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ELECTRON PARAMAGNETIC RESONANCE STUDIES ON NITROGENASE

II. INTERACTION OF ADENOSINE 5'-TRIPHOSPHATE WITH AZOFERREDOXIN*

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SUMMARY

The interaction of ATP with both iron–sulfur proteins of nitrogenase from *Clostridium pasteurianum*, azoferredoxin and molybdoferredoxin, has been studied by low-temperature EPR spectroscopy. ATP in the presence of Mg^{2+} changes the rhombic EPR signal of azoferredoxin with g-values of 2.06, 1.94 and 1.87 to an axial signal, with g values of 2.04 and 1.93. The binding of two molecules of ATP per azoferredoxin dimer (mol. wt 55000) is suggested. Comparative data with other purine and pyrimidine nucleotides and ATP analogues demonstrate the involvement of structural elements of the substrate in the conversion of the EPR signal of azoferredoxin. A similar effect is induced by 5 M urea, which suggests that ATP causes a conformation change of the protein. In contrast, no effect of ATP was observed on the EPR signal of molybdoferredoxin.

INTRODUCTION

Nitrogenase-catalyzed electron transport from a reductant to dinitrogen depends upon the hydrolysis of ATP^{1-3} and both components of the enzyme, azoferredoxin (iron protein) and molybdoferredoxin (molybdenum–iron protein) are required[4]. Several suggestions have been made to explain the energy requirement for the reduction of dinitrogen to ammonia. Mortenson[2] proposed an activation of reduced nitrogenase by ATP which subsequently would reduce dinitrogen directly. The Du Pont group[5,6] postulated an electron activation by ATP to form a metal hydride with a molybdenum phosphate or molybdenum–ATP intermediary complex. It was suggested that this complex reduced dinitrogen at a site containing iron and molybdenum. Other possibilities discussed were an ATP-dependent formation of hydrated electrons[7] and ATP as a proton source (H. Brintzinger, in ref. 8). Bulen *et al.*[9] proposed a model for nitrogenase in which ATP induced a conformation

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change of the protein at the substrate binding site. Such a change of the enzyme should lead to a stretching and consequently facilitate reduction of the nitrogen molecule. Most of the above mentioned hypotheses, although unproven because of the lack of experimental data, assume that the ATP-interacting site of nitrogenase is close to the substrate (N_2) binding site, presumably the molybdenum–iron protein.

Gel filtration studies by Bui and Mortenson[10] indicated a binding of ATP and ADP to azoferredoxin but not to molybdoferredoxin. As a conclusion the interaction of an ATP–azoferredoxin complex with a N_2–molybdoferredoxin complex was postulated. The validity of the binding of ATP to azoferredoxin was questioned by Biggins and Kelly[11] who found an equal binding of ATP to the iron protein and molybdenum–iron protein of *Klebsiella* by a similar technique to that previously used[10]. No binding at all was demonstrable by equilibrium dialysis[11].

In the present paper, which is part of a series of electron paramagnetic resonance investigations on nitrogenase from *Clostridium pasteurianum*[12,13] we employed EPR as a probe to study qualitatively the interaction of ATP with azoferredoxin and molybdoferredoxin. The results support some previous findings from this laboratory and yield new insight in the mechanism of nitrogenase. A preliminary report has appeared[14].

EXPERIMENTAL

Azoferredoxin and molybdoferredoxin were prepared by a modified method[15] and had specific activities of 2200 and 2300 nmoles acetylene reduced per min per mg protein, respectively. EPR spectra were recorded at 23 °K with a Varian V4500 spectrometer[12]. Molybdoferredoxin solutions contained approx. 40 mg protein/ml and were 0.05 M in Tris–HCl buffer (pH 7.5), 1 mM in $Na_2S_2O_4$ and 0.2 M in KCl. Azoferredoxin with a concentration of 20 to 30 mg protein/ml buffer was 0.25 M with respect to NaCl. Protein concentrations were estimated by the biuret method of Gornall et al.[16]. The protein sample added to the EPR tube had a volume of 0.3 ml and nucleotides were added to make the final concentration 10 mM. Prior to thawing and making additions, the tubes were refilled with deoxygenated argon or nitrogen gas. This precaution is necessary since air is admitted through the serum cap during freezing of the tube and oxidation of the thawed proteins can result. ATP, ADP, AMP, UTP, CTP, GTP and NAD were of the highest available purity (Sigma Chemical Co.) and were dissolved in 1 M Tris–HCl (pH 8.0). α,β-Methylene ATP and β,γ-methylene ATP were purchased from Miles Laboratories. Urea (Schwarz-Mann, ultra pure) was dissolved in 0.05 M Tris–HCl (pH 7.5). A molecular weight of 55000 for azoferredoxin was used for calculations[17].

RESULTS AND DISCUSSION

Effect of ATP on azoferredoxin

The EPR spectrum of dithionite-reduced azoferredoxin (Fig. 1A) approximates a rhombic 1.94-type signal with g values of 2.06, 1.94 and 1.87. A small line is still present at $g=4.3$ (ref. 12). Upon addition of $MgCl_2$ and ATP in a molar ratio of one (Fig. 1B) the spectrum undergoes substantial changes in line shape. However, the integrated intensity still accounts for 0.2 mole of electrons per mole of protein
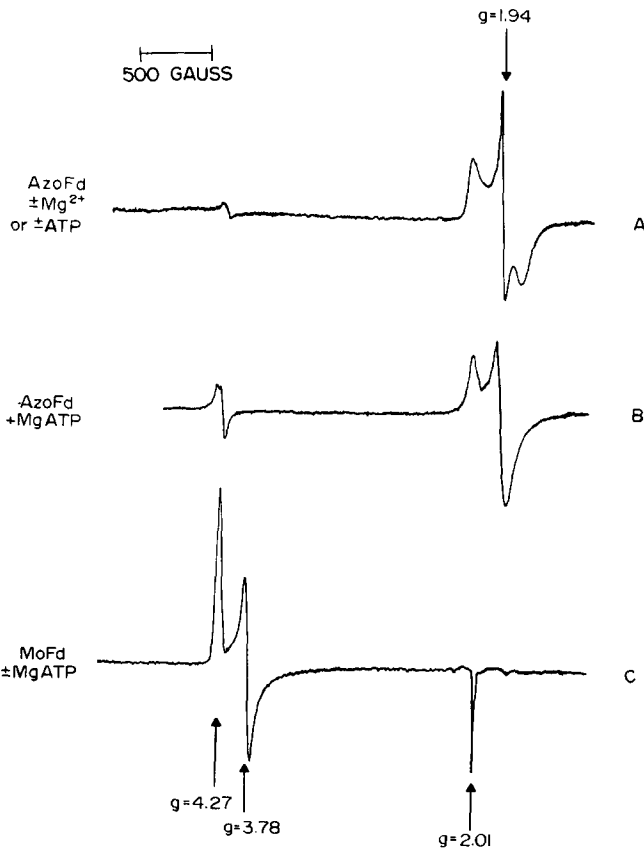
Fig. 1. Effect of ATP on the EPR spectrum of azoferredoxin (AzoFd) and molybdoferredoxin (MoFd). A, azoferredoxin 36 mg/ml in 0.05 M Tris–HCl (pH 7.5), 0.25 M with respect to NaCl and 1 mM in $Na_2S_2O_4$; no change in the spectrum after addition of $MgCl_2$ (10 mM) or ATP (10 mM) alone; B, like A after addition of MgATP; C, molybdoferredoxin 42 mg/ml in buffer containing 0.2 M KCl, spectrum recorded in the presence or absence of MgATP (10 mM). Sweep rate 500 G/min, time constant 0.3 s; gain 50, modulation amplitude 4.5 G, frequency 9.22 GHz, microwave power 3 mW, temperature 23 °K, Varian standard cavity.

of activity 2200, a number identical with that of reduced azoferredoxin without MgATP[*][12]. Despite the rather high specific activity of azoferredoxin the possibility that the preparation contains only 20% of an active electron carrier species has to be seriously considered. After the addition of MgATP the signal of azoferredoxin is nearly axial with g values of 1.93 and 2.04. The signal amplitude has decreased by approx. 22%. The $g = 4.3$ resonance intensity increases but its shape appears to be identical to the untreated control. The effect is specific for the magnesium complex of ATP. The addition of ATP or $MgCl_2$ alone is ineffective (Fig. 1A). The order of addition of ATP and Mg^{2+} (or both simultaneously) to azoferredoxin is without importance. Because oxidized azoferredoxin does not possess an EPR signal[13], these

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[*] Although under the conditions employed the magnesium–ATP complex exists negatively charged ($MgATP^{2-}$) we use throughout the paper the simpler notation (MgATP).

observations are restricted to the reduced form of the protein. A kinetic study of the signal change by means of a rapid quench technique[13] indicates a half time between 10 and 40 ms.

The requirement for magnesium ions in addition to ATP to produce the change in the EPR spectrum of azoferredoxin agrees with the previous finding that Mg^{2+} is required for nitrogenase activity even in the absence of an ATP-generating system[18]. Bui and Mortenson[10] also showed that Mg^{2+} was required for the binding of ATP to azoferredoxin.

Effect of ATP on molybdoferredoxin

The EPR spectrum of molybdoferredoxin remains the same both in the $g=4$ and $g=2$ region after addition of ATP, Mg^{2+} or MgATP (Fig. 1C).

Titration of azoferredoxin with ATP

The amount of MgATP necessary to convert the signal of azoferredoxin to the MgATP form was determined by the addition of increasing quantities of the nucleotide. In order to avoid complications during the thawing and refreezing of the extremely oxygen sensitive protein, each titration point was obtained separately and corrected for dilution. Fig. 2 shows the plot of the decreasing signal height *versus* the relative amount of MgATP added, together with EPR recordings of 3 samples from which the points were obtained. Two intermediary states (B and C) show the successive decrease of the amplitude of the signal(s), the disappearance of the $g=1.87$ resonance and the gradual appearance of the $g=2.04$ resonance. The curve of Fig. 2 levels off at a ratio of 1.9 moles MgATP per mole of azoferredoxin
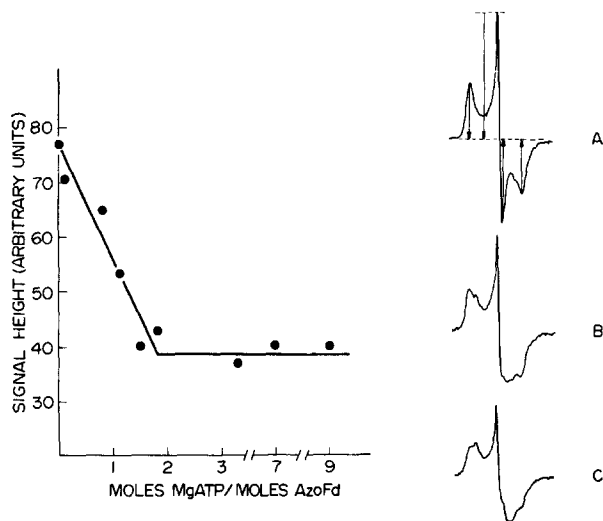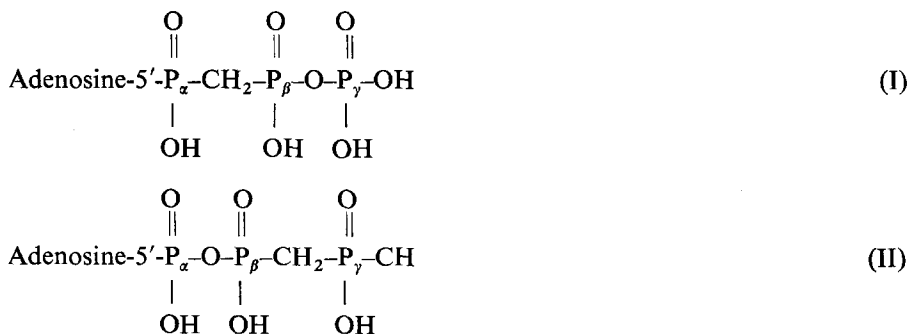

Fig. 2. Titration of the azoferredoxin $g=1.94$ resonance with MgATP. 102 nmoles of azoferredoxin were titrated with a 0.1 M MgATP solution. Each point was obtained with a separate EPR tube and corrected for dilution. A, spectrum of the initial state indicating the type of spectral change; the maximal signal amplitude was used for the measurements; B, state with an ATP/protein molar ratio of 1.0; C, state with an ATP/protein molar ratio of 1.8. For final state see Fig. 1B. Spectrometer settings as in Fig. 1.

(mol. wt 55000). Two separate sets of experiments yielded values of 1.6 and 1.8. It is interesting to compare these values obtained by monitoring a physical property of the protein with previous data of Moustafa and Mortenson[18]. These authors have shown that a Hill plot using initial reaction rates and varying the ATP concentration from 2 to 10 mM, gave a slope of 1.9. This number can be interpreted as the number of substrate binding sites and agrees with the EPR titration which indicates the binding of two molecules of MgATP per azoferredoxin.

Specificity of the signal change

We have investigated the specificity of the effect of MgATP on azoferredoxin. The nucleotides AMP, CTP, UTP, and NAD, either in the presence or absence of Mg^{2+} do not produce any change in the EPR spectrum of azoferredoxin (Fig. 3A). Addition of inorganic phosphate or pyrophosphate is equally ineffective. Guanosine triphosphate (Fig. 3B) caused a decline of approx. 25% of the signal intensity and produced a spectrum somewhat intermediate between the reduced form and the MgATP form of azoferredoxin. However, all these nucleotides are ineffective in the nitrogenase assay[18,19]. The observed weak interaction of GTP with azoferredoxin suggests that GTP might inhibit nitrogenase and corresponding experiments are in progress.

Interestingly MgADP produces the same change in the EPR spectrum as MgATP (Fig. 3C) whereas ADP alone is ineffective. We also have tested the effect of two ATP analogues, α,β-methylene ATP (I) and β,γ-methylene ATP (II) on the EPR spectrum of azoferredoxin.

$$
\begin{array}{ccc}
\text{O} & \text{O} & \text{O} \\
\parallel & \parallel & \parallel
\end{array}
$$

$$\text{Adenosine-5'-}P_\alpha\text{-CH}_2\text{-}P_\beta\text{-O-}P_\gamma\text{-OH} \qquad\qquad (I)$$

$$
\begin{array}{ccc}
\mid & \mid & \mid \\
\text{OH} & \text{OH} & \text{OH}
\end{array}
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$$
\begin{array}{ccc}
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\parallel & \parallel & \parallel
\end{array}
$$

$$\text{Adenosine-5'-}P_\alpha\text{-O-}P_\beta\text{-CH}_2\text{-}P_\gamma\text{-CH} \qquad\qquad (II)$$

$$
\begin{array}{ccc}
\mid & \mid & \mid \\
\text{OH} & \text{OH} & \text{OH}
\end{array}
$$

Addition of either analogue (I, II) caused no signal change in the $g=2$ region but yields a pronounced split signal at $g=4.3$ (Fig. 4B, cf. also Fig. 1B). Upon addition of magnesium ions, changes identical to those caused by MgATP or MgADP were observed only with Compound II but not with Compound I (Fig. 4C).

That structural features of ATP, ADP and analogue (II) cause these signal changes is obvious. Common to the effective compounds are the purine and pentose rings and the α,β-anhydridic diphospho bond. These elements need to be in a proper spacial arrangement which probably explains the requirement of Mg^{2+}. The ATP and ADP molecules have an intrinsic tendency to fold the phosphate chain towards the adenine ring[20,21]. Mg^{2+} binds either to the β- and γ-phosphates of ATP or the α- and β-phosphates of ADP[22], but there is no direct metal ion–adenine ring interaction[22–24]. However, recently the formation of an outer-sphere type metal–ATP
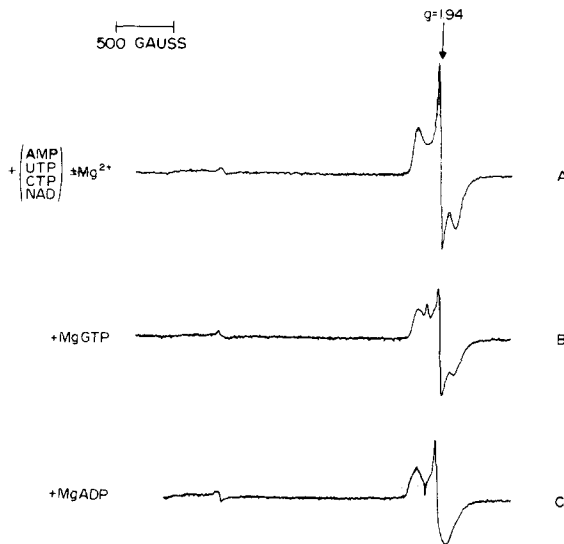
Fig. 3. Effect of various purine and pyrimidine nucleotides on the EPR spectrum of azoferredoxin Azoferredoxin concentration 25.4 mg/ml; final nucleotide concentration 10 mM. A, control or after addition of AMP, UTP, CTP or NAD in the presence or absence of Mg^{2+}; B, after addition of MgGTP; C, after addition of Mg^{2+} and ADP. Gain 100, other settings as in Fig. 1.

complex has been demonstrated for manganese–ATP[24]. It is likely that this is also true for the MgATP complex. We assume that such a structure is necessary for a nucleotide to interact with azoferredoxin. In the case of AMP and NAD no complex formation would be possible. Guanosine triphosphate, whose structure is close to that of adenosine triphosphate shows a weak interaction with azoferredoxin. This behaviour most likely can be explained by substitution of the amino group in position 6 by oxygen and introduction of an amino group in position 2 which alters the affinity of the purine ring to its binding site on azoferredoxin and/or changes the folding of the phosphate chain. The terminal phosphate group with its high bond energy is not required for the conversion of the signal since MgADP and the β,γ-methylene MgATP also cause the effect. For the α,β-methylene ATP, the P_α–C–P_β bond angle because it is smaller than the P_α–O–P_β bond angle may prevent proper folding of the molecule, which then is excluded from the binding site at the protein.

Although most of the interpretation assumes the interaction of a metal ion–ATP complex with the protein, the experiments described here have not been designed to rule out the formation of a metal–protein complex[25] which might be the active form and specifically bind certain nucleotides.

Effect of urea

A possible explanation of what causes the change in the EPR spectrum of azoferredoxin comes from an observation of the effect of protein denaturing agents. Figs. 5A and 5B show the EPR spectra of molybdoferredoxin and azoferredoxin in the presence of 5 M and 0.5 M urea, respectively. As one would expect the signal of molybdoferredoxin disappears because of unfolding of the protein chain and distortion of the iron–sulfur centre(s). The signal of azoferredoxin even disappears
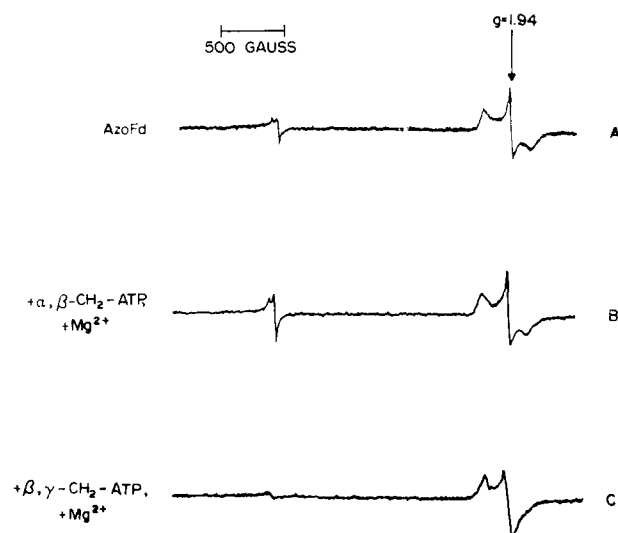
Fig. 4. Effect of ATP analogues on the EPR spectrum of azoferredoxin. Azoferredoxin concentration 25.4 mg/ml, final nucleotide concentration 1.65 mM; A, azoferredoxin without addition; B, azoferredoxin *plus* α,β-methylene ATP with or without magnesium or also *plus* β,γ-methylene ATP without magnesium; C, azoferredoxin *plus* magnesium salt of β,γ-methylene ATP. Spectrometer settings as in Fig. 1.
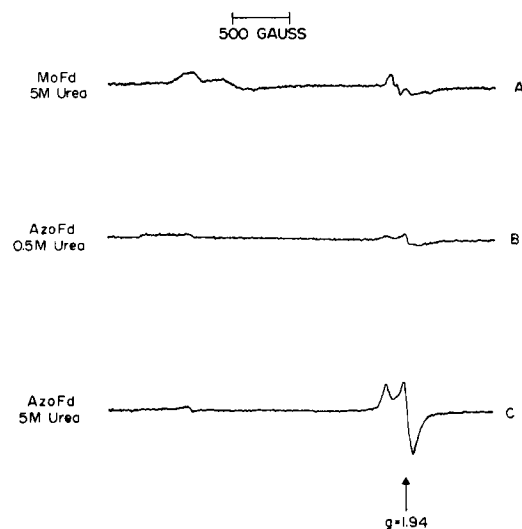

Fig. 5. EPR spectra of molybdoferredoxin and azoferredoxin in the presence of urea. A, 31 mg/ml molybdoferredoxin in 5 M urea; B, 10 mg/ml azoferredoxin in 0.5 M urea; 10 mg/ml azoferredoxin in 5 M urea. NaCl concentration in A, 0.1 M; in B and C, 0.125 M. Spectrometer settings as in Fig. 1.

at 0.5 M urea. Surprisingly however, in the presence of 5 M urea the EPR spectrum of azoferredoxin is virtually the same as in the presence of MgATP (Fig. 5C). An intriguing explanation for this phenomenon is that azoferredoxin in 5 M urea undergoes a conformation change which leads to a different arrangement of the iron–

sulfur centre(s) and which is demonstrable by the change in the EPR spectrum. A similar or identical conformation change of the protein would then have to be assumed for the effects of MgATP, MgADP and the magnesium salt of β,γ-methylene ATP.

At the moment it is not clear what causes the differential effect between the final urea concentration of 0.5 and 5 M. Investigations with spinach ferredoxin[26] emphasized the importance of the ionic strength of the solvent in such experiments. Therefore it is likely that the 0.125 M NaCl present with azoferredoxin cannot be disregarded and more systematic investigations are in progress.

The results presented here show that MgATP interacts with azoferredoxin and suggest that it does not interact with molybdoferredoxin. It is generally assumed that the dinitrogen binding site is on the molybdenum–iron protein. However, in contrast to the majority of the nitrogenase reaction models a direct involvement of MgATP at a substrate binding site on molybdoferredoxin is unlikely. The signal change of the EPR spectrum of azoferredoxin is induced by ATP, ADP and the β,γ-methylene ATP and requires in each case Mg^{2+}. Interestingly both the activator (ATP) and inhibitor (ADP) of the nitrogenase reaction have the same effect on the EPR spectrum of the protein.

We propose that this effect is a conformational change in analogy to the EPR spectrum of azoferredoxin in the presence of urea. Measurements of the ultraviolet absorption and circular dichroism of azoferredoxin should yield more evidence for this assumption. It is possible that the site(s) on azoferredoxin that interacts with ATP or ADP is regulatory or allosteric. There is no doubt that the conversion of the EPR signal of azoferredoxin is physiologically significant because the same observation was made with a catalytically active nitrogenase using either protons or acetylene as substrate[13].

ACKNOWLEDGEMENTS

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