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Effect of Magnesium Adenosine 5′-Triphosphate on the Accessibility of the Iron of Clostridial Azoferredoxin, a Component of Nitrogenase†

Gustavus A. Walker and Leonard E. Mortenson*

ABSTRACT: Azoferredoxin has been shown to have a minimum of three different interconvertible states: an oxidized state (one in which its activity is not destroyed), a reduced state, and a reduced state in the presence of magnesium ATP. Each of these states could function in the reductions catalyzed by nitrogenase. The rapid removal of iron from azoferredoxin by α,α'-dipyridyl, only when azoferredoxin is complexed with magnesium ATP, is directly correlated with a loss in the electron paramagnetic resonance spectrum of the reduced azoferredoxin–magnesium ATP state. In the absence of α,α'-dipyridyl, the effect of magnesium ATP is reversible. The magnesium ATP effect is highly specific since no other magnesium nucleotide examined catalyzed the rapid transfer of iron from azoferredoxin to α,α'-dipyridyl. In addition, the iron of molybdoferredoxin, the other nitrogenase component, is not made accessible to α,α'-dipyridyl upon addition of magnesium ATP. Although ADP has been shown to compete with ATP in the nitrogenase system and to inhibit its activity, it does not mimic ATP in allowing the iron of azoferredoxin to be removed by added iron chelators. In fact, ADP inhibits the ability of ATP to make the iron of azoferredoxin accessible to chelators. Significantly, reduced molybdoferredoxin which itself reacts very slowly with α,α'-dipyridyl also hinders the removal of iron from the magnesium ATP complex of azoferredoxin. Oxidation of azoferredoxin in such a way that most of it is not inactivated disrupts its α,α'-dipyridyl-resistant structure. However, if dithionite is immediately added to the protein after oxidation, that protein not inactivated returned to its α,α'-dipyridyl-resistant structure. The amount of restoration correlated with the amount of activity recovered after the same treatment.

ATP hydrolysis is required in the catalytic transfer of electrons by nitrogenase from a reductant to dinitrogen (McNary and Burris, 1962; Mortenson, 1964; Hardy and D'Eustachio, 1964). Neither component of the enzyme, azoferredoxin (iron–protein) or molybdoferredoxin (molybdenum–iron protein), is enzymatically active in the absence of the other (Mortenson *et al.*, 1967). It is not understood how ATP functions in the reduction of substrates by nitrogenase, although several hypotheses have been advanced. Among these postulates are: (1) electron activation by ATP (Mortenson, 1964; Hardy *et al.*, 1965), (2) ATP mediated electron transfer (Mortenson, 1964; Hardy and Burns, 1968), (3) ATP supported substrate binding (Kelly and Lang, 1970), and (4) ATP induced changes in the conformation of one or both components of nitrogenase (Bulen *et al.*, 1965b; Silverstein and Bulen, 1970; Yates, 1972; Zumft *et al.*, 1973).

Zumft *et al.* (1973) proposed that ATP induces a conformational change in azoferredoxin because the changes that it caused in the electron paramagnetic resonance (epr) spectrum of azoferredoxin were similar to those spectral changes obtained with azoferredoxin in the presence of 5 M urea. These observations suggested that a urea- or ATP-induced conformational change would probably involve a reorientation of the iron–sulfur center(s) within azoferredoxin. Walker and Mortenson (1973) recently reported that the iron in reduced azoferredoxin is accessible to an iron chelator in the presence of MgATP[1] but not in its absence. The latter provided strong evidence that MgATP alters the conformation of azoferredoxin.

This study elaborates the effect of MgATP on azoferredoxin in the presence of the ferrous iron chelator, α,α'-dipyridyl.

Materials and Methods

Azoferredoxin (AzoFd) and molybdoferredoxin (MoFd) were prepared from *Clostridium pasteurianum* W5 by the method of Zumft and Mortenson (1973). Final preparations of AzoFd and MoFd were in 0.05 M Tris-HCl (pH 7.4) and were 0.35 and 0.25 M, respectively, in NaCl and 1 mM with

† From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907. *Received October 8, 1973.* This investigation was supported by grants from the National Institutes of Health (AI-04865) and the National Science Foundation (GB-35589X).

[1] Abbreviations used are: MgATP, for the negatively charged complex ($Mg \cdot ATP^{2-}$) which exists under the conditions employed in these experiments; AzoFd, azoferredoxin; MoFd, molybdoferredoxin.

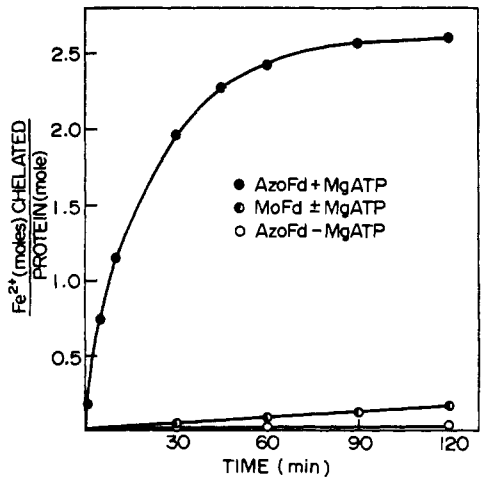

FIGURE 1: The reaction of α,α'-dipyridyl with *C. pasteurianum* AzoFd and MoFd. Either 21.3 nmol of AzoFd (final concentration, 0.0085 mM) or 7.75 nmol of MoFd (final concentration, 0.003 mM) in 0.1 ml was added anaerobically (to the experimental and control cuvettes) through a syringe to 1.8 ml of 0.1 M Tris-HCl buffer (pH 7.4) in (1-cm light path) cuvets fitted with rubber serum stoppers. The contents of the blank cuvets were the same as the experimental and control cuvets except that 0.1 ml of 0.05 M Tris-HCl (pH 7.4) was added instead of protein. The absorbance at 520 nm was read against the blank to establish a base line; 0.1 ml of 4.3 mM MgATP and 0.1 ml of 0.1 M Tris-HCl (pH 7.4) were added to the experimental and control cuvets, respectively. Next 0.5 ml of 25 mM α,α'-dipyridyl was quickly added to each cuvet to make the total volume 2.5 ml. The absorbance at 520 nm was immediately read. An initial iron complex corresponding to 0.18 Fe^{2+} per AzoFd dimer and 0.71 Fe^{2+} per MoFd tetramer was subtracted from each point on the AzoFd and MoFd curves, respectively. This AzoFd preparation had 3.3 Fe per dimer of AzoFd and the MoFd preparation had 24 Fe per tetramer.

respect to dithionite. Both proteins were judged to be pure with respect to polypeptides by disc gel electrophoresis in the presence of sodium dodecyl sulfate. The AzoFd preparations used in these experiments had an acetylene reduction activity from 1600 to 2100 nmol of acetylene reduced per min per mg of protein; the MoFd preparation had a specific activity of 1600 nmol of acetylene reduced per min per mg of protein. Reactions with α,α'-dipyridyl and *o*-phenanthroline showed that the AzoFd preparations contained from 3.0 to 3.5 Fe atoms per dimer and that the MoFd preparations contained about 24 Fe per tetramer. Because fully active AzoFd is believed to have 4.0 Fe per dimer (Nakos and Mortenson, 1971), some apo-AzoFd was probably present in the AzoFd preparations. Molecular weights of 55,000 per AzoFd dimer and 220,000 per MoFd tetramer were used for calculations (Nakos and Mortenson, 1971; Huang *et al.*, 1973).

Chemicals were purchased from commercial sources: α,α'-dipyridyl from Fisher Scientific; ATP, ADP, AMP, UTP, and CTP from Sigma Chemical Co.; and α,β-methylene-ATP and β,γ-methylene-ATP from Miles Laboratories.

Iron was determined by complexing it with either *o*-phenanthroline (Lovenberg *et al.*, 1963) or α,α'-dipyridyl and measuring the absorbance of the resulting colored complex. In the latter procedure the absorbance was read immediately. Under our conditions molar extinctions of 8400 at 520 mμ for the ferrous α,α'-dipyridyl complex and 11,000 at 512 mμ for the ferrous *o*-phenanthroline complex were found. A Cary-14 recording spectrophotometer was used for all absorption measurements.

All reactions were performed anaerobically under argon at 25°. Unless specified all reagents (*e.g.*, nucleotides, α,α'-dipyridyl) contained 1 mM $Na_2S_2O_4$ in 0.1 M Tris-HCl at pH 7.4. The reduction of methyl viologen was used to confirm the presence of dithionite at the end of all reactions. The α,α'-dipyridyl stock solution also contained 5% by volume of ethanol.

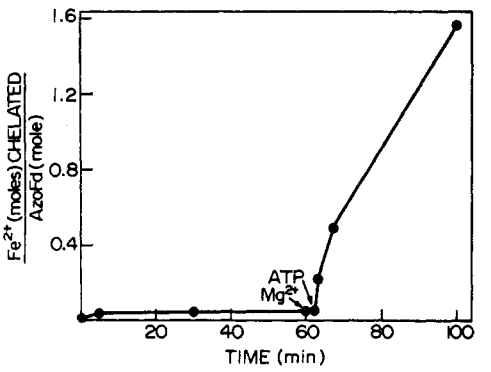

FIGURE 2: The reversibility of the MgATP effect on AzoFd. AzoFd (74.5 nmol) was incubated anaerobically with 1500 nmol of MgATP in a volume of 0.75 ml at 25° for 2 min (ample time to put the protein in theMgATP form) and was then put on a Sephadex G-25 column (13 cm × 1.6 cm) which had been equilibrated with 0.05 M Tris-HCl at pH 7.4. The ATP free protein was collected from the column and 21.3 nmol of AzoFd in 0.28 ml was reacted with 0.5 ml of 25 mM α,α'-dipyridyl (as in Figure 1) for 1 hr. While continuing to monitor the absorbance at 520 nm we added 21.3 nmol of Mg^{2+} in 0.1 ml to the experimental and blank cuvets and the mixture was allowed to react for 2 min. Next, 21.3 nmol of ATP in 0.1 ml was added to each cuvet and the absorbance at 520 nm quickly recorded. The total volume after all additions was 2.78 ml. The initial amount of iron chelated at "zero" time (0.25 Fe^{2+} per AzoFd dimer) was subtracted from each point.

Results

Reaction of α,α'-Dipyridyl with MoFd. Figure 1 compares the reactions of MoFd and AzoFd with α,α'-dipyridyl. Like AzoFd, MoFd also reacts extremely slowly with the reagent under dithionite-reduced conditions. Unlike AzoFd, MoFd does not change its reactivity with α,α'-dipyridyl when magnesium ATP is added. For example, after 20 hr with or without magnesium ATP, only 20% of the iron of MoFd reacted. This is to be contrasted to AzoFd where in 20 hr, in the absence of magnesium ATP, 11% of its iron reacted whereas, in the presence of ATP, 80% reacted in 1 hr.

There is a small amount of iron in preparations of both molybdoferredoxin and azoferredoxin that reacts rapidly with α,α'-dipyridyl at "zero" time and this has been subtracted from each point in Figure 1. This accounts for a large percentage of the 11 and 20% figures mentioned above and probably results from the small fraction of the AzoFd and MoFd that either is present in a precursor form (see Zumft and Mortenson, 1973) or that has been denatured at some point in the purification procedure or during handling.

The accessibility of the iron of reduced AzoFd to external chelators is dependent upon the presence of MgATP but if MgATP is separated from the protein, the structure of AzoFd returns to its α,α'-dipyridyl-resistant conformation. Figure 2 shows that when AzoFd and MgATP were incubated together anaerobically for 2 min, ample time to form the complex, and then separated on a Sephadex G-25 column, the AzoFd collected from the column did not "release" its iron to α,α'-dipyridyl. Little complex was found during incubation for 1 hr with α,α'-dipyridyl. Magnesium was added to the AzoFd from the column and still no reaction with α,α'-dipyridyl was observed, but when ATP was added in addition to Mg^{2+}, 0.5 Fe/AzoFd was transferred to α,α'-dipyridyl within 5 min.

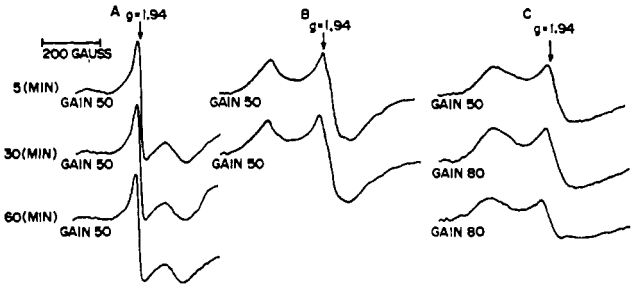

FIGURE 3: Effect of α,α'-dipyridyl on the epr spectrum of AzoFd. Three serum vials, sealed with rubber stoppers, were evacuated and filed with argon. Into each was introduced the following reactants with a final volume of 1.32 ml: AzoFd, 9.6 mg; dithionite, 2.64 μmol; NaCl, 28 μmol; Tris-HCl (pH 7.4), 92 μmol; (where indicated) α,α'-dipyridyl, 12.8 μmol, and MgATP, 12 μmol. Samples (0.3 ml) from each mixture were anaerobically introduced to separate epr tubes, previously evacuated and filled with argon. After the indicated incubation time each epr tube was frozen in liquid nitrogen and stored until they could be read. Each epr spectrum (g = 2 region) was obtained with a separate tube: (A) AzoFd and α,α'-dipyridyl; (B) AzoFd and MgATP; (C) AzoFd, α,α'-dipyridyl, and MgATP. Sweep rate, 200 G/min; time constant, 0.3 sec; gain 50 or 80, modulation amplitude 1000 G, frequency, 9.22 GHz, microwave power, 3 mW, temperature, 23°K, Varian standard cavity. This experiment was done in collaboration with Graham Palmer (now at Rice University, Houston, Texas).

Specificity of the Effect of MgATP on AzoFd. The specificity of the effect of MgATP on AzoFd has been investigated by observing changes in the epr spectrum of AzoFd (Zumft *et al.*, 1973). Various purine and pyrimidine nucleotides were tested, and of those tested only MgADP (in high concentration) and Mgβ,γ-methylene-ATP caused an effect comparable to that found with MgATP. Is the same specificity found when α,α'-dipyridyl is used as a probe to detect the MgATP effect? Table I shows that the "α,α'-dipyridyl effect" is specific for MgATP. Under the conditions indicated, ATP, ADP, MgADP, and Mgβ,γ-methylene-ATP show only a slight effect on AzoFd but this effect is not more than 13% of that found with MgATP. Other compounds such as MgAMP, MgGTP, MgCTP, and MgUTP are inactive.

TABLE I: Effect of Nucleotides on Iron Removal from AzoFd by α,α'-Dipyridyl.

Expt	Additions[a]	Fe (mol) Chelated after 1 hr[b]/ AzoFd (mol)
1	0	0.01
2	Mg^{2+}	0.03
3	ATP	0.20
4	MgATP	1.62
5	ADP	0.11
6	MgADP	0.20
7	MgAMP	0.00
8	$MgPP_i$	0.01
9	MgGTP	0.07
10	MgCTP	0.06
11	MgUTP	0.06
12	Mgβ,γ-methylene-ATP	0.12
13	Mgα,β-methylene-ATP	0.04

[a] Mg(nucleotide) is used to designate the species where equimolar concentrations of magnesium and nucleotide are mixed at pH 7.4. All conditions are the same as in Figure 1 except the final concentration of all additions was 0.085 mM. [b] All values have been corrected for the initial amount of iron chelated at "zero" time (see text).

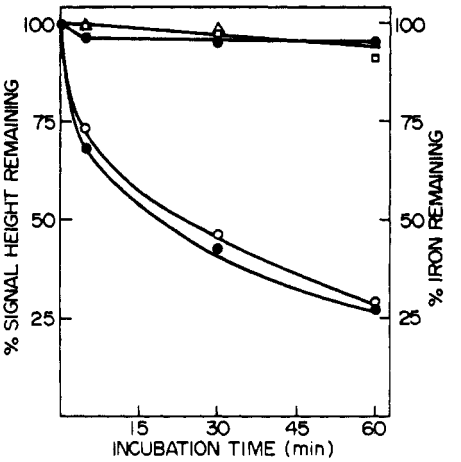

FIGURE 4: Relationship between the loss of electron paramagnetic resonance and loss of iron through chelation by α,α'-dipyridyl in the presence of MgATP. The data from these curves are included as part of Figure 3. The maximum signal height (g = 1.94 type) was arbitrarily made to equal the crest to trough distance of the AzoFd plus MgATP epr tube or the AzoFd plus α,α'-dipyridyl tube at 5-min incubation: (□) AzoFd plus α,α'-dipyridyl; (△) AzoFd plus MgATP; (●) AzoFd plus MgATP and α,α'-dipyridyl. In the parallel experiment where the chelation of iron was followed with time by observing the absorbance at 520 nm, 0.3-ml samples from vials, with mixtures identical with those used in Figure 3, were anaerobically introduced to cuvets (1-mm light path) fitted with rubber serum stoppers: (⊙) AzoFd plus α,α'-dipyridyl; (○) AzoFd plus MgATP and α,α'-dipyridyl. This AzoFd preparation had 3.0 Fe per dimer.

Effect of α,α'-Dipyridyl on the Epr Spectrum of AzoFd. The results in Figure 3A show that after incubation for 1 hr α,α'-dipyridyl made no contribution and caused no appreciable changes in the "1.94" epr signal of reduced AzoFd. The addition of MgATP to AzoFd produced in less than 1 min both qualitative and quantitative changes in the epr spectrum of reduced AzoFd as previously reported (Zumft *et al.*, 1972; Orme-Johnson *et al.*, 1972) but no additional change occurred after 30-min incubation (Figure 3B). However, the reaction of α,α'-dipyridyl with reduced AzoFd in the presence of MgATP (Figure 3C) caused substantial decreases in signal amplitude after incubation for 1 hr. Figure 4 shows that the removal of iron from AzoFd by α,α'-dipyridyl correlates with the decrease in epr signal height.

Effect of the Concentration of MgATP on Iron Removal from AzoFd. The rate of transfer of iron from AzoFd to α,α'-dipyridyl increases with increasing concentrations of MgATP in the manner described in Figure 5. Although AzoFd appears to bind 2 mol of MgATP (Moustafa and Mortenson, 1967; Tso and Burris, 1973; Zumft *et al.*, 1973), under the conditions of this experiment a ratio of 20:1 (MgATP to AzoFd) is required to maximize the rate of iron chelation by α,α'-dipyridyl. This undoubtedly reflects the dissociation constant for the MgATP–AzoFd complex (Tso and Burris report a constant of 17 μM) as an excess of MgATP over AzoFd would be required so that all the AzoFd would be in the form in which its iron is accessible to α,α'-dipyridyl.

The concentration of MgATP that is required to maximize the rate of iron removal from AzoFd depends on the time at which the reaction is measured. For example, although the rate of iron removal leveled off at about 0.16 mM MgATP when the reaction was measured at 1 hr, for maximum reaction

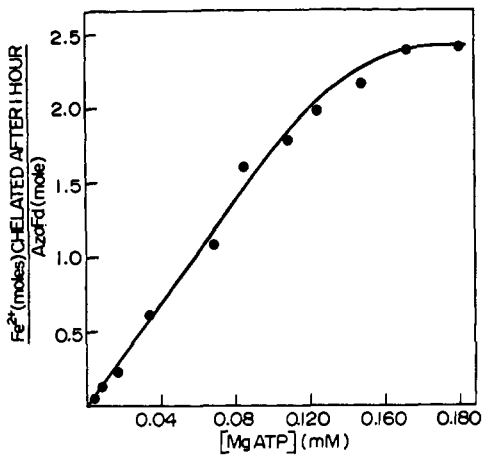

FIGURE 5: Effect of the concentration of MgATP on the rate of iron removal from AzoFd by α,α'-dipyridyl. All conditions are the same as Figure 1 except that the concentration of MgATP was varied as indicated. An initial rapid iron chelation of 0.22 Fe^{2+} per AzoFd dimer was subtracted from each point.

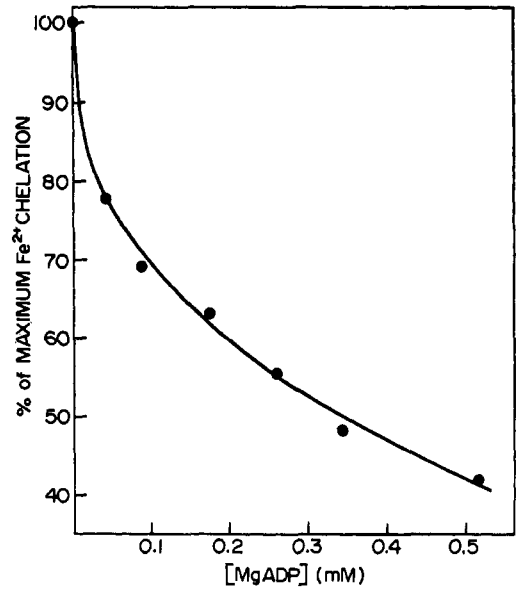

FIGURE 6: MgADP inhibition of the rate of Fe^{2+} transfer from AzoFd to α,α'-dipyridyl in the presence of MgATP. All conditions are the same as in Figure 1 except that the MgADP in 0.1 ml was added to the experimental and blank cuvets before the addition of 0.43 μmol of MgATP (0.17 mM). The total volume in each cuvet was 2.5 ml. After α,α'-dipyridyl was added, the rate of formation of the Fe^{2+}–α,α'-dipyridyl complex was followed for 15 min and compared with the control without ADP. Differences in the 520-nm absorbance at 1 min and at 15 min were used to determine the rate. Maximum complex formation in 15 min in the absence of ADP was 1.2 Fe^{2+} per AzoFd dimer. A second group of controls with the same concentrations of ADP, but no ATP, was run. The amount of iron chelated under these conditions, although it was neglible (0.06 Fe^{2+}/AzoFd dimer) and varied little with ADP concentration, was subtracted from the above results before the percentage of maximum complex formation was determined.

at 30 min a higher concentration than 0.18 mM MgATP was required.

Effect of MgADP. ADP is an inhibitor of nitrogenase-mediated ATP hydrolysis, electron transport, and substrate reduction (Bulen *et al.*, 1965a; Moustafa and Mortenson, 1967; Bui and Mortenson, 1969; Mortenson *et al.*, 1973). Data of Moustafa and Mortenson (1967) and Tso and Burris (1973) provided evidence that MgADP competes with at least one of the two MgATP sites. Data from the present paper (Table I) show that MgADP does not induce the conformation change in AzoFd that exposes the iron of AzoFd to α,α'-dipyridyl.

Because of the latter finding it was of interest to determine if the prior addition of MgADP to AzoFd would interfere with iron chelation induced by ATP in the presence of α,α'-dipyridyl. Figure 6 shows that MgADP does interfere. For example, at an ADP/ATP ratio of 1:1 about 40% inhibition of the iron removal was observed.

Effect of MoFd on the MgATP-Induced Conformational Change in AzoFd. Information on how MoFd affects the ATP-induced accessibility of the iron in AzoFd could give insight into the nature of the interaction between these two components of the nitrogenase system and into the role that ATP plays in the formation of the nitrogenase complex. Figure 7 shows the results of this experiment. A molar ratio of two AzoFd to one MoFd was chosen because this ratio gives optimal AzoFd specific activity (Mortenson *et al.*, 1973). The reactions were followed for only 5 min to avoid the possible effects of physiological oxidation of AzoFd that would occur if the reductant became exhausted (Mortenson *et al.*, 1973; Orme-Johnson *et al.*, 1972). The number of nmoles of iron transferred from AzoFd in the presence of MoFd was 48% of that transferred in its absence. When the concentration of MoFd was doubled so that the ratio of MoFd to AzoFd was 1:1, the amount of iron transferred from AzoFd at the end of 5 min decreased to 35% of the amount transferred in the absence of MoFd.

Effect of Oxygen. The sensitivity of AzoFd to oxygen has been reported (Moustafa and Mortenson, 1969). It was recently reported that oxidized AzoFd also reacts with α,α'-dipyridyl (Walker and Mortenson, 1973). It was necessary now to determine whether the effects of systematic oxidation of the protein could be reversed by reduction with dithionite.

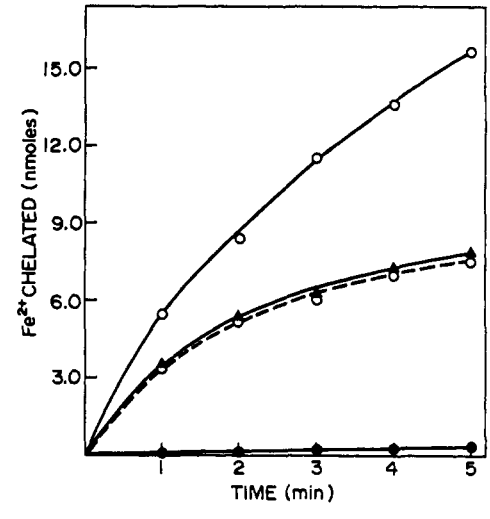

FIGURE 7: Effect of MoFd on chelation of iron from AzoFd; 21.3 nmol of AzoFd (74.6 nmol of Fe), or 10.65 nmol of MoFd (255.6 nmol of Fe), or both were added anaerobically to 1.56 ml of 0.1 M Tris-HCl buffer (pH 7.4) in (1-cm light path) cuvets fitted with rubber serum stoppers. A blank contained all additions except protein. To each cuvet was added 0.1 mg of creatine kinase, 20 μmol of dithionite, 20 μmol of creatine phosphate, and 0.43 μmol of MgATP; 12.5 μmol of α,α'-dipyridyl was quickly added to each cuvet and the absorbance at 520 nm was followed for 5 min. The final volume was 2.7 ml. The iron chelated from MoFd alone (●) was subtracted from the total iron chelated from AzoFd and MoFd (▲) and the resultant (O- - -O) compared with that chelated from AzoFd alone (-O-O). The "zero" time iron was subtracted from each value; for AzoFd it was 3.85 nmol of Fe^{2+} and for MoFd it was 8.55 nmol of Fe^{2+}.

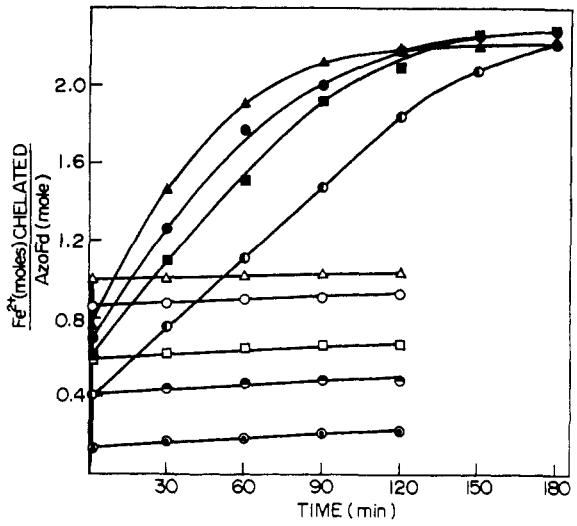

FIGURE 8: Effect of oxidation of AzoFd on the removal of its iron by α,α'-dipyridyl; 23.3 nmol of AzoFd in 0.1 ml was added anaerobically (to the experimental and control cuvets) to 2.0 ml of 0.1 M Tris-HCl (pH 7.4) under an atmosphere of argon containing a small concentration of air. No extra dithionite was added. This batch of AzoFd contained 3.0 Fe per dimer. The blank cuvet contained the same additions as the experimental and control cuvets except that 0.1 ml of 0.05 M Tris-HCl (pH 7.4) was added instead of protein. The absorbance at 440 nm was monitored and the protein was observed to be in the fully reduced state. The experimental cuvet was gently agitated to dissolve a small amount of the oxygen present in the gas phase. This process was repeated until varying percentages of the AzoFd were oxidized as measured by the absorbance at 440 nm. AzoFd (in separate cuvets) at the following oxidation states was obtained: (▲) 100% oxidized; (●) 80% oxidized; (■) 50% oxidized; (◐) 30% oxidized. To each of the above cuvets 0.5 ml of 25 mM α,α'-dipyridyl (no dithionite) was added to make the final volume 2.6 ml. The absorbance at 520 nm was recorded at 0 time and at each 30 min interval thereafter up to 180 min. The controlled oxidation of AzoFd was repeated with a second group of cuvets containing the same components as above. Dithionite (1 μmol) in 0.01 ml was immediately added after the appropriate oxidation and prior to the addition of α,α'-dipyridyl: (△) 100% oxidized; (○) 80% oxidized; (□) 50% oxidized; (◓) 30% oxidized. The α,α'-dipyridyl added to the control (⊙) which was not oxidized contained 1 mM dithionite.

Is the protein still active? The protein was oxidized 30, 50, 80, and 100% (measured spectrally) as described in the legend for Figure 8. The more the protein was oxidized the greater was the initial amount of iron chelated and the more apparent was the hyperbolic character of the curves which descibe the rate of chelation that occurred after the initial burst. In spite of the variation in the initial extent of iron chelated and the subsequent rate of chelation, all reactions were virtually complete within 3 hr with about 77% of the total iron reacted as ferrous iron. An interpretation of the amount of total iron found as ferrous or ferric is complicated by the possibility that when the structure of iron–sulfur proteins is disrupted, any ferric iron released could be reduced by liberated sulfide and exposed sulfhydryl groups (Lovenberg *et al.*, 1963; Malkin and Rabinowitz, 1967).

The above experiment was repeated with the exception that dithionite was added immediately after the controlled oxidation. The initial amount of complexable iron was approximately the same as it was when no dithionite was added but now little "secondary" chelation of iron resulted (Figure 8). The initial amount of iron chelated in a control experiment in which AzoFd was not oxidized was 0.14 Fe^{2+}/AzoFd dimer. If this is subtracted from the initial amount of iron chelated in the dithionite-reversed group, the remaining amount of iron chelated at "zero" time is proportional to the degree of oxidation as measured spectrally. After this correction the percentage of iron chelated rapidly at "zero" time after 30, 50, 80, and 100% oxidation was 9, 15, 25, and 30%, respectively, a constant ratio (per cent iron removed/per cent oxidation) of 0.3. This irreversible loss of iron corresponded with the loss in activity (Table II).

TABLE II: Effect of Air Oxidation of AzoFd on the Reduction of Acetylene by Nitrogenase.[a]

% Oxidation[b]	% Loss of Activity
0.0	0
17.7	0
44.7	6.7
84.7	30.0
100.0	34.3

[a] Walker (1974). [b] Measured as % increase in 440-nm absorbance.

Discussion

Previous findings that MgATP can bind to AzoFd but not appreciably to MoFd (Bui and Mortenson, 1968; Tso and Burris, 1973) and that MgATP causes substantial changes in the epr spectrum of AzoFd (Zumft *et al.*, 1972; Orme-Johnson *et al.*, 1972) but not of MoFd are consistent with the observation in this paper that MgATP increases extensively the accessibility of the iron of AzoFd but not of MoFd.

There is no correlation in the length of time it takes for MgATP to effect a conformational change in AzoFd as measured by epr and the length of time it takes for α,α'-dipyridyl to remove all the iron of AzoFd in the presence of MgATP. The effect that MgATP induces in AzoFd is thought to occur between 1 and 3 sec because that is the time required for MgATP to effect the maximum change in the epr spectrum of AzoFd (the time is in the order of 10 msec if MoFd is also added; W. G. Zumft, L. E. Mortenson, and G. Palmer, unpublished data). The change in the epr signal is thought to be a result of a conformational change and in this paper we give evidence that this ATP induced conformational change in AzoFd is reversible. When α,α'-dipyridyl is employed to demonstrate the effect of MgATP on AzoFd, the transfer of iron from AzoFd to the chelator is a secondary effect of the ATP induced conformational change and its rate depends on both the concentration of α,α'-dipyridyl and MgATP. MgATP is required in sufficient excess over AzoFd to keep it in a state where its iron is accessible to the chelator. If the incubation time of MgATP with AzoFd is extended from about 15 sec to 1 hr before α,α'-dipyridyl is added, it still takes about 1 hr to chelate 80% of the iron from AzoFd. Therefore, little if any iron is released from AzoFd in the presence of MgATP.

There exists discrepancies in the results from the different techniques used to show the specificity of the MgATP effect. The results from the epr data (Zumft *et al.*, 1973) show that MgADP and Mgβ,γ-methylene-ATP cause changes in the epr spectrum of AzoFd which are like those caused by MgATP. Thorneley and Eady (1973) have recently observed that the addition of MgATP or MgADP to AzoFd of *Klebsiella* in the presence of 5,5′-dithiobis(2-nitrobenzoate) can increase the reactivity of its thiol groups, although MgATP is ap-

preciably more effective. On the other hand, the results of this paper show that none of the nucleotides tested except MgATP can effectively induce changes in the accessibility of the iron of AzoFd to α,α'-dipyridyl. In addition, prior incubation of AzoFd with MgADP inhibits the effect of MgATP, which suggests that MgADP and MgATP are competing for the same site or that MgADP binds at an allosteric site and prevents binding of MgATP.

If one assumes that all of the above techniques are probing the same effect of MgATP on AzoFd, then each technique seems to "see" different aspects possibly because of the restrictive nature of each technique and because the effect induced in AzoFd seems to involve a complex realignment and reorientation of catalytic centers, notably the iron-sulfur group(s). MgADP and Mgβ,γ-methylene-ATP may effect minor, local changes in the protein which are enough to convert the epr signal but not enough to effect the precise realignment of catalytic groups that may be required for activity. Quite possibly α,α'-dipyridyl is a more sensitive tool to gauge the specific but overall conformational changes that MgATP induces in AzoFd.

If AzoFd is systematically oxidized (but not overoxidized) by trace amounts of oxygen present in argon, and if α,α'-dipyridyl is subsequently introduced, the kinetics of iron chelation (Figure 8) show that some iron is chelated rapidly whereas other iron is slowly chelated by α,α'-dipyridyl. The accessibility of the iron which can be rapidly chelated at "zero" time cannot be reversed by the addition of dithionite. That iron is released as a result of oxygen denaturation of AzoFd. The accessibility of the remaining iron, which is slowly chelated by α,α'-dipyridyl, can be reversed if dithionite is added immediately following oxidation. This latter iron is associated with active AzoFd, since when AzoFd is oxidized 100% (measured spectrally) by oxygen and then dithionite added, 70% of the protein returns to its α,α'-dipyridyl-resistant structure and about 70% of the activity is present when measured by acetylene reduction.

The results of these experiments show that AzoFd has a minimum of three different, interconvertible states: the oxidized (not O_2 damaged) state, the reduced state, and the reduced state in the presence of MgATP. Another possible state, the oxidized state in the presence of MgATP, has not been demonstrated, although the fact that ATP is hydrolyzed (at a low rate) by nitrogenase in the absence of a reductant (Bui and Mortenson, 1969) suggests that AzoFd does not have to be in the reduced form to bind ATP. Each of these states could correspond to a different conformation of AzoFd, which could function in the reductions catalyzed by nitrogenase. Although there is no definitive evidence presented by the data in this paper that the conformation of physiologically oxidized AzoFd is different from that conformation induced by MgATP, different changes in the epr spectrum of AzoFd, loss of the spectrum upon oxidation, and change in the spectrum upon the addition of MgATP to reduced AzoFd suggest that the two conformations are distinct (Palmer *et al.*, 1972; Orme-Johnson *et al.*, 1972; Mortenson *et al.*, 1973).

Yates (1972) has suggested that magnesium ATP interacts with AzoFd through a metal ion which remains associated with the protein. Other than nonspecifically bound metal ions, iron is the only metal that has been reported to be a part of AzoFd. If, as suggested, iron were to provide a site for interaction with ATP, it seems likely that the iron also would be available to α,α'-dipyridyl, a result opposite to that shown in this paper. One could allow for an interaction of MgATP with the iron of AzoFd, if the interaction resulted from MgATP first binding with specific amino acid groups to effect a conformational change which then allowed it or another MgATP to interact with the iron.

The fact that MgATP causes substantial changes in the epr spectrum of AzoFd in the range of $g = 1.94$ and that loss of iron from AzoFd to α,α'-dipyridyl can be correlated with decreases in amplitude of the epr signal around $g = 1.94$ makes reasonable the assumption that MgATP binding causes a change in the iron–sulfur centers in AzoFd.

How AzoFd interacts with MoFd to effect substrate reduction is a central question in trying to understand the mechanism of dinitrogen fixation. The preponderance of the evidence based on epr studies suggests that the formation of the reduced MgATP–AzoFd complex must precede the transfer of electrons from AzoFd to MoFd (Orme-Johnson *et al.*, 1972; Smith *et al.*, 1972; Mortenson *et al.*, 1973). Whether or not MgATP induces dissociation of AzoFd into subunits before a catalytically active unit can be formed with MoFd is not known. Attempts in this laboratory to promote the dissociation of reduced AzoFd dimer into monomer by passing low concentrations of AzoFd through a Sephadex G-200 column equilibrated with MgATP and dithionite were not successful; only dimer was collected. It is possible that the iron complex or complexes of AzoFd require ligands of both subunits and if so it would be impossible to obtain a subunit that contained iron and was active.

MoFd interferes with the accessibility of the iron of AzoFd (in the presence of α,α'-dipyridyl). This observation is consistent with the idea that an ATP induced conformational change in AzoFd is required before an active complex can be formed with MoFd and that the iron–sulfur center(s) in AzoFd constitute part of the active site in conjunction with the appropriate ligands of MoFd. The reduced AzoFd–MgATP complex could interact close to the substrate binding site of MoFd and result in an altering of the ligand conformation around the molybdenum or iron atoms of MoFd. This would allow electron transfer from AzoFd to MoFd with its concomitant ATP hydrolysis.

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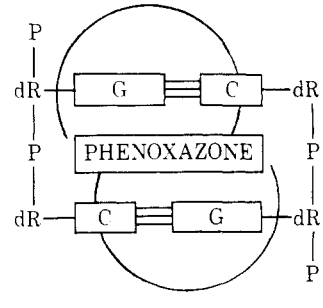
Peptide Antibiotic–Dinucleotide Interactions. Nuclear Magnetic Resonance Investigations of Complex Formation between Actinomycin D and d-pGpC in Aqueous Solution†

Dinshaw J. Patel

ABSTRACT: This study reports on the high resolution ^{1}H and ^{31}P nuclear magnetic resonance (nmr) spectra of 1:2 actinomycin D (Act-D)–d-pGpC in aqueous solution. A 1.6 ppm downfield ^{31}P shift of the internucleotide phosphate of d-pGpC on binding to Act-D in aqueous solution supports the Sobell–Jain proposal (H. M. Sobell, and S. C. Jain (1972), *J. Mol. Biol. 68*, 21) that the antibiotic intercalates between GC and CG Watson–Crick base pairs. The proton chemical shift changes for the Act-D and d-pGpC resonances on complex formation compare favorably with those predicted to arise from the ring currents and magnetically anisotropic groups of the stacked residues in the Sobell–Jain model of the complex. The guanine 2-amino exchangeable proton(s) shift downfield on complex formation consistent with their participation in hydrogen bond formation in aqueous solution.

Ribonucleic acid synthesis is inhibited when the peptide antibiotic actinomycin D (Act-D),[1] an antitumor agent, binds to double helical DNA (for a review, see Reich and Goldberg, 1964). The structure of the Act-D–DNA complex has been suggested to involve a hydrogen-bonding recognition between the guanine 2-amino proton (G–NH_2) and an acceptor group on Act-D on the basis of chemical studies of deoxy polynucleotides possessing or lacking the G–NH_2 group (Reich *et al.*, 1962). On the other hand, an intercalation model has been proposed based on spectroscopic, hydrodynamic, and kinetic studies (Müller and Crothers, 1968) and on the sedimentation behavior of supercoiled closed circular DNA in the presence of Act-D (Waring, 1970).

A detailed stereochemical model for the binding of Act-D to DNA has been proposed (Sobell and Jain, 1972; Sobell, 1973) on the basis of an X-ray study of the 1:2 Act-D–d-G complex in the crystal (Sobell *et al.*, 1971; Jain and Sobell, 1972). Act-D is suggested to intercalate between base-paired d-pGpC sequences such that the phenoxazone ring is stacked between GC and CG Watson–Crick base pairs. The complex is further stabilized by strong intermolecular hydrogen bonds involving the guanine 2-amino group and hydrophobic interactions between groups on the sugar and peptide rings. The pseudotwofold symmetry of the 1:2 Act-D–d-pGpC results from the twofold symmetry of the dinucleotide duplex coinciding with the pseudotwofold symmetry of the Act-D molecule.



† From Bell Laboratories, Murray Hill, New Jersey 07974. *Received November 2, 1973.*

[1] Abbreviation used is: Act-D, actinomycin D.