AN EFFECT OF MAGNESIUM ADENOSINE 5'-TRIPHOSPHATE ON THE STRUCTURE OF AZOFERREDOXIN FROM CLOSTRIDIUM PASTEURIANUM

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SUMMARY

Azoferredoxin from Clostridium pasteurianum has been treated anaerobically with 65 fold excess of α,α'-dipyridyl in the presence and absence of various nucleotides. Under reduced conditions 1% of the iron of AzoFd is chelated by α,α'-dipyridyl between 1 minute and 1 hour. However, when ATP is added in the presence of Mg^{2+}, 80% of the iron in azoferredoxin is chelated within an hour. This effect is reproducible and nonenzymatic. The lack of this effect with other purine and pyrimidine nucleotides demonstrates that it is specific for magnesium ATP. Treatment of azoferredoxin with 4 M urea or oxygen in the presence of α,α'-dipyridyl induces a similar effect. An ATP-induced change in the availability of the iron in azoferredoxin to the chelator, α,α'-dipyridyl, is evidence that a conformational change has occurred.

INTRODUCTION

Many hypotheses have been made to explain the requirement of ATP for the reduction of substrates by nitrogenase[1-8], but comparatively little supporting data have been produced. In this report we describe recent findings which elucidate a possible role ATP may have in the mechanism of N_2 fixation. For a comprehensive discussion of N_2 fixation the reader is referred to a recently published review[9].

It has been previously postulated by several researchers that ATP may induce a change in the conformation in one or both components of nitrogenase[6-8]. Zumft et al.[8] found that the addition of magnesium ATP to azoferredoxin (AzoFd) caused a change in the electron paramagnetic resonance (EPR) spectrum similar to that obtained with AzoFd in the presence of 5 M urea. By analogy they proposed that a change in the conformation of AzoFd had been induced by magnesium ATP. No spectral changes were seen after the addition of magnesium ATP to molybdoferredoxin (MoFd).

These observations led to the idea that a conformational change in AzoFd induced by magnesium ATP or urea would probably involve a shift of groups within the protein, notably Fe-S center(s). If such a shift did occur, it was reasoned that maybe the Fe-S center(s) would be exposed so that an iron chelator could compete favorably for the iron. For this study α,α'-dipyridyl was chosen because it immediately forms a stable red complex with ferrous iron[10].

The results of this report provide evidence that magnesium ATP alters the conformation of AzoFd.

MATERIALS AND METHODS

AzoFd was prepared from Clostridium pasteurianum by a modified method of Mortenson[11,12]. The preparations used in these experiments had an acetylene reduction activity from 1,600 to 2,100 nmoles of acetylene reduced per min per mg protein and was shown to be pure by disc gel electrophoresis in the presence of sodium dodecyl sulfate. Reactions with α,α'-dipyridyl showed that these AzoFd preparations contained between 3.0 and 3.5 Fe per dimer. Because fully active AzoFd is believed to have 4.0 Fe per dimer[13], some apo-AzoFd was probably present.

Iron was determined by using either o-phenanthroline (Lovenberg et al., 1963) or α,α'-dipyridyl. In the latter procedure the absorbancy was read immediately. A molar extinction of 8,400 at 520 mμ was used for the ferrous α,α'-dipyridyl complex and 11,000 at 512 mμ for the ferrous-o-phenanthroline complex. A Cary-14 recording spectrometer was used to measure the α,α'-dipyridyl reaction with AzoFd.

All reactions were performed anaerobically under argon at 25°C. Unless specified all reagents contained 1 mM $Na_2S_2O_4$ in 0.1 M Tris-HCl at pH 7.4 (e.g. nucleotides, urea, α,α'-dipyridyl). The α,α'-dipyridyl stock solution also contained 5% ethanol.

RESULTS

The reaction of α,α'-dipyridyl with AzoFd. The results in Fig. 1 show

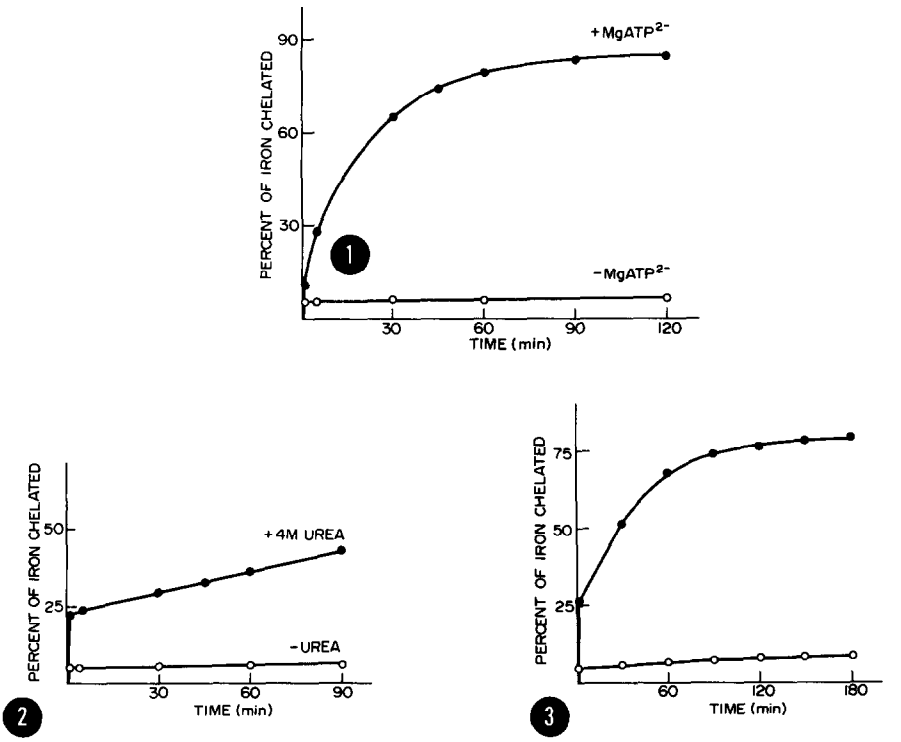

Figure 1: The reaction of α,α'-dipyridyl with C. pasteurianum AzoFd. 21.3 nmoles of AzoFd in 0.1 ml were added anaerobically (to the experimental and control cuvettes) through a syringe to 1.8 ml of 0.1 M Tris-HCl buffer (pH 7.4) in 3 cm cuvettes, fitted with serum rubber stoppers. A blank contained the same volume of buffer except a 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 magnesium ATP (●-●-●) and 0.1 ml of 0.1 M Tris-HCl (pH 7.4) (0-0-0) was added to the experimental and control cuvettes, respectively. Next 0.5 ml of 25 mM α,α;-dipyridyl was quickly added to each cuvette to make the total volume 2.5 ml. The adsorbancy at 520 nm was immediately read. The curves have been corrected for dilution. This preparation of AzoFd has 3.3 Fe per dimer of AzoFd.

Figure 2: Effect of urea upon iron chelation from AzoFd by α,α'-dipyridyl. All cuvettes contain 0.914 ml of 0.1 M Tris-HCl, pH 7.4 and 1 ml of 10 M urea (●-●-●) or 1 ml of 0.1 M Tris-HCl (0-0-0). Blanks contained identical concentrations of urea or Tris-HCl. 21.3 nmoles of AzoFd in 0.086 ml was added anaerobically to the experimental and control cuvettes. A comparable volume of 0.05 M Tris-HCl, pH 7.4, was added to the blanks. 0.5 ml of 25 mM α,α'-dipyridyl was next added to each cuvette to make the total volume 2.5 ml. The absorbancy at 520 nm was quickly read. Within 20 hr all the iron of AzoFd (3.0 Fe per dimer) had been chelated in the presence of 4 M urea.

that under strictly anaerobic conditions in the presence of dithionite AzoFd reacted extremely slowly with excess α,α'-dipyridyl but on addition of magnesium ATP a dramatic increase in the rate of the reaction occurred. An accurate determination of the order of this reaction must await further investigations, however, under conditions of this experiment the ratio of the time for 3/4 of the reaction to occur to the time for 1/2 of the reaction to occur is 2.8. This suggests that the order of the reaction is more than first order[14].

Under the conditions specified 80% of the total iron has reacted within an hour. All the iron reacted within 20 hours. This does not show that all the iron is in the ferrous state, since as the structure of AzoFd is disrupted, dithionite could reduce any ferric iron. In the control experiment without magnesium ATP only 11% of the total iron has reacted after 20 hours and 45% of that occurs within the first minute. The initial burst is probably due to a small fraction of AzoFd which has had its structure disrupted by oxidation during some point in the purification procedure.

The above results have been repeated several times and are reproducible with no more than 10% deviation.

It was necessary to determine if the effect of magnesium ATP on AzoFd in the presence of α,α'-dipyridyl was specific. Other nucleotides as well as ATP and magnesium alone were tested. They included the magnesium salts of ADP,

Figure 3: Effect of oxidation of AzoFd on the chelation of its iron by α,α'-dipyridyl. 23.3 nmoles of AzoFd in 0.1 ml was added anaerobically to the experimental and control cuvettes which contained 2.0 ml of 0.1 M Tris-HCl, pH 7.4. No extra dithionite was added. The blank cuvette contained a comparable volume with 0.1 ml of 0.05 M Tris-HCl, pH 7.4 added instead of protein. The absorbancy at 440 nm was monitored and the protein was observed to be in the fully reduced state. The experimental cuvette was gently agitated to disolve the small amount of oxygen present in argon into the solution. This process was repeated until the absorb ancy at 440 indicated that the protein was 100% oxidized. Next 0.5 ml of 25 mM α,α'-dipyridyl with no dithionite was quickly added to make the total volume 2.6 ml. The absorbancy at 520 nm was immediately read (●-●-●). The α,α'-dipyridyl added to the control (0-0-0) which was not oxidized, contained 1 mM dithionite. This AzoFd preparation had 3.0 Fe per dimer of AzoFd.

AMP, GTP, CTP, and UTP. After a reaction time of one hour the results were compared and found to be less than 13% of the effect produced by magnesium ATP under identical conditions.

It was of interest to determine if urea, a protein denaturant, and oxygen which can irreversibly inactivate AzoFd, could disrupt the α,α'-dipyridyl resistant structure of AzoFd. In the presence of 4 M urea (Fig. 2) α,α'-dipyridyl immediately reacts with about 22% of the total iron and then reacts with the remaining iron at a slower rate. The kinetics of iron chelation are hyperbolic when AzoFd is first oxidized 100% as described in the legend for Fig. 3. After 3 hours the reaction is virtually complete with about 80% of the iron reacted as ferrous iron.

DISCUSSION

An ATP-induced change in the availability of the iron of AzoFd to the chelator, α,α'-dipyridyl is evidence that a conformational change has occured. Magnesium ATP apparently causes AzoFd to change in conformation so that its Fe-S center(s) are exposed. Alternatively, the binding of ATP might alter the position of hydrophobic groups on AzoFd, making them more vulnerable to disruption by α,α'-dipyridyl, which in effect would make the iron more accessible.

The reaction of 4 M urea with AzoFd in the presence of α,α'-dipyridyl suggests that the Fe-S center(s) of AzoFd are hidden within hydrophobic pockets when the protein is in its reduced state. The Fe-S clusters within other iron sulfur protein such as bacterial ferredoxin and high potential iron protein are surrounded with nonpolar amino acid side chains[15]; therefore, it is reasonable to make this assumption with AzoFd.

The results of the oxidation of azoferredoxin followed immediately by reaction with α,α'-dipyridyl suggest that most of the iron is in the ferrous state but an interpretation of these results is complicated by the possibility that on disruption of AzoFd its iron may be reduced by liberated sulfide and exposed sulfhydryl groups, as suspected with other iron sulfur proteins[16].

However, these results do show that the conformation of oxidized AzoFd is different from that of reduced AzoFd which in turn is different from that of reduced AzoFd in the presence of magnesium ATP.

Unique use of the ferrous iron chelator, α,α'-dipyridyl, has been employed to demonstrate the specific, nonenzymatic effect of magnesium ATP on AzoFd as well as to provide insight into the structural integrity of this protein. Other experiments are in progress to more fully characterize the effect magnesium ATP induces on AzoFd.

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