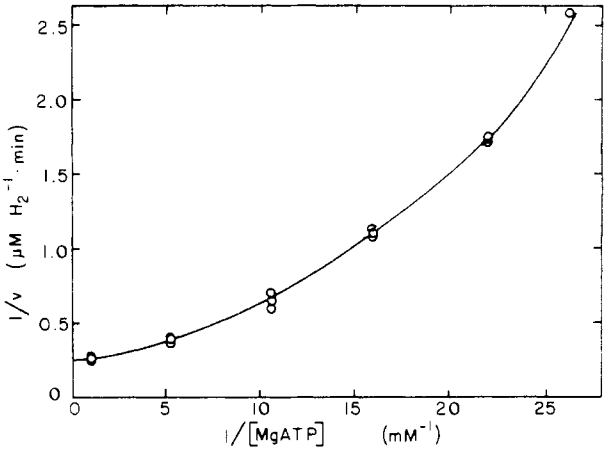


FIGURE 6: Dependence of H_2 evolution on the MgATP concentration. Reaction conditions were 50 mM buffer, 25 mM creatine phosphate, 0.15 mg/mL creatine kinase, 3 mM magnesium acetate, 20 mM dithionite, 8.61 μM dinitrogenase (12.5 μM molybdenum), and 2.36 μM dinitrogenase reductase (1.25 μM [4Fe–4S] clusters), with MgATP added as indicated to initiate the reaction. Velocities are expressed in μM H_2 evolved per min.

electron per [4Fe–4S] basis). An alternative estimation of k_2 can be made from the extent of oxidation of dinitrogenase reductase in the stopped-flow experiment. The steady-state oxidation level of dinitrogenase reductase is governed by the relationship

$$\frac{\text{dinitrogenase reductase}^{ox}}{\text{dinitrogenase reductase}^{red}} = \frac{k_1}{k_2} \qquad (5)$$

As $k_1 + k_2 = k_{app}$ and dinitrogenase reductase^{ox} + dinitrogenase reductase^{red} = dinitrogenase reductase^{tot}, the rate constants can be evaluated if dinitrogenase reductase^{ox} and dinitrogenase reductase^{tot} are known. Dinitrogenase reductase^{ox} is proportional to the observed absorbance change, and dinitrogenase reductase^{tot} will be proportional to the absorbance change at infinite MgATP, if k_1 is much greater than k_2 at high MgATP levels. This restriction holds for the experiment described, and the ratio of dinitrogenase reductase^{ox}/dinitrogenase reductase^{tot} is then equal to the ratio of the measured absorbance change to the extrapolated absorbance change at infinite MgATP concentration. By this method k_2 can be evaluated at all MgATP concentrations, but in our stopped-flow experiment the error in the determination of the absorbance change caused very large errors in the calculated value for k_2 above 100 μM MgATP. The calculation of k_2 from the absorbance change, therefore, was restricted to MgATP concentrations below 100 μM MgATP, and the turnover estimate of $k_2 = 3.8$ s^{-1} was used at MgATP concentrations of 100 μM and above. The correction for k_2 has a relatively small effect on k_1 above 100 μM MgATP, and in this range the method used to evaluate k_1 and k_2 from k_{app} does not have a significant effect on the kinetic constants for MgATP derived from the MgATP dependence of k_1.

The k_1 values calculated from k_{app} and k_2 then were used to evaluate the MgATP dependence of the electron transfer rate by fitting to the equation

$$k = \frac{k_1'[\text{MgATP}]^2}{K_A'K_B' + K_B'[\text{MgATP}] + [\text{MgATP}]^2} \qquad (6)$$

This yielded $k_1' = 320$ s^{-1}, $K_A'K_B' = 0.215$ mM^2, $K_A' = 0.22$ mM, and $K_B' = 0.97$ mM with an overall standard error of the fit of 11%. The dependence of k_1 on the MgATP concentration is shown in Figure 5.

H_2 Evolution. To compare the pre-steady-state kinetic constants for MgATP with the steady-state values, we evaluated the MgATP dependence of the H_2 evolution reaction (Figure 6). The reaction has a sigmoidal dependence on MgATP concentration and clearly yields a parabolic double-reciprocal plot. The data were fitted to eq 2 in which $V_{max} = 400$ μM min^{-1}, $K_A''K_B'' = 0.0117$ mM^2, $K_A'' = 0.39$ mM, and $K_B'' = 0.030$ mM with an overall error of the fit of 7%. For comparison, reaction conditions were designed to yield a maximum value for dinitrogenase reductase turnover, which is $\sim$10 s^{-1}; we assume one electron per [4Fe–4S] and a high concentration of MgATP. Conditions were established to ensure that the slow step preceding the reduction of dinitrogenase reductase (step k_2) would remain constant and not be subject to reversal by a buildup of bisulfite or excess dinitrogenase.

Discussion

Workers studying the nitrogenase enzyme system accept a role for MgATP in the electron transfer between the two component proteins of nitrogenase, primarily because of the absolute requirement for MgATP in the reaction (Orme-Johnson et al., 1972; Smith et al., 1973) and because of the modification of the physical characteristics of dinitrogenase reductase upon the binding of MgATP (Zumft et al., 1974; Walker & Mortenson, 1973; Thorneley & Eady, 1973). Thorneley (1975) had demonstrated the dependence of the rate of electron transfer on the concentration of MgATP and thus has provided support for a role of MgATP in the electron transfer. Eady et al. (1978) have demonstrated a role for MgATP in electron transfer by showing that ATP is hydrolyzed at the same rate as the rate of the initial electron transfer between the proteins. A second role for MgATP has been suggested based on the kinetics of substrate reduction (Thorneley & Eady, 1977; Smith et al., 1976; Rennie et al., 1978) wherein activation toward substrate reduction at the expense of H_2 evolution is observed at high concentrations of MgATP. However, a fuller understanding of the substrate reduction kinetics shows that this activation is not a specific property of the MgATP but rather is associated with the rate of electron transfer through dinitrogenase (Burris & Hageman, 1980). Therefore, we perceive no role for MgATP in substrate reduction beyond that of electron transfer.

What then of a second role for MgATP after the electron transfer between the nitrogenase proteins but before the evolution of H_2? Eady et al. (1978) demonstrated that some ATP is hydrolyzed at the same time as electrons are trans-

ferred between the proteins, but they concluded that their data actually support a second hydrolytic role for MgATP. They state that they observe the hydrolysis of two ATP's per molecule of dinitrogenase present in the reaction mixture and this corresponds to 0.4 ATP per molecule of dinitrogenase reductase present. The dinitrogenase that they used in this experiment had approximately half to two-thirds the specific activity (1300 nmol of C_2H_2 min^{-1} mg^{-1}) reported previously for dinitrogenase from *K. pneumoniae* [2100 nmol of C_2H_2 min^{-1} mg^{-1} (Thorneley et al., 1975)]. If the protein they used in the ATP hydrolysis experiment was only half to two-thirds active, then the observed hydrolysis would correspond to three to four ATP's per active dinitrogenase molecule. In short, for the observed stoichiometry of ATP hydrolysis to be meaningful, it must be related to the active-site concentration of dinitrogenase or dinitrogenase reductase and not to the bulk protein concentrations.

Eady et al. (1978) further argue that the hydrolysis of two ATP's is only half that required for H_2 evolution, with the implication that sufficient electrons have been transferred to dinitrogenase in the step accompanying hydrolysis of two ATP's for H_2 evolution to occur, but present no data on the stoichiometry of electron transfer, depriving this conclusion of plausibility. The lack of data on the stoichiometry of the electron transfer again points out the need to know the active-site purity of the enzyme.

Finally, they state that at 10 °C the hydrolysis of ATP is uncoupled from H_2 evolution in the steady state. This is a futile hydrolysis of ATP as discussed by Orme-Johnson & Davis (1977), but Eady et al. (1978) argue that this represents a second hydrolytic role for ATP. The hydrolysis of ATP upon electron transfer between the two proteins does not require that all the ATP hydrolyzed at that site support net electron transfer. Orme-Johnson & Davis (1977), as well as Hageman & Burris (1978b), have proposed that the futile hydrolysis of ATP occurs in the complex of oxidized dinitrogenase reductase with dinitrogenase. Under these circumstances ATP hydrolysis could occur at the site normally coupled to electron transfer without leading to net electron transfer. This would be futile hydrolysis but not a second role for MgATP.

Figure 1 shows a burst of ATP hydrolysis on a time scale comparable to the time scale of electron transfer between the component proteins. Even when compared on a total protein basis, there is more than one ATP hydrolyzed per dinitrogenase reductase molecule or more than three ATP per dinitrogenase molecule. To estimate the active-site concentration of dinitrogenase, we measured the Mo content of the protein, and it was assumed that there are two Mo's per dinitrogenase molecule and that there is one active site per Mo (Münck et al., 1975). As there were 1.45 Mo's per dinitrogenase molecule in the protein used in Figure 1, the observed ATP hydrolysis corresponds to 2.2 ± 0.5 ATP's hydrolyzed per Mo. Münck et al. (1975) have shown that the paramagnetic center corresponding to the FeMo cofactor (Rawlings et al., 1978) is reduced by a single electron in the fast (Smith et al., 1973) step of the electron transfer reaction. If, as we believe, the Mo content measures the active-site concentration of dinitrogenase, there are two ATP's hydrolyzed per electron transferred. The active-site purity of dinitrogenase reductase was measured by the BPS reaction (Ljones & Burris, 1978a,b), which measures its [4Fe–4S] cluster content. As there was 0.53 [4Fe–4S] cluster per dinitrogenase reductase molecule, the observed ATP hydrolysis corresponds to 2.5 ± 0.4 ATP's hydrolyzed per [4Fe–4S] cluster. Ljones & Burris (1978a) have shown that dinitrogenase reductase can donate one electron per [4Fe–4S] cluster, so calculating from the active-site concentration of dinitrogenase reductase indicates that there are 2.5 ATP's hydrolyzed per electron transferred. The active-site concentration of each protein thus indicates that within the precision of the estimations there are two ATP's hydrolyzed per electron transferred. This ATP hydrolysis is coupled to electron transfer from dinitrogenase reductase to dinitrogenase and not at some subsequent step in the reaction, for the following reason: the minimum observed hydrolysis in the steady state is two ATP's per electron (Ljones & Burris, 1972; Watt et al., 1975), and thus ATP hydrolysis coupled to the electron transfer between the component proteins of nitrogenase accounts for all of the observed steady-state hydrolysis of ATP. On the basis of present data, there is thus no reason to postulate a second hydrolytic role for ATP in the nitrogenase reaction. Futile hydrolysis of ATP has been observed under some conditions, and we (Hageman & Burris, 1978b; Orme-Johnson & Davis, 1977) and Ljones (1979) have discussed mechanisms that could account for this excess ATP hydrolysis.

Thorneley & Cornish-Bowden (1977) also argued that their data provide support for a second role for MgATP in the H_2 evolution reaction. Our interpretation of the stopped-flow experiment presented here differs fundamentally from that of Thorneley (1975) and Thorneley & Cornish-Bowden (1977). We believe that their data do not support the existence of a second role for MgATP, if one takes account of the back-reaction rate constant k_2, as well as the apparent first-order rate constant used by the above authors in their analysis. For the pseudoequilibrium described by eq 3, the apparent first-order rate constant that is observed is given by eq 4. It is important to note that k_1 and k_2 do not necessarily describe single chemical steps but only reactions that are apparently first-order overall. For proper evaluation of the MgATP dependence of the oxidation reaction, k_1 must be evaluated from k_{app} and k_2 as described under Results.

The occurrence of a back-reaction suggests that the extent of the oxidation of dinitrogenase reductase will be dependent on the concentration of MgATP. Indeed, this is observed in our experiment (Table I and Figure 4). Thorneley (1975), on the other hand, did not observe such an effect. However, the lowest concentration of MgATP that he used (63 μM) gives a rate constant for the oxidation that is comparable to our MgATP concentration of 167 μM. Most of the change in the extent of oxidation we observe occurs at MgATP concentrations of 100 μM and less. It may be that the studies of Thorneley (1975) were carried out at MgATP concentrations too high for the evaluation of k_2. The difference in MgATP concentrations required to give comparable k_{app} values probably represents species differences, as we utilized nitrogenase from *A. vinelandii* and Thorneley (1975) utilized nitrogenase from *K. pneumoniae*. The higher apparent K_m for MgATP in the *A. vinelandii* system makes it especially suitable for studies of MgATP dependence, as the sigmoidal effects are observable at higher MgATP concentrations so that more useful concentrations of enzyme can be employed.

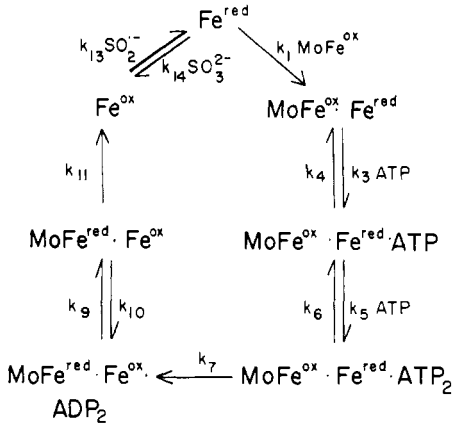
When the contribution of the back-reaction (k_2) is incorporated into the apparent first-order rate constant, a clearly sigmoidal dependence of the oxidation rate constant on the MgATP concentration is evident. The kinetic constants for MgATP were obtained by fitting the data to eq 6. It has also been suggested elsewhere (Watt & Burns, 1977) that Thorneley (1975) did not utilize sufficiently low concentrations of MgATP to observe this sigmoidal dependence. As discussed, we believe this argument has merit, but a factor

Table II: Summary of MgATP Kinetic Constants

reaction[a]	V_{max}	K_AK_B (mM²)	K_A (mM)	K_B (mM)
BPS reaction	6.8 ± 0.3	0.094 ± 0.005	0.43 ± 0.06	0.220 ± 0.006
electron transfer	320.0 ± 30.0	0.215 ± 0.020	0.22 ± 0.02	0.970 ± 0.100
H_2 evolution	395.0 ± 14.0	0.0117 ± 0.0006	0.39 ± 0.09	0.030 ± 0.006

[a] BPS reaction is the MgATP-dependent release of iron from dinitrogenase reductase to the chelator bathophenanthrolinedisulfonate; V_{max} is in nmol of Fe min^{-1}. Electron transfer is the MgATP-induced electron transfer between dinitrogenase reductase and dinitrogenase; V_{max} is the limiting first-order rate constant for this reaction in s^{-1}. H_2 evolution from Figure 6; V_{max} is in μM H_2 min^{-1}. The equation fitted to was $v = V_{max}[MgATP]^2/(K_AK_B + K_B[MgATP] + [MgATP]^2)$.

Scheme I



perhaps of greater importance is that Thorneley (1975) and Thorneley & Cornish-Bowden (1977) did not correct their observed rate constants for the rate constant of the back-reaction. The correct MgATP dependence also would have been observed if the initial velocities of the oxidation reactions had been measured rather than the first-order rate constants.

The agreement between the values for k_2 calculated from the extent of oxidation at low MgATP levels with the value of k_2 estimated from the turnover time of the protein at high MgATP levels shows that MgATP does not significantly affect the reduction of the complex between oxidized dinitrogenase reductase and dinitrogenase. Thus, a second role for MgATP seems plausible neither for the dissociation of the proteins that precedes reduction nor in the actual reduction process.

The BPS reaction developed by Ljones & Burris (1978b) was utilized to estimate the binding of MgATP to dinitrogenase reductase. A point of interest is that there appear to be differences among species, as Ljones & Burris (1978b) obtained a K_d of 85 μM for MgATP in *C. pasteurianum*, whereas this work reports ~300 μM in *A. vinelandii*. The binding constants were evaluated by fitting to eq 2 which gives a sigmoidal MgATP dependence, without forcing a relationship between the two binding constants as does the analysis of Ljones & Burris (1978b). The MgATP dependence of the H_2 evolution reaction also was measured. Conditions were chosen such that the turnover time of dinitrogenase reductase was 0.1 s, at high MgATP concentrations. These conditions ensured that the unimolecular slow step preceding the reduction always would be limiting in the reduction process, whereas the actual reduction of dinitrogenase reductase would not become limiting. This gives a value for k_2, as discussed in the evaluation of the stopped-flow experiment, independent of the MgATP concentration and having a well-defined value. The MgATP dependence of the reaction was evaluated by fitting to eq 2, and the various kinetic constants are summarized in Table II.

Table III: Comparison of Kinetic Constants

	K_A	K_B
ATP binding	k_4/k_3	k_6/k_5
electron transfer	k_4/k_3	$(k_6 + k_7)/k_5$
H_2 evolution	k_4/k_3	$[(k_6 + k_7)/k_5]k_{11}/k_7$

A possible model for H_2 evolution by nitrogenase is shown in Scheme I. In this scheme, k_1 (k_1 as used here should not be confused with k_1 used in the previous discussion) represents the binding between the two proteins of nitrogenase, a reaction that occurs more rapidly than electron transfer between the proteins (Thorneley, 1975). k_3 and k_4 describe the binding of the first MgATP to dinitrogenase reductase, k_5 and k_6 describe the binding of the second MgATP to dinitrogenase reductase, k_7 is the first-order rate constant for electron transfer between the MgATP complex of dinitrogenase reductase to dinitrogenase, k_9 and k_{10} express the rapid dissociation of MgADP from the complex, k_{11} represents the slow step that yields a species of oxidized dinitrogenase reductase that can be reduced by dithionite, and k_{13} is the constant for the known fast reaction of dithionite with oxidized dinitrogenase reductase. k_{11} may represent the dissociation of the two nitrogenase proteins that occurs after every electron transfer between them (Hageman & Burris, 1978a), as the reduction of free dinitrogenase reductase occurs very rapidly (Thorneley et al., 1976). The system must cycle twice to supply the requisite two electrons for production of H_2. The binding between the two nitrogenase proteins and the binding of MgATP to the complex is depicted in Scheme I as an ordered process. In fact, MgATP is known to bind to dinitrogenase reductase in the absence of dinitrogenase (Ljones & Burris, 1978b; Emerich et al., 1978), and the proteins can interact both in the presence (Thorneley, 1975) and in the absence (Emerich et al., 1978) of MgATP. This supports a random mechanism for the first three steps. The rate of the initial electron transfer is independent of the order of mixing of dinitrogenase reductase, dinitrogenase, and MgATP (Thorneley, 1975), and this further supports a random mechanism. The scheme is shown ordered for convenience only, and a random mechanism does not change the derived kinetic equations if the association and dissociation reactions in the first three steps are fast relative to the electron transfer reaction (k_7).

The binding of MgATP is expressed by the second and third steps of the model, and, if dinitrogenase does not perturb this binding, then the dissociation constants are given by

$$K_A = k_4/k_3 \quad (7)$$

$$K_B = k_6/k_5 \quad (8)$$

where K_A describes the binding of the first MgATP and K_B describes the binding of the second MgATP. If the first three steps are fast, the Michaelis constants for MgATP in the electron transfer reaction are given by

$$K_A' = k_4/k_3 \quad (9)$$

$$K_B' = (k_6 + k_7)/k_5 \quad (10)$$

If the reaction described by k_{11} is irreversible, the kinetic constants for the dependence of H_2 evolution on MgATP also can be evaluated. This condition was achieved by having

present an excess of dinitrogenase and a high level of reductant so that the released, oxidized dinitrogenase reductase did not have an opportunity to rebind to dinitrogenase. Under these conditions, the MgATP Michaelis constants for the H_2 evolution reaction are given by

$$K_A'' = k_4/k_3 \tag{11}$$

$$K_B'' = \frac{k_6 + k_7}{k_5}\frac{k_{11}}{k_7} \tag{12}$$

The kinetic constants are summarized in Table III.

Scheme I predicts that, if k_6 is much greater than k_7, the MgATP kinetic constants evaluated from the stopped-flow experiment should be equivalent to the constants for binding of MgATP to dinitrogenase reductase. The observed differences are small and likely result from the change in the binding constant between MgATP and dinitrogenase reductase when dinitrogenase reductase is complexed to dinitrogenase and to experimental error and to the fact that k_6 is comparable in magnitude to k_7. Thorneley & Cornish-Bowden (1977) have observed that the dissociation of MgADP from dinitrogenase reductase occurs at a rate comparable to electron transfer between the proteins.

Scheme I also predicts the kinetic constants for MgATP in the H_2 evolution reaction. A numerical estimate can be made for conditions such that the slow step preceding reduction of dinitrogenase reductase is rate limiting in the reduction. The maximum turnover number observed for dinitrogenase reductase is 10 s^{-1} (for one electron per [4Fe–4S] cluster), k_7 has a value of 320 s^{-1}, and the ratios of the other rate constants are defined by eq 9 and 10 describing the stopped-flow experiment. With eq 11 and 12 and the kinetic constants from the rapid reaction experiment to predict the numerical values, the kinetic constants for the H_2 evolution reaction become K_AK_B = 0.007 mM^2, K_A = 0.22 mM, and K_B = 0.030 mM. These values are remarkably close to the observed values for the H_2 evolution reaction. It is important to recognize that these predictions are only valid under the conditions described above. The ability to predict accurately the kinetic constants for the H_2 evolution reaction from the pre-steady-state values with the model described argues strongly against any second role for MgATP.

The model presented in Scheme I predicts ping-pong kinetics between MgATP and dithionite. In fact, the data of Watt & Burns (1977) indicate sequential patterns. The prediction of ping-pong kinetics requires that the reduction of dinitrogenase reductase by dithionite be irreversible. However, dithionite is known to react reversibly with electron transfer proteins (Mayhew, 1978), and the reaction with dinitrogenase reductase probably is reversible. The measurement of the MgATP and dithionite kinetic patterns was made by analyzing progress curves of dithionite oxidation (Watt & Burns, 1977). This procedure requires the presence of large amounts of bisulfite at the lower concentrations of dithionite. It is likely that the kinetic patterns between MgATP and dithionite observed (Watt & Burns, 1977) were distorted by the reversible nature of the dithionite reaction and the high concentrations of the oxidant bisulfite at the end of the progress curves. The reversible connection present at the end of the progress curve would produce a sequential pattern rather than the predicted ping-pong pattern. An inhibition by bisulfite observed at low MgATP concentrations but not at high MgATP concentrations (R. V. Hageman and R. H. Burris, unpublished experiments) supports the reversible nature of the dithionite reaction and Scheme I. This model predicts the known patterns of MgATP and dithionite kinetics for H_2 evolution, but a more rigorous quantitative understanding would require establishment of more of the rate constants in the scheme.

The data as presented here strongly favor the function of MgATP in the electron transfer between the two proteins of nitrogenase from *A. vinelandii* as the sole role of MgATP in the nitrogenase reaction. The hydrolysis of ATP coupled to electron transfer can account for all of the observed ATP hydrolysis in the steady-state reaction. It also is possible to predict the steady-state MgATP kinetic constants in the H_2 evolution reaction from pre-steady-state measurements during electron transfer. The data also allow one to eliminate a role for MgATP in events after electron transfer but before reduction of dinitrogenase reductase. Therefore, there seems to be no logical reason to postulate a second role for MgATP in the functioning of nitrogenase.

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