Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Roeder, R. G. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 285-329, Cold Spring Harbor, New York, N.Y.
Schaller, H., Nüsslein, C., Benhoeffer, F. J., Rusz, C., & Nuitzschmann, J. (1972) *Eur. J. Biochem. 26*, 474-481.
Spiegel, S., Obendorf, F. L., & Marcus, A. (1975) *Plant Physiol. 56*, 502-507.

Nitrogenase: The Reaction between the Fe Protein and Bathophenanthrolinedisulfonate as a Probe for Interactions with MgATP†

Torbjørn Ljones‡ and R. H. Burris*

ABSTRACT: The reaction between the Fe(II) chelating agent, bathophenanthrolinedisulfonate, and the iron-sulfur cluster in the Fe protein of nitrogenase from *Clostridium pasteurianum* has been studied. This reaction is greatly accelerated by the presence of MgATP. Analysis of the relationship between reaction rate and concentration of MgATP supports a model in which both of two binding sites for MgATP on the Fe protein must be occupied before the protein undergoes a conformational change, allowing the iron-sulfur site to react rapidly with chelator. This model is also consistent with presently available data on equilibrium binding of MgATP to the Fe protein. MgADP inhibits the effect of MgATP on the chelator reaction in a manner which suggests that MgADP binds strongly to one of the MgATP sites and more weakly to the other. Loss of enzymic activity due to exposure to O_2 or 0 °C is accompanied by a decrease in the ATP-specific chelator reaction. Hence, this reaction was used to estimate the concentration of active iron-sulfur centers for the purpose of computing the extinction coefficient of the Fe protein, giving the value $\Delta\epsilon_{430nm}$(ox-red) = 6600 M^{-1} cm^{-1}.

Nitrogenase, the enzyme system that is responsible for biological nitrogen fixation, requires ATP for its catalysis and hydrolyzes the ATP to ADP and orthophosphate (for reviews, see Zumft & Mortenson, 1975; Winter & Burris, 1976; Orme-Johnson & Davis, 1977). There is experimental evidence that the Fe protein of nitrogenase interacts with MgATP, followed by detectable changes in physical and chemical properties of the protein. No observations supporting direct interaction between MgATP and the MoFe protein have so far been reported.

Tso & Burris (1973) used a gel equilibration method to study the binding of MgATP and MgADP to purified Fe protein and found that the protein possesses two binding sites for MgATP. MgADP binds to one of these sites. The EPR[1] spectrum of the Fe protein undergoes a change when MgATP binds (Orme-Johnson et al., 1972; Smith et al., 1973; Zumft et al., 1973), suggesting that MgATP binding alters the conformation of the Fe protein. Zumft et al. (1973) concluded, on the basis of EPR spectra of the Fe protein with different levels of MgATP, that the Fe protein binds 2 molecules of MgATP per protein molecule. The binding of MgATP lowers the redox potential of the Fe protein (Zumft et al., 1974; Ljones, Tso, Orme-Johnson, & Burris, cited in Burris & Orme-Johnson (1974) and in Orme-Johnson & Davis (1977)).

G. A. Walker & L. E. Mortenson (1973, 1974) have presented a simple and elegant approach to studies of the interaction between the Fe protein and MgATP. Earlier work had shown that iron-sulfur clusters in ferredoxins react with chelating agents for iron, thus disrupting the clusters (Malkin & Rabinowitz, 1967). This reaction is rather slow unless the ferredoxin is denatured, and both of the nitrogenase proteins display similar behavior. Walker & Mortenson discovered that addition of MgATP would dramatically increase the rate of reaction between the chelating agent 2,2′-bipyridyl and the Fe protein. The MoFe protein showed no such response to MgATP. All the requirements and characteristics of the interaction between the Fe protein and MgATP that have been inferred from other types of experiments were observed: requirement for Mg^{2+} in addition to ATP, inhibition by ADP, little or no response to other nucleoside triphosphates. These observations were interpreted by Walker & Mortenson as evidence that the binding of MgATP changes the conformation of the Fe protein such that the iron sulfur site becomes more accessible to reaction with chelators.

Walker & Mortenson (1974) did not measure initial velocities of the reaction with 2,2′-bipyridyl, thus precluding detailed kinetic analysis. Mortenson et al. (1975) later determined initial velocities in similar experiments and proposed a model for binding of MgATP to the Fe protein, but their model is not consistent with the data and model of Tso & Burris (1973). We have, therefore, further pursued the approach of Walker & Mortenson. On the basis of our chelator experiments we propose a model for interaction between the binding of MgATP and reactions at the iron-sulfur site of the Fe protein; this model is consistent both with the model of Tso

† From the Department of Biochemistry and Center for Studies of N_2 Fixation, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received October 31, 1977.* This investigation was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, by Public Health Service Grant AI-00848 from the National Institute of Allergy and Infectious Diseases, by National Science Foundation Grant PCM-74-17604, and by a travel grant to T.L. from the Norwegian Research Council for Science and the Humanities.

‡ Permanent address: Department of Biochemistry, University of Bergen, N-5000 Bergen, Norway.

[1] Abbreviations used: Bes, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; EPR, electron paramagnetic resonance.

0006-2960/78/0417-1866$01.00/0

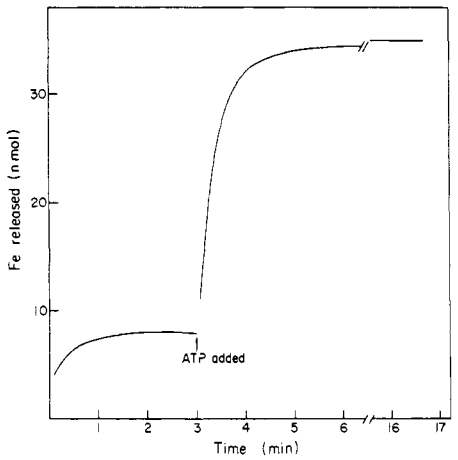


FIGURE 1: Reaction between the Fe protein and bathophenanthrolinedisulfonate. Reaction mixtures contained 50 mM Bes^+, pH 7.0, 2 mM $Na_2S_2O_4$, 1 mM bathophenanthrolinedisulfonate, and 2 mM $MgCl_2$. To this was added 0.6 mg of Fe protein, and, after 3 min, 1 μmol of ATP (in 10 μL, final concentration 1 mM). Temperature was 25 °C and final volume was 1.00 mL.

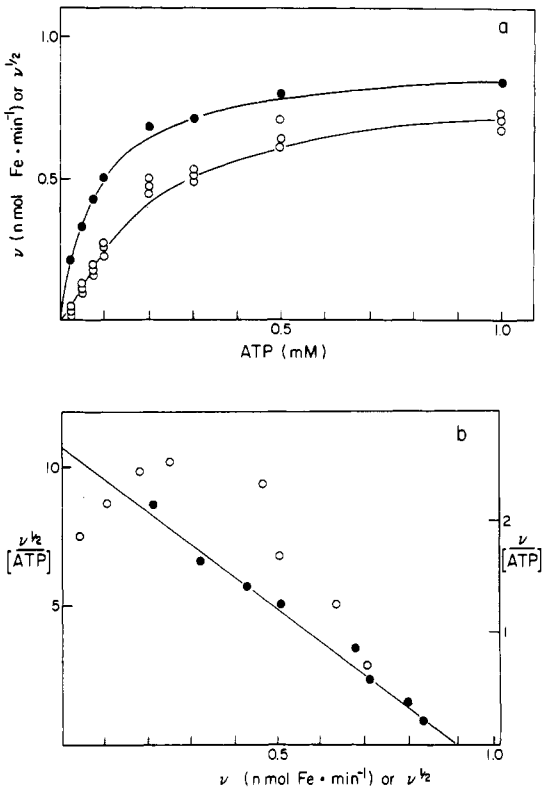


FIGURE 2: Rate of reaction between the Fe protein and bathophenanthrolinedisulfonate as a function of ATP concentration. Reaction mixtures contained 50 mM Bes at pH 7.0, 5 mM $Na_2S_2O_4$, 0.1 mM bathophenanthrolinedisulfonate, 2 mM $MgCl_2$, and 120 μg of Fe protein. These mixtures were incubated for 3 min and different volumes of ATP solutions containing equimolar amounts of $MgCl_2$ were added to start the reaction. Final volumes were 1.00 mL. Temperature was 25 °C. (a) Plots of v vs. [ATP] (○) and $v^{1/2}$ vs. [ATP] (●); (b) plots of v/[ATP] vs. v (○) and $v^{1/2}$/[ATP] vs. $v^{1/2}$ (●) from the same experiment. The replicates are indicated in the plot of v vs. [ATP]; in the other plots, only the median value at each ATP level is shown.

& Burris for equilibrium binding and the model of Watt & Burns (1977) for steady-state kinetics. For these experiments we have used the water soluble Fe(II) chelating agent bathophenanthrolinedisulfonate (4,7-diphenyl-1,10-phenanthrolinedisulfonate; Blair & Diehl, 1961). This reaction can be used to measure the concentration of active, as opposed to inactivated iron-sulfur centers in the Fe protein, and we also report results of applying the reaction with chelator to measurements of the extinction coefficient and of cold inactivation of the Fe protein from *Clostridium pasteurianum*.

Experimental Procedure

The nitrogenase proteins were purified from cells of *C. pasteurianum* W5 as previously described (Tso et al., 1972) but with two modifications: The cell paste was not dried, but was frozen and stored in liquid nitrogen, and the cell-free extracts were prepared by lysis with lysozyme (Witz et al., 1967; M. Henzl, personal communication). Frozen cell paste (500 g) was mixed anaerobically with 1000 mL of 40 mM Tris buffer, pH 8.5, containing 500 mg of crystalline egg white lysozyme and 20 mg of deoxyribonuclease (Sigma, type I), thawed rapidly, and incubated under H_2 with stirring at 30 °C for 30 min.

Rates of formation of the Fe(II)-bathophenanthrolinedisulfonate complex during the reaction between the chelator and the Fe protein were continuously recorded with a Beckman DU-Gilford 222 spectrophotometer and Honeywell Electronic 194 recorder. The absorbance increase was monitored at 535 nm, and results were calculated using a value of 22 140 M^{-1} cm^{-1} for the extinction coefficient of the complex (Blair & Diehl, 1961). Cuvettes with a 10-mm light path were sealed with rubber serum stoppers and evacuated and flushed with N_2 through hypodermic needles. Unless indicated otherwise, ATP was the last component to be added.

Techniques for anaerobic handling of extracts and analytical procedures were as previously described (Ljones, 1973, and references therein).

Results

Reaction between the Fe Protein and Bathophenanthrolinedisulfonate. Figure 1 shows that the Fe protein reacts with bathophenanthrolinedisulfonate in much the same way as with 2,2′-bipyridyl (Walker & Mortenson, 1974). An initial rapid phase in the absence of ATP is probably due to reaction with iron in inactive Fe protein, followed by a slow phase which is probably due to the slow reaction between the chelator and active Fe protein in the absence of ATP. Addition of ATP then leads to a large increase in reaction rate. The main difference in the behavior of the two chelators is that the Fe protein + ATP reacts much more rapidly with bathophenanthrolinedisulfonate than with 2,2′-bipyridyl (50% completion in less than 30 s with 1 mM bathophenanthrolinedisulfonate and 1 mM ATP (Figure 1) vs. ca. 15 min with 5 mM 2,2′-bipyridyl and 0.17 mM ATP (Walker & Mortenson, 1974)). In agreement with Walker & Mortenson, we found that the reaction with chelator requires the presence of both Mg^{2+} and ATP. MoFe protein and bathophenanthrolinedisulfonate did not show any response to MgATP.

Variation of MgATP Concentration. The rate of reaction between the Fe protein and bathophenanthrolinedisulfonate was measured at different levels of MgATP. Cuvettes containing all components except ATP were preincubated for 3 min before the addition of ATP. Initial rates were measured as the tangents to the initial parts of the curves for absorbance changes. By using 0.1 mM chelator, compared with 1 mM in Figure 1, we could measure the initial rates with reasonable precision even at the higher levels of ATP.

Plots of rates (v) vs. ATP concentration were clearly sigmoidal, whereas plots of the square roots of rates vs. ATP concentration appeared to be hyperbolic (Figure 2a). We have

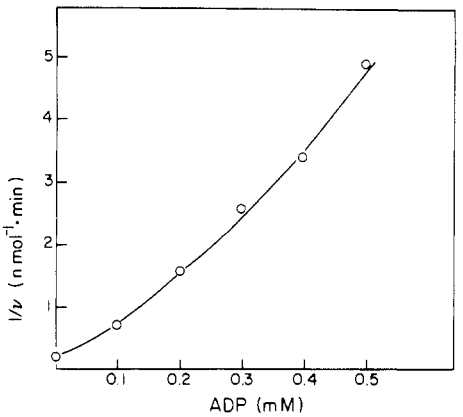


FIGURE 3: ADP inhibition of the reaction between the Fe protein and bathophenanthrolinedisulfonate. Experimental conditions were as described in the legend for Figure 2, except that the bathophenanthrolinedisulfonate concentration was 1.0 mM and the ATP concentration was 0.5 mM. The stock solutions of ADP and ATP contained equimolar amounts of $MgCl_2$. The reaction was started by addition of ATP 3 min after addition of the other components.

also presented the data from such an experiment in Figure 2b as v vs. $v/[MgATP]$ and $v^{1/2}$ vs. $v^{1/2}/[MgATP]$. According to Walter (1974), this type of linearized plot most clearly detects deviations from hyperbolic equations of the type $r = v/V_{max} = S/(K + S)$. Figure 2b shows that the data points when plotted as v vs. $v/[MgATP]$ definitely do not fall on a straight line; thus the chelation reaction does not follow the rate law $v/V_{max} = [MgATP]/(K_s + [MgATP])$. A clear trend, which is most notable at low ATP levels, can be seen. When plotted as $v^{1/2}$ vs. $v^{1/2}/[MgATP]$, however, the data points give a reasonably good fit to a straight line. K_s and V_{max} were estimated to be 85 μM and 0.84 nmol min^{-1} by a direct linear plot (Eisenthal & Cornish-Bowden, 1974) to the equation $v^{1/2} = v_{max}{}^{1/2}[MgATP]/(K_s + [MgATP])$, and the line in Figure 2b was drawn according to these values.

We have carried out a number of similar experiments and have in each case found that plots of v vs. $v/[MgATP]$ show a similar trend in their deviation from a straight line, whereas plots of $v^{1/2}$ vs. $v^{1/2}/[MgATP]$ only show the deviation expected from experimental error without any apparent trend in the deviations.

Effects of ADP. In agreement with Walker & Mortenson (1974), we have found that ADP inhibits the effect of ATP on the accessibility of the iron–sulfur site in the Fe protein. Walker & Mortenson reported a small increase in the rate of chelation with MgADP but with no MgATP present, but we have not been able to detect any such increase in rate due to MgADP with bathophenanthrolinedisulfonate as the chelator.

The results in Figure 3 reveal that ADP is a rather strong inhibitor of the ATP-induced chelation reaction. The curvature in the plot of $1/v$ vs. [ADP] was observed in each of several other experiments of this type. ADP appears to bind more tightly than ATP, in agreement with the equilibrium binding experiments of Tso & Burris (1973) and the kinetic experiments of Ljones (1973) and Thorneley & Cornish-Bowden (1977).

Effect of Free ATP. Thorneley (1974) reported that free ATP (not complexed to Mg^{2+}) is a competitive inhibitor of MgATP in catalysis with *Klebsiella pneumoniae* nitrogenase, whereas Davis & Orme-Johnson (1976) found no such inhibition with *C. pasteurianum* nitrogenase. We have examined the effect of free ATP on the chelation reaction with *C. pasteurianum* Fe protein, using constant levels of Mg^{2+} and a variable excess of ATP and have found no evidence for inhibition by free ATP (data not shown).

Reaction of Enzymically Oxidized Fe Protein with Bathophenanthrolinedisulfonate. Walker & Mortenson (1974) reported results of chelation studies with Fe protein that had been oxidized partially or fully by low levels of O_2, such that part of the enzymic activity was retained. These experiments revealed that oxygen-inactivated protein reacted very rapidly with 2,2′-bipyridyl, both before and after addition of dithionite, whereas oxidized, but not inactivated, protein reacted at an intermediate rate without dithionite and very slowly after reduction with dithionite. We have used bathophenanthrolinedisulfonate for similar experiments, but we preferred to use enzymic oxidation of the Fe protein (Ljones, 1973; M. Walker & Mortenson, 1973) with low levels of MoFe protein in the presence of MgATP, as this method does not lead to inactivation. In agreement with Walker & Mortenson (1974), we found a rather high rate of reaction with oxidized Fe protein, i.e., about five times higher than the rate with reduced Fe protein in the presence of 1 mM MgATP.

It is not known whether ATP interacts with the oxidized Fe protein, and information as to whether ATP influences the reaction between oxidized Fe protein and the chelator seemed pertinent. Control experiments without ATP were impossible when enzymic oxidation was used, but there was no evidence for any increase in the rate of chelation when ATP was varied from 25 μM to 1 mM. The ATP level in each experiment was kept constant by a creatine phosphate–creatine kinase system. Two alternative interpretations of these observations are: (1) ATP has no effect on the accessibility of the iron–sulfur site of oxidized Fe protein or (2) ATP binds much more tightly to oxidized than to reduced Fe protein. Alternative (2) seems unlikely, because there was no effect of decreasing the ATP level from 1 mM to 25 μM (compare with Figure 2 for behavior of reduced Fe protein), and we therefore favor alternative 1.

Extinction Coefficient of the Fe Protein. Because of its extreme O_2 sensitivity, most preparations of the Fe protein contain substantial proportions of O_2-inactivated protein. Thus, estimates of extinction coefficients are likely to be in error (Ljones, 1973; M. Walker & Mortenson, 1973; Thorneley, 1975). A comparison of rates in the steady state and during oxidation of excess Fe protein (Ljones, 1973) has, indeed, suggested that the extinction coefficient at 430 nm for the difference spectrum between oxidized and reduced Fe protein must be closer to the corresponding value for 4Fe-4S clusters in ferredoxins (ca. 7000 M^{-1} cm^{-1}: Hong & Rabinowitz, 1970; Stombaugh et al., 1976) than the value of 4500 M^{-1} cm^{-1} that was computed on the basis of protein content and molecular weight.

Walker & Mortenson (1974) and Walker (1974) reported that the decrease in the enzymic activity of the Fe protein after partial O_2 inactivation was proportional to the increase in iron that reacted with 2,2′-bipyridyl in the absence of ATP and proportional to the decrease in the ATP-dependent chelation reaction. We have observed similar behavior using bathophenanthrolinedisulfonate; thus the ATP-dependent reaction with chelator measures the concentration of iron in active, as opposed to O_2-inactivated (or cold-inactivated, see below) Fe protein. Comparison of absorbance changes during enzymic oxidation of the Fe protein (Ljones, 1973) and the ATP-specific reaction with bathophenanthrolinedisulfonate should therefore provide better estimates of the extinction coefficient than calculations based on total protein or total iron. In one such experiment, 3.5 mg of Fe protein in 1.00 mL of 50 mM Bes, pH 7.0, with 0.2 mg of MoFe protein, 5 μmol of ATP, 10 μmol of $MgCl_2$, 20 μmol of creatine phosphate, and 0.2 mg of

TABLE I: Cold Inactivation of the Fe Protein from *C. pasteurianum*.[a]

Time of exposure to 0 °C (min)	Fe released upon addition of ATP (nmol)	
	With glycerol	Without glycerol
0	3.7	3.6
195	4.0	3.6
270	3.9	2.3
340	3.7	2.2
610	3.8	1.6
685	3.6	1.4
1470	3.6	0.1

[a] Solutions of *C. pasteurianum* Fe protein, 1.24 mg/mL in 50 mM Tris, pH 8.0, with 1 mM $Na_2S_2O_4$ (and 25% glycerol, when present), were kept on ice in serum bottles submerged in a solution of dithionite and methylviologen. For assays, 100 μL of protein solution was added to cuvettes with 890 μL of 50 mM Bes, pH 7.0, containing 2 μmol of $Na_2S_2O_4$, 2 μmol of $MgSO_4$, and 1 mM bathophenanthrolinedisulfonate at 25 °C. Absorbance was recorded for 5 min, 1 μmol of ATP in 10 μL was added, and the absorbance was recorded for another 10 min. The Fe protein had a specific activity of 1.7 μmol of C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$.

creatine kinase gave $A_{430nm} = 0.22$ upon oxidation, whereas the ATP-specific bathophenanthrolinedisulfonate reaction gave $A_{535nm} = 0.59$ with 0.7 mg of Fe protein (see Figure 1). Assuming 4Fe-4S clusters, these data indicate an extinction coefficient, $\Delta\epsilon_{430nm}$(ox-red) = 6600 M^{-1} cm^{-1}. A calculation on the basis of a protein determination indicates a value of 3770 M^{-1} cm^{-1}. These observations suggest that this particular preparation contains 57% active Fe protein. The specific activity was 1.7 μmol of $S_2O_4^{2-}$ oxidized min^{-1} (mg of protein)$^{-1}$ at pH 6.6 and 30 °C; thus there is reasonable correspondence between the estimated percentage of active protein, and the ratio of the specific activity of this preparation to the highest specific activity reported for *C. pasteurianum* Fe protein (3.1 μmol min^{-1} mg^{-1}; Tso et al., 1972).

Four other similar experiments with enzymic oxidation and the ATP-specific chelation reaction gave these values of $\Delta\epsilon_{430nm}$ (ox-red) (in M^{-1} cm^{-1}): 6020 (one measurement); 6500, 6840, 6770 (three measurements with the same Fe protein preparation on the same day). The median value was 6600.

Cold Inactivation of the Fe Protein. Nitrogenase from *C. pasteurianum* is cold labile, i.e., inactivated by storage at or near 0 °C (Dua & Burris, 1963), and this is due to inactivation of the Fe protein (Moustafa & Mortenson, 1969). This loss of enzymic activity is accompanied by an increase in the accessibility of the iron-sulfur site in the Fe protein to reaction with bathophenanthrolinedisulfonate, such that ATP is no longer necessary for rapid reaction (Table I). The data in Table I show the amount of iron that reacted with chelator only in the presence of ATP; a decrease in this fraction of the total iron was always matched by a corresponding increase in the amount of iron that reacted with chelator in the absence of ATP. Preincubation of cold-inactivated protein for 2 h at 25 °C before addition of chelator did not reactivate the protein; i.e., it did not increase the proportion of the total iron that reacted only in the presence of ATP. The time course of the decrease of active iron-sulfur sites as shown in Table I is quite similar to the data for loss of enzymic activity of purified Fe protein as reported by Moustafa & Mortenson (1969). These authors also reported that part of, but not all, the iron in the Fe protein would react with 2,2′-bipyridyl after cold inactivation. In Table I we also report that glycerol protects completely against cold inactivation.

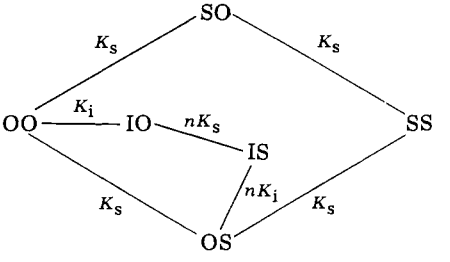
Walker (1974) reported that the rate of reaction between the Fe protein and 2,2′-bipyridyl in the absence of ATP increased with decreasing temperatures in the range 24–0 °C. We have observed similar behavior using 1 mM bathophenanthrolinedisulfonate, 0.6 mg/mL Fe protein, and 50 mM Bes, pH 7.0. The ratio between the rates at 0 °C to rates at 25 °C of the second, slow phase of the reaction (no ATP, see Figure 1) was 2.3. On the other hand, the corresponding ratio for the reaction between bathophenanthrolinedisulfonate and the MoFe protein, which is not cold labile, was 0.16. Reaction mixtures with Fe protein at 0 °C were heated at 25 °C, and the reaction rates then slowed down to the previously observed rates at 25 °C, apparently as quickly as temperature equilibration was attained. Hence, these experiments have discerned two separate effects of exposing the Fe protein from *C. pasteurianum* to 0 °C: A rapid, freely reversible increase in the accessibility of the iron–sulfur site and a much slower cold inactivation.

Discussion

The use of bathophenanthrolinedisulfonate instead of 2,2′-bipyridyl for studies on the iron–sulfur site of the Fe protein of nitrogenase offers several advantages. The higher extinction coefficient of the Fe(II) complex with bathophenanthrolinedisulfonate compared with 2,2′-bipyridyl (22 140 M^{-1} cm^{-1} at 55 nm (Blair & Diehl, 1961) vs. 8400 M^{-1} cm^{-1} at 520 nm (Walker & Mortenson, 1974)) increases the sensitivity; the higher reaction rates with bathophenanthrolinedisulfonate give added convenience and permit the use of lower concentrations of chelator; the high solubility of bathophenanthrolinedisulfonate in water allows the use of concentrated stock solutions without organic solvents. Experiments can be designed such that the reaction is nearly completed in a few minutes, enabling its use in estimating the concentration of active iron–sulfur centers in the Fe protein.

Tso & Burris (1973), on the basis of their equilibrium binding data, proposed the model shown in Scheme I for binding of MgATP and MgADP to the Fe protein.

SCHEME I



O denotes an open binding site
S = MgATP
I = MgADP
K_s = site-specific dissociation constant for binding of MgATP
K_i = site-specific dissociation constant for binding of MgADP

When ADP is absent, the number of moles of bound MgATP per mole of protein is given by:

$$R = 2S/(K_s + S)$$

The fraction of the total Fe protein population with 2 molecules of MgATP bound per protein molecule is:

$$SS = \left(\frac{S}{K_s + S}\right)^2$$

Two alternative, limiting models are possible for the coupling between binding of MgATP and the increase in reactivity of the iron–sulfur cluster: (1) binding at each site increases the reactivity by a constant amount and independently of binding at the other site; and (2) both sites must be occupied by MgATP before there is an increase in reactivity. Assuming that there is proportionality between the rate of ATP-induced chelation and the proportion of Fe protein molecules in the reactive, ATP-liganded state, which is supported by our observation that rates are proportional to the Fe protein level at a constant ATP level (data not shown), we can write for alternative 1

$$\frac{v}{V_{max}} = \frac{[MgATP]}{K_s + [MgATP]}$$

and for alternative 2

$$\frac{v}{V_{max}} = \left(\frac{[MgATP]}{K_s + [MgATP]}\right)^2$$

[MgATP], the concentration of MgATP not bound to protein, is considered to be equal to total MgATP because of the low levels of protein used.

The results in our present report clearly support alternative 2 (see Figure 2). This equation predicts sigmoidal curves for plots of v vs. [MgATP] and hyperbolic curves for plots of $v^{1/2}$ vs. [MgATP].

The data of Tso & Burris (1973) show that the dissociation constants at the two ATP sites are identical or close to identical; thus, these equilibrium binding data do not support cooperative effects in the absence of ADP. On the other hand, observations of sigmoidal kinetics when MgATP was the variable substrate for nitrogenase (Silverstein & Bulen, 1970) have been the basis for models of interaction between the binding sites. We believe that our model contributes toward a reconciliation of these seemingly conflicting views: MgATP binds to each of two identical sites on the Fe protein; these sites do not interact with each other, but they interact simultaneously with a third site, namely, the iron–sulfur site. Thus, the sigmoidal plots are not due to interaction between the binding sites, but to interaction with a third and different site, the iron–sulfur site, and to the requirement that both binding sites must be occupied before they interact with the iron–sulfur site.

Mortenson et al. (1975) presented results of chelator assays with 2,2′-bipyridyl which indicated a sigmoidal dependence upon concentration of MgATP, but their interpretation differs from ours. They reported that plots of $1/v$ vs. $1/[MgATP]^2$ were linear and that Hill plots were linear with slopes of about 2. Although not explicitly stated by the authors, both of these claims are equivalent to the proposal that the rate law for the chelator reaction is:

$$\frac{v}{V_{max}} = \frac{[MgATP]^2}{K_s + [MgATP]^2}$$

Mortenson et al. gave no theoretical justification for selecting this model, which predicts that the concentration of complexes with one MgATP per Fe protein is vanishingly small compared with complexes with two MgATP (Cornish-Bowden, 1976); i.e., the cooperativity between the binding sites for MgATP is complete. This is in direct conflict with the observations and model of Tso & Burris (1973).

With ADP present, SS in the model of Tso & Burris (1973; see also Tso, 1973) can be expressed as:

$$SS = \frac{S^2}{K_s(1 + I/K_i) + K_s(2 + I/nK_i)S + S^2}$$

ADP inhibits the ATP stimulation of the reaction with bathophenanthrolinedisulfonate and does not itself stimulate the reaction. Thus, it seems reasonable to assume that v/V_{max} = SS, also in the presence of ADP. The equation for SS predicts that ADP is a competitive inhibitor of ATP and that plots of $1/v$ vs. [ADP] at constant [ATP] are linear. Data fitting to this equation is more difficult than data fitting to our model for the behavior in the absence of [ADP], because plots of $v^{1/2}$ vs. ATP are no longer expected to be simple hyperbolas. We have, therefore, not attempted to rigorously decide whether ADP is a competitive inhibitor, but the data of Mortenson et al. (1975) and our own results (data not shown) indicate competitive inhibition. Plots of $1/v$ vs. [ADP] curve upwards (Figure 3), suggesting that there are higher order terms for I in the denominator of the expression for SS, caused by binding of ADP to the second ATP site. The curvature is slight, which is compatible with weak binding at the second site, something not easily detected in equilibrium binding studies of the type reported by Tso & Burris (1973).

Data from equilibrium binding and steady-state kinetic studies with a constant ADP level, measuring either ATP binding or steady-state rates at different ATP levels, are seemingly contradictory. Equilibrium binding data indicate positive cooperativity between the binding of ADP at one site and ATP at another site and apparent negative cooperativity in the binding of ATP at both sites (Tso & Burris, 1973), whereas steady-state kinetic data indicate apparent positive cooperativity in the binding of ATP at multiple sites (Ljones, 1973; Thorneley & Cornish-Bowden, 1977). Once more, our model for interaction between the ATP/ADP sites and the Fe–S site on the Fe protein provides reconciliation: Binding of ATP to one site is enhanced when ADP is bound to the other site, but this binding of ATP does not contribute toward reactions at the Fe–S site as long as the other binding site is not occupied by ATP. Thus, with ADP present and at low levels of ATP, a fairly large proportion of the Fe protein molecules will bind 1 ATP, but a very small proportion will bind 2 ATP. Because the first ATP molecule binds more tightly than the second, equilibrium binding data indicate negative cooperativity, whereas rate measurements with chelator reactions or steady-state catalysis only indicate the fraction of the Fe protein with 2 ATP molecules bound and therefore appear consistent with positive cooperativity.

Thorneley & Cornish-Bowden (1977) have presented an analysis of nitrogenase kinetics based on current models for allosteric behavior. These models are formulated for equilibrium binding studies, however, and our analysis suggests that the assumptions that are needed for application of these models to steady-state kinetic data are not valid for nitrogenase.

The stoichiometry between ATP hydrolysis and electron transfer during nitrogenase catalysis is variable and depends upon temperature (Hadfield & Bulen, 1969; Watt et al., 1975) and component ratio (Ljones & Burris, 1972). Nevertheless, there seems to be a lower limit close to 2 for the ATP/e^- ratio. In the present report we propose that 2 molecules of MgATP must bind simultaneously to the Fe protein to induce the conformational change that precedes electron transfer to the MoFe protein. The Fe protein contains 1 iron–sulfur center of the 4Fe-4S type per protein molecule (Gillum et al., 1977; Orme-Johnson & Davis, 1977; Ljones, 1974) and thus probably transfers 1 electron per redox cycle. Our model is therefore consistent with a lower limit of 2 for the ATP/e^- ratio and accounts for all of the ATP hydrolyzyed and all of the observed sites for ATP binding in coupling with electron transfer from the Fe protein to the MoFe protein. We certainly do not wish to exclude the possibility of other functions for ATP in nitrogenase catalysis (Smith et al., 1976), but available data at the

present time can equally well be explained without such a proposition.

The value of the dissociation constant for MgATP from the data in Figure 2, $K_s = 85\ \mu M$, is considerably higher than the value reported by Tso & Burris (1973), $K_s = 17\ \mu M$. A possible explanation for this discrepancy is that bathophenanthrolinedisulfonate interacts with the Fe protein and changes the affinity for MgATP. To test this hypothesis, experiments similar to the one depicted in Figure 2 were performed at different levels of chelator, and values for K_d were estimated. There was some tendency toward higher values for K_s at levels of chelator higher than 1 mM, but, at levels from 0.05 to 1 mM, there were no significant differences in the K_s values (data not shown). Thus, secondary effects due to interaction between the protein and chelator are probably not the reason for the discrepancy between the K_s values, unless bathophenanthrolinedisulfonate binds to the Fe protein with a very high affinity. Recent work by Emerich (1978) with the gel equilibration method indicated a dissociation constant of 53 μM for the binding of MgATP to the Fe protein of *C. pasteurianum* and 104 μM for the Fe protein of *Bacillus polymyxa*. Hence, the discrepancy between K_s values found by the chelation method and by direct binding measurements may be less than previously indicated (Mortenson et al., 1975). In this context, it seems pertinent to point out that the concentration of MgATP that gives half-maximal increase in reactivity of the iron–sulfur center is not equal to K_s, but rather to $2.44K_s$. It is, therefore, more meaningful to compare apparent Michaelis constants with the value $2.44K_s$, rather than with K_s.

Watt & Burns (1977) have recently presented a thorough discussion of the arguments and experimental data in favor of sigmoidal rather than hyperbolic kinetics with MgATP as variable substrate for nitrogenase. They suggested that other investigators (Ljones, 1973; Thorneley & Willison, 1974) may have overlooked the more obvious sigmoidal parts of the kinetic curves because of failure to measure rates at sufficiently low concentrations of MgATP. Our present report supports this contention. Using the chelator assay to study MgATP binding, we found obvious deviations from hyperbolic behavior only at MgATP levels below 0.1 mM (Figure 2). The presence of MgADP, however, shifts the value of the apparent K_s for MgATP upwards and reveals the obvious sigmoidal character of the plots at higher MgATP levels, thus explaining why the sigmoidal character of kinetic data is more easily detected in the presence of MgADP (Ljones, 1973; Thorneley & Cornish-Bowden, 1977).

As opposed to their steady state kinetic observations, Thorneley & Cornish-Bowden (1977) reported that ADP did not induce sigmoidal kinetics when rates of pre-steady-state electron flow from Fe protein to MoFo protein were measured with stopped-flow spectrophotometry. We are not able to offer any explanation for this apparent discrepancy in the context of our model for interactions between the ATP/ADP sites and the Fe-S cluster on the Fe protein. In light of the comments of Watt & Burns (1977), however, the report of Thorneley & Cornish-Bowden may not include sufficient data at low ATP levels. Furthermore, the data were not plotted in the way that would most clearly distinguish between sigmoidal and hyperbolic kinetics (Walter, 1974).

Watt & Burns (1977) proposed an empirical kinetic equation for *Azotobacter vinelandii* nitrogenase. With MgATP as the only variable substrate, their equation can be written like this:

$$\frac{v}{V_{max}} = \frac{[MgATP]^2}{[MgATP]^2 + K_1[MgATP] + K_2}$$

At 25 °C, they found $K_1 = 5.05 \times 10^{-5}\ M^{-1}$ and $K_2 = 2.8 \times 10^{-9}\ M^{-2}$. Their empirical equation is analogous to our proposed equation for the coupling between the binding of MgATP at two sites and the increase in reactivity of the iron–sulfur site of the Fe protein, except that we propose identical dissociation constants, which means that in our equation $K_1 = 2K_s$ and $K_2 = K_s^2$, or $K_1^2/K_2 = 4$. The values reported by Watt & Burns give $K_1^2/K_2 = 0.91$. When experimental error and the increased complexity of kinetic parameters compared with equilibrium parameters are considered, this comparison suggests that the affinities for MgATP at the two different binding sites during steady state catalysis are rather similar.

The MgATP-induced shift in the EPR spectrum of the Fe protein suggests that the binding of MgATP causes a conformational change in the protein. Recent observations on the Fe protein from *C. pasteurianum* (Orme-Johnson & Davis, 1977) cast some doubt on the significance of this effect to the catalytic mechanism, as the EPR effect can only be observed at pH values above 7.5 and not at pH values closer to the pH optimum of 6.5. On the other hand, Walker (1974) observed the MgATP effect on the reaction with 2,2′-bipyridyl over the pH range from 6.0 to 9.0 with constant rates over this range. Hence, the chelation reaction may be a more generally applicable probe for MgATP-induced conformational changes of the Fe protein than is EPR spectroscopy.

References

Blair, D., & Diehl, H. (1961) *Talanta 7*, 163.

Burris, R. H., & Orme-Johnson, W. H. (1974) in *Microbial Iron Metabolism* (Neilands, J. B., Ed.) p 200, Academic Press, New York, N.Y.

Cornish-Bowden, A. (1976) *Principles of Enzyme Kinetics*, p 120, Butterworths, London.

Davis, L. C., & Orme-Johnson, W. H. (1976) *Biochim. Biophys. Acta 452*, 42.

Dua, R. D., & Burris, R. H. (1963) *Proc. Natl. Acad. Sci. U.S.A. 50*, 169.

Eisenthal, R., & Cornish-Bowden, A. (1974) *Biochem. J. 139*, 715.

Emerich, D. W. (1978) *Diss. Abstr.* (in press).

Gillum, W. O., Mortenson, L. E., Chen, J.-S., & Holm, R. H. (1977) *J. Am. Chem. Soc. 99*, 584.

Hadfield, K. L., & Bulen, W. A. (1969) *Biochemistry 8*, 5103.

Hong, J.-S., & Rabinowitz, J. C. (1970) *J. Biol. Chem. 245*, 4982.

Ljones, T. (1973) *Biochim. Biophys. Acta 321*, 103.

Ljones, T. (1974) in *The Biology of Nitrogen Fixation* (Quispel, A., Ed.) p 622, North Holland Publishing Co., Amsterdam.

Ljones, T., & Burris, R. H. (1972) *Biochim. Biophys. Acta 275*, 93.

Malkin, R., & Rabinowitz, J. C. (1967) *Biochemistry 6*, 3880.

Mortenson, L. E., Walker, M. N., & Walker, G. A. (1975) in *Proceedings of the 1st International Symposium on Nitrogen Fixation* (Newton, W. E., & Nyman, C. J., Eds.) Vol. 1, p 117, Washington State University Press, Pullman, Wash.

Moustafa, E., & Mortenson, L. E. (1969) *Biochim. Biophys. Acta 172*, 106.

Orme-Johnson, W. H., & Davis, L. C. (1977) in *Iron-Sulfur Proteins* (Lovenberg, W., Ed.) Vol. 3, p 15, Academic Press, New York, N.Y.

Orme-Johnson, W. H., Hamilton, W. D., Ljones, T., Tso, M.-Y. W., Burris, R. H., Shah, V. K., & Brill, W. J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 3142.
Silverstein, R., & Bulen, W. A. (1970) *Biochemistry 9,* 3809.
Smith, B. E., Lowe, D. J., & Bray, R. C. (1973) *Biochem. J. 135,* 331.
Smith, B. E., Thorneley, R. N. F., Eady, R. R., & Mortenson, L. E. (1976) *Biochem. J. 157,* 439.
Stombaugh, N. A., Sundquist, J. E., Burris, R. H., & Orme-Johnson, W. H. (1976) *Biochemistry 15,* 2633.
Thorneley, R. N. F. (1974) *Biochim. Biophys. Acta 358,* 247.
Thorneley, R. N. F. (1975) *Biochem. J. 145,* 391.
Thorneley, R. N. F., & Cornish-Bowden, A. (1977) *Biochem. J. 165,* 255.
Thorneley, R. N. F., & Willison, K. R. (1974) *Biochem. J. 139,* 211.
Tso, M.-Y. W. (1974) *Diss. Abstr. 34B,* 4263.
Tso, M.-Y. W., & Burris, R. H. (1973) *Biochim. Biophys. Acta 309,* 263.
Tso, M.-Y. W., Ljones, T., & Burris, R. H. (1972) *Biochim. Biophys. Acta 267,* 600.
Walker, G. A. (1974) *Diss. Abstr. 35B,* 2633.
Walker, G. A., & Mortenson, L. E. (1973) *Biochem. Biophys. Res. Commun. 53,* 904.
Walker, G. A., & Mortenson, L. E. (1974) *Biochemistry 13,* 2382.
Walker, M., & Mortenson, L. E. (1973) *Biochem. Biophys. Res. Commun. 54,* 669.
Walter, C. (1974) *J. Biol. Chem. 249,* 699.
Watt, G. D., & Burns, A. (1977) *Biochemistry 16,* 264.
Watt, G. D., Bulen, W. A., Burns, A., & Hadfield, K. L. (1975) *Biochemistry 14,* 4266.
Winter, H. C., & Burris, R. H. (1976) *Annu. Rev. Biochem. 45,* 409.
Witz, D. F., Detroy, R. W., & Wilson, P. W. (1967) *Arch. Mikrobiol. 55,* 369.
Zumft, W. G., & Mortenson, L. E. (1975) *Biochim. Biophys. Acta 416,* 1.
Zumft, W. G., Palmer, G., & Mortenson, L. E. (1973) *Biochim. Biophys. Acta 292,* 413.

DNA Unwinding Protein from Meiotic Cells of *Lilium*†

Yasuo Hotta* and Herbert Stern

ABSTRACT: An ATP-dependent DNA unwinding protein is present at a high level of activity in meiotic cells of lilies. The protein also acts as a DNA-dependent ATPase, the single strand form being the preferred cofactor. It binds in the absence of ATP to single-strand DNA and to ends or nicks in duplex DNA. A 3′-OH terminus is required for binding at duplex ends; such binding is highly stable. Unwinding occurs in the presence of ATP, and it is limited to about 50 base pairs per end or 400–500 base pairs per nick. The ATP hydrolyzed during unwinding is distinguishable from ATP hydrolysis in the presence of single-strand DNA.

Several types of DNA unwinding proteins which require ATP for activity have been prepared from *Escherichia coli* (Abdel-Monem & Hoffmann-Berling, 1976; Abdel-Monem et al., 1976, 1977a,b; Richet & Kohiyama, 1976). The proteins bind to ss-DNA[1] (Abdel-Monem & Hoffmann-Berling, 1976) and, when so bound, function as ATPases. The biological role of the ATPase activity is unknown, but the activity has been useful as an assay in purifying proteins of this kind. By using the assay, we have identified and partially purified a DNA unwinding protein ("U-protein") in meiotic cells of *Lilium.* Among the four chromatographically separable DNA-dependent ATPase activities present in meiotic cells, only U-protein increases markedly during meiosis. As will be discussed fully in a separate study, the increase coincides with the pairing and crossing-over of the chromosomes and is low during premeiotic S phase. Its behavior thus contrasts with the behavior of the *E. coli* "rep protein" which has been assigned a specific unwinding role in ΦX-174 replication (Scott et al., 1977). Although U-proteins are probably involved in DNA replication of higher eukaryotes, this paper is confined to the meiotic U-protein which, because of its prominence during meiotic prophase, is probably involved in recombination.

In general, the U-protein of lily meiocytes is similar in its properties to those of the *E. coli* U-proteins I and II described by Hoffmann-Berling and his collaborators (Abdel-Monem et al., 1977c). There are, nevertheless, several differences in property between the *E. coli* and *Lilium* proteins which may be related to their respective biological roles. The *E. coli* U-proteins I and II do not bind to duplex DNA and require an appreciable but still undetermined length of ss-DNA to unwind an adjacent duplex region (Abdel-Monem et al., 1977a,b). Lily U-protein can bind to duplex DNA and can initiate unwinding from the ends of linear molecules, or from nicks in linear and circular forms. Except for very short DNa duplexes (less than 100 bp), the lily U-protein does not fully unwind ds-DNA; it initiates and effects unwinding over an apparently fixed length of DNA from the point of initiation.

Materials and Methods

1. Isolation of Cells. Meiotic cells ("microsporocytes") were extruded from anthers by squeezing individual flower buds of

† From the Department of Biology, B-022, University of California, San Diego, La Jolla, California 92093. *Received August 16, 1977; revised manuscript received February 10, 1978.* This work was supported by grants from the National Science Foundation (PCM 76-09660) and the National Institutes of Health (HD 03015).

[1] Abbreviations used: ss-DNA, single stranded DNA; ds-DNA, double stranded DNA; bp, base pairs.

0006-2960/78/0417-1872$01.00/0