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ELECTRON PARAMAGNETIC RESONANCE STUDIES ON NITROGENASE

III. FUNCTION OF MAGNESIUM ADENOSINE 5'-TRIPHOSPHATE AND ADENOSINE 5'-DIPHOSPHATE IN CATALYSIS BY NITROGENASE

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SUMMARY

The electron paramagnetic resonance spectra of azoferredoxin and molybdoferredoxin, components of the nitrogenase of *Clostridium pasteurianum*, disappear when the proteins are oxidized by certain dyes. When molybdoferredoxin and azoferredoxin were mixed in a 1 to 2 molar ratio, the electron paramagnetic resonance spectrum of the mixture was the sum of the two spectra with the exception of a slight change in the azoferredoxin signal. Addition of magnesium ATP and dithionite to this reconstituted nitrogenase resulted in a rapid change in the spectrum of both nitrogenase components; the molybdoferredoxin spectrum at all *g*-values decreased with a half-life less than 70 ms to 40% of its original size whereas the azoferredoxin signal changed in shape and size with a half-life of less than 40 ms. If an ATP-generating system was added instead of MgATP so that no ADP accumulated, then the molybdoferredoxin signal almost completely disappeared and the azoferredoxin signal changed in shape and slightly in size. These changes occurred at molar ratios of molybdoferredoxin to azoferredoxin from 1:14 to 1:0.2. If the reaction was allowed to consume the reductant, then the molybdoferredoxin signal(s) was restored but the azoferredoxin signal disappeared. The signal of azoferredoxin was restored and the signal of molybdoferredoxin again disappeared on addition of more reductant. The data suggest that for nitrogenase to catalyze the reduction of substrates, the magnesium ATP-reduced azoferredoxin complex is formed first and this complex then reacts with molybdoferredoxin to allow electron flow. In addition the data suggests that the rate-limiting reaction is an ATP-mediated electron flow from azoferredoxin to molybdoferredoxin. Finally the results show that no flow of electrons from azoferredoxin or molybdoferredoxin occurs when a mixture of ADP and ATP in a molar ratio of 2:1 is added initially or is reached by conversion of ATP to ADP and inorganic phosphate during reduction of protons. A mechanism consistent with these findings is proposed.

INTRODUCTION

At the present state of our understanding, nitrogenase of *Clostridium pasteurianum* consists of two dissociating components with a minimal composition of a dimer of molybdoferredoxin (MoFe protein) and a dimer of azoferredoxin (Fe protein). The dimer of molybdoferredoxin contains two subunits of molecular weights of 50700 and 59500 and the dimer of azoferredoxin two similar subunits of 27500 each[1–3]. The minimal molecular weight of the total complex would be 165200. It has not yet been rigorously shown that this minimal unit is a functioning one and a multiple of this unit of 330400 molecular weight may be the true nitrogenase. The most pure nitrogenase components have activity in the acetylene reduction assay of 2600 when based on molybdoferredoxin and 2700–3100 when based on azoferredoxin[1,4,5]. The molybdenum content[1] of this highly purified molybdoferredoxin is one per dimer (110000 mol. wt).

Previous publications[6,7] have shown that both azoferredoxin and molybdoferredoxin of the nitrogenase of *C. pasteurianum* have characteristic electron paramagnetic resonance spectra. In addition it was shown[7] that azoferredoxin complexed with MgATP* to produce a distinct and reproducible change in its EPR spectrum. Magnesium ADP also complexed with azoferredoxin to give a change identical to that of MgATP but no other purine and pyrimidine nucleotides with the exception of an ATP analogue produced such a change[7]. Magnesium ATP or ADP had no effect on the EPR spectrum of molybdoferredoxin. These results agree with the results of the binding studies of Bui and Mortenson[8] in which it was shown that MgATP and ADP bind to azoferredoxin but not to molybdoferredoxin.

Since it is essential in studies of catalysis by nitrogenase to know how ATP functions and to know how electrons are transferred in the process, we have investigated these functions by observing changes in the EPR spectra of reconstituted nitrogenase upon adding the components required for N_2 fixation. In addition we have investigated the changes in spectra obtained by oxidation of the individual components with dyes and of the reconstituted nitrogenase components by physiological oxidation.

EXPERIMENTAL

The nitrogenase components of *C. pasteurianum* were purified by previously described procedures[1,9,10]. Molybdoferredoxin and azoferredoxin with activities of 2300 nmoles and 2200 nmoles acetylene reduced per min per mg protein, respectively, were used. Azoferredoxin and molybdoferredoxin with activities as high as 2500 and 3100 have recently been reported by Tso *et al.*[5]. We have obtained azoferredoxin with activities of 2700 (ref. 4) and molybdoferredoxin of 2600 (ref. 1). All proteins used were homogeneous[1]; however, because of the lability of the proteins, some inactive species could have been present. Based on our experience this is more likely for azoferredoxin than for molybdoferredoxin.

EPR spectra were recorded at about 20 °K on a Varian V4500 spectrometer

* MgATP, the species where equimolar concentrations of magnesium and ATP are mixed at pH 7.5.

modified to allow a greater dynamic range for microwave power[6]. Samples were prepared as previously described[6,7]. The stop freeze experiment was performed in a substantially modified version of the Bray apparatus[11]. Nitrogenase with a 1:2 molar ratio of molybdoferredoxin to azoferredoxin was used in these studies so we could see maximum change of the azoferredoxin EPR signals (the highest specific activity of azoferredoxin is seen at ratios close to 1:2). Large changes of the molybdoferredoxin EPR spectrum also occur within 20 s at molar ratios of molybdoferredoxin to azoferredoxin from 1:14 to 1:0.2. Nitrogenase was placed in one syringe and MgATP or a MgATP/ADP mixture was placed in a second syringe. 10 mM sodium dithionite was added to the nitrogenase mixture and 1 mM to the MgATP solution or to the MgATP/ADP mixture. Approx. 0.25-ml portions of nitrogenase and 0.05 ml of MgATP were shot at times ranging from 10 to 1050 ms through a mixing chamber into isopentane at 130 °K (*cf.* ref. 11). The frozen nitrogenase–magnesium ATP reaction mixture was then carefully packed into an EPR tube and the spectra recorded.

All chemicals, Tris, $MgCl_2$, sodium dithionite, ATP, ADP, creatine phosphate, creatine kinase (EC 2.7.3.2) and dyes were of the best quality available. The pH of all solutions (including the enzymes) was adjusted to 7.5 and 0.05 M Tris buffer was present with all solutions. For other experimental details see previous papers[6,7].

RESULTS AND DISCUSSION

Effect of magnesium ATP on the EPR spectrum of nitrogenase

Since we have already established the individual EPR spectra of azoferredoxin and molybdoferredoxin[6,7], we had to establish whether the two components when mixed had an additive spectrum or interacted in such a way that their EPR spectra were not additive. The results can be seen in Fig. 1 by comparing A and C with D. There was a slight effect of the combination of molybdoferredoxin with azoferredoxin on the "doublet" of azoferredoxin at the high field range (lowest g-value), otherwise the two spectra are additive and this spectrum now will be referred to as the EPR spectrum of nitrogenase. The narrow "spike" of molybdoferredoxin at $g=2.01$ does not obscure changes in the azoferredoxin signal at the $g=1.94$ range. We also recorded the EPR spectra of the additional controls, azoferredoxin *plus* Mg^{2+}, azoferredoxin *plus* ATP, azoferredoxin *plus* MgATP and molybdoferredoxin *plus* MgATP (Figs 1A, 1B, 1C). These spectra were discussed in the previous paper of this series[7].

We examined the effect of MgATP on the EPR spectra of nitrogenase and observed a large change particularly in the magnitude of the molybdoferredoxin signal(s)) (compare Fig. 1D with 1E). After incubation for less than 1 min, the signal(s) of the molybdoferredoxin at all g-values decreased to 40% of its original amplitude. The contents of the EPR tube were then thawed and after 30 min incubation at 22 °C to convert the ATP to an ATP/ADP ratio of 1:2, the contents of the tube were refrozen and the spectrum was again recorded (Fig. 1F). Now the amplitude of the EPR signal(s) of molybdoferredoxin returned to that of the original state. The amplitude of the azoferredoxin spectrum decreased probably as a result of some denaturation caused by repetitive freezing and thawing. When

more MgATP was added to this nitrogenase, the molybdoferredoxin signal(s) at all *g*-values again decreased (Fig. 1G).

If instead of adding MgATP an ATP-generating system was added, the signals

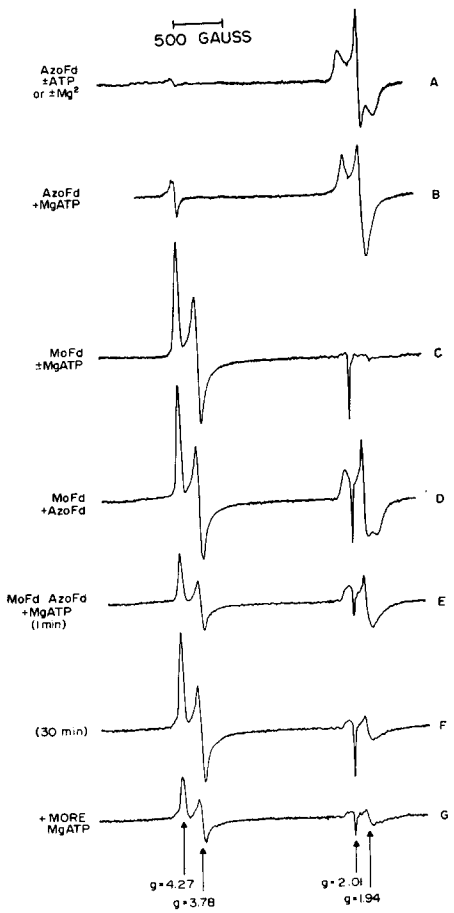

Fig. 1. Effect of MgATP on the EPR spectra of nitrogenase and its components. A. The EPR tube contained 0.3 ml of azoferredoxin (AzoFd) (36.0 mg protein/ml). Similar tubes of azoferredoxin made 10 mM in Mg^{2+} or 10 mM in ATP gave the same spectrum. B. A similar tube of azoferredoxin made 10 mM in MgATP. The amplitude should be increased by a factor of 1.1 to correct for dilution. C. The tube contained 0.3 ml molybdoferredoxin (MoFd) (36 mg/ml). A similar tube with 10 mM MgATP added gave the same spectrum. D. This tube contained 0.3 ml molybdoferredoxin and 0.15 ml azoferredoxin. E. This tube was similar to tube D but 10 mM MgATP also was added. It was allowed to react for 1 min and then frozen and recorded. The amplitude should be increased by a factor of 1.07 to correct for dilution. F. This tube is the same as tube E except that tube E was made anaerobic again, thawed, mixed and incubated for 30 min before recording. Dithionite was still present. G. This tube is tube F made anaerobic again, thawed, and an additional 30 μl of 0.1 M MgATP added. The contents were mixed for one min, frozen and the EPR spectrum recorded. The amplitude should be increased by a factor of 1.14 to correct for dilution. The settings of the instrument were: gain 50; sweep rate 1000 G/min; time constant 0.3 s; frequency 9.22 GHz. In A through C modulation amplitude was 4.5 G and in D through G it was 8.9G. Temperature was 23 °K; microwave power 3 mW; Varian standard cavity assembly.

of molybdoferredoxin decreased even further, *i.e.* they almost completely disappeared in less than the 20 s necessary to mix the tube and freeze it for measurement. Within this time limit the same result was obtained with ratios of azoferredoxin to molybdoferredoxin varying from 1:14 to 1:0.2. Thus all the molybdoferredoxin was converted to a form that had no EPR signal even at widely different ratios of molybdoferredoxin to azoferredoxin. The optimum ratio of molybdoferredoxin to azoferredoxin required for maximum substrate reduction based on molybdoferredoxin is greater than 1 to 4 (based on azoferredoxin, it is about 1 to 2).

This suggests that even though electrons flow from azoferredoxin to molybdoferredoxin and the electron flow requires the utilization of ATP, only that reduced molybdoferredoxin bound to azoferredoxin functions in substrate reduction.

Oxidation of the EPR-positive species of molybdoferredoxin and azoferredoxin

In a previous paper[6] we showed the effects of oxidation of molybdoferredoxin and azoferredoxin by oxygen and potassium ferricyanide. These oxidations, unless carried out for a very short time with O_2 or with a low ferricyanide concentration, destroy the activity of the proteins. Such oxidations gave interesting but complicated EPR spectral changes and an explanation of such spectra (not yet available) could yield valuable information on the structure of the metal–sulfur chromophore(s) of these proteins. In the present study, however, information is needed on the final state of the spectra of nitrogenase (or its individual components) when oxidized in such a way that the proteins are not inactivated. Two oxidation–reduction dyes have proven successful for these studies, phenazine methosulfate and methylene blue[12]. In addition a technique was used to observe the physiological oxidation state of nitrogenase, *i.e.* an ATP-generating system (*cf.* Fig. 3) was added to various mixtures of molybdoferredoxin and azoferredoxin and the system was allowed to exhaust only the dithionite. One would expect that in the absence of reductant the final state of nitrogenase would be the oxidized form.

The results of the oxidation of the EPR-positive species of molybdoferredoxin and azoferredoxin with phenazine methosulfate and methylene blue are given in Fig. 2. The signals of both components almost disappeared on addition of these dyes. No additional signals appeared; in particular signals like those observed when molybdoferredoxin was oxidized with O_2 or ferricyanide[6], were not detected. When methylene blue was added a free-radical species of methylene blue was produced so only the spectrum at low field ($g=4$ region) was recorded. Neither of these dyes at the concentrations used inactivated the components. Addition of dithionite to dye-oxidized molybdoferredoxin restored about 50% of its signal but only after a period of about 10 min. In other words it was a very slow reaction and can not be considered a functional part of the reduction mechanism. On the other hand the EPR signals of reduced azoferredoxin are restored immediately when dithionite is added to dye-oxidized azoferredoxin.

Based on a molecular weight of molybdoferredoxin of 110000 (ref. 1) (the dimer) and a previously reported technique of titration of reduced molybdoferredoxin with methylene blue[12] the number of electrons that were removed from molybdoferredoxin was about 2. This was based on complete decolorization of methylene blue and little of the free-radical species was present. Similar titrations with azoferredoxin were much more difficult because of the extreme sensitivity

of the protein[12]. However, based on a molecular weight of 55000 (the dimer) and an activity of 2200 nmoles acetylene reduced per min per mg protein, the number is about 2 (previous experiments were done with azoferredoxin of activity about 900 and indicated about 0.5 to 0.6 electron per dimer). Absolute numbers can not be established until the optimum activity and purity of these components is established.

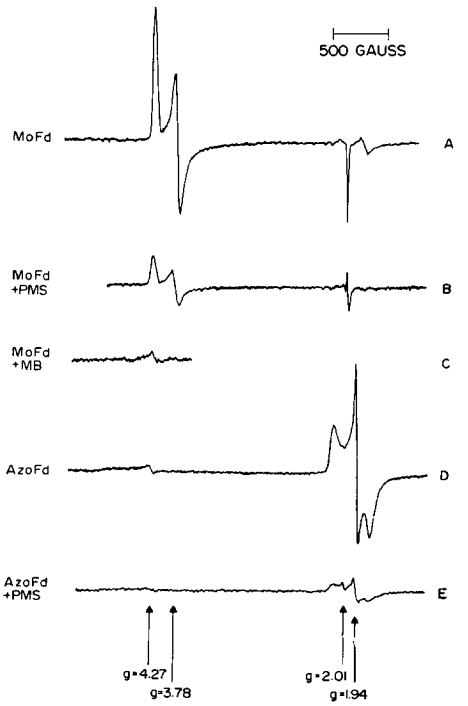

Fig. 2. Oxidation of molybdoferredoxin and azoferredoxin by methylene blue (MB) and phenazine methosulfate (PMS). A. Spectrum of reduced molybdoferredoxin (0.3 ml of 42.4 mg/ml) before oxidation. B. Spectrum of the same molybdoferredoxin after addition of 50 μl of 0.1 M phenazine methosulfate. The amplitude should be increased by a factor of 1.17 to correct for dilution. C. Spectrum ($g=4$ region only) of molybdoferredoxin after addition of 10 μl of 0.1 M methylene blue. D. Spectrum of untreated reduced azoferredoxin (0.3 ml of 36 mg/ml). E. Spectrum of the same azoferredoxin after addition of 10 μl of 0.1 M phenazine methosulfate. The settings of the instrument were as for Figs 1A–1C except that the sweep rate was 500 G/min.

The results of a physiological oxidation of molybdoferredoxin and azoferredoxin are shown in Fig. 3. MgATP and an ATP-generating system were added to 1:14 and 1:2 molar mixture of molybdoferredoxin and azoferredoxin and the reductant (dithionite) present with the proteins was consumed completely in the production of H_2 from protons (ATP dependent H_2 evolution)[13]. The EPR spectra that result showed that molybdoferredoxin was converted almost completely to the EPR-positive species and that the EPR spectrum of azoferredoxin disappeared when the dithionite was consumed (Fig 3B and 3E) like the results with phenazine methosulfate oxidation (Fig. 2E). Addition of $S_2O_4^{2-}$ to the reductant depleted

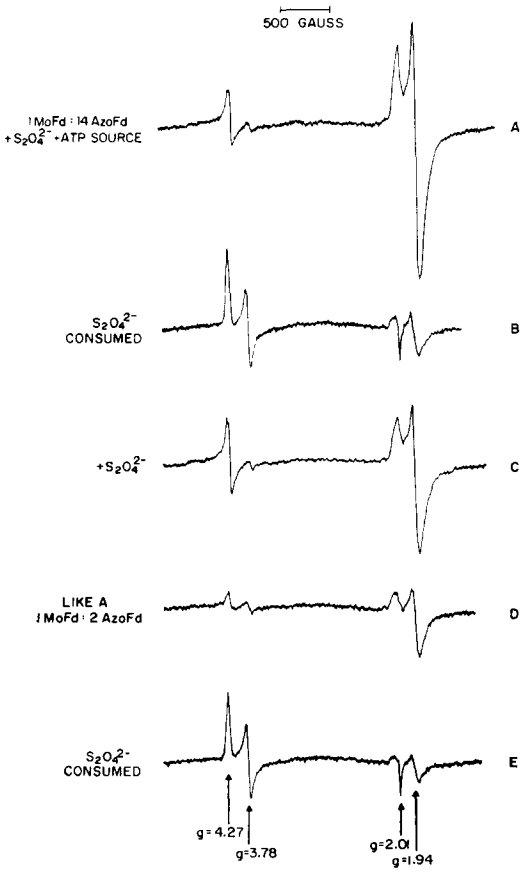

Fig. 3. Physiological oxidation of nitrogenase. A. EPR spectrum of a mixture of 1.1 mg of molybdoferredoxin (35 μl), 3.8 mg of azoferredoxin (100 μl) and 165 μl containing 1.0 mg of creatine kinase (EC 2.7.3.2, 105 units/mg), 4 μmoles of $S_2O_4^{2-}$, 2.5 μmoles of ATP, 30 μmoles of creatine phosphate and 5 μmoles of $MgCl_2$. The total volume was 0.3 ml and the pH was 7.5 buffered by 50 mM Tris. The nitrogenase components were syringed anaerobically into the EPR tube and the reaction was initiated by adding the ATP-generating system. The contents of the tube were mixed thoroughly for 30 s, frozen in liquid N_2 and the spectrum recorded. The signal amplitudes for 35 μl of molybdoferredoxin and 100 μl azoferredoxin in 0.3 ml with no ATP added was the same as that in (A) for azoferredoxin and the same as that in (B) for molybdoferredoxin. The molybdoferredoxin signal is almost completely gone but the azoferredoxin·MgATP signal has not decreased. B. The tube in A was thawed rapidly, mixed, allowed to incubate for 5 min to exhaust the dithionite, frozen in liquid N_2 and the spectrum again recorded. The signals of molybdoferredoxin are restored but the signals of azoferredoxin are now almost completely gone (there is some molybdoferredoxin II present in the molybdoferredoxin[6] that accounts for some of the small residual "1.94" signal). C. The contents of tube B were thawed, 20 μl of 0.1 M $S_2O_4^{2-}$ was added, the contents mixed and frozen within 20 s. The recorded spectrum shows that the molybdoferredoxin signal disappears again and the azoferredoxin signal reappears. D. The same as tube A except that only 20 μl azoferredoxin was added (the ratio of molybdoferredoxin to azoferredoxin is approx. 1:2). Note that again the signal of molybdoferredoxin is almost gone and the signal of azoferredoxin is still present. E. The contents of tube D were treated as tube B. Again note the restoration of the molydboferredoxin signal and the loss of the azoferredoxin signal. The settings for the instrument were: gain 100; sweep rate 500 G/min; time constant 0.3 s; frequency 9.22 GHz; modulation amplitude 10.5 G; temperature was 23 °K.

system, almost completely restored the original state (Fig. 3C). The combined effects of the exhaustion of reductant suggest that electrons flow from azoferredoxin to molybdoferredoxin to substrate. Since the electrons of the species of molybdoferredoxin with the EPR signal, were not used when MgATP and physiologically oxidized azoferredoxin were present (Figs 3B and 3E), one could conclude that MgATP complexed to reduced azoferredoxin is required for their use or that the electrons of this species are not used for substrate reduction. It was reported previously that in the absence of MgATP, the electrons from molybdoferredoxin do not reduce substrate[15,16].

When dithionite was added to the physiologically oxidized nitrogenase described above, the EPR spectrum of azoferredoxin reappeared and the spectrum of molybdoferredoxin decreased to its previous level (compare Fig. 3C with Fig. 3A or 1E).

There are several possibilities for the loss of the molybdoferredoxin EPR signal(s). One is an oxidation (the loss of an electron or electrons) and another is a reduction (the gain of an electron or electrons). The former would be similar to dye oxidation of molybdoferredoxin and the latter could be an MgATP-mediated electron flow that results in electrons being transferred from reduced azoferredoxin to partially reduced molybdoferredoxin (the species with an EPR signal) to produce a fully reduced molybdoferredoxin that is capable of reducing substrate and that has no EPR spectrum. Only the partially reduced molybdoferredoxin would have an EPR signal. If the loss of the EPR signal is by reduction and if only those electrons transferred to molybdoferredoxin from azoferredoxin *via* an ATP-mediated mechanism are used for substrate reduction, one could argue that when reductant is completely consumed, partially reduced molybdoferredoxin and the full EPR signal should be restored. This argument would suggest that the rate limiting reaction is the reduction of substrate. Likewise, when all ATP is consumed and the ratio of ATP/ADP reaches 1:2, all molybdoferredoxin would be in its EPR-positive state and the azoferredoxin would be in its reduced (EPR) state.

Stop freeze measurement of EPR spectral changes on addition of magnesium ATP

Since it was established that characteristic changes in the EPR spectra of molybdoferredoxin and azoferredoxin occur on addition of MgATP, it was of interest to follow the kinetics of these changes to determine which component changed first. The stop freeze technique was used to establish this order. Nitrogenase (1 mole molybdoferredoxin to 2 moles azoferredoxin) was mixed with MgATP for times ranging from 10 to 1050 ms, the samples were frozen rapidly and EPR spectra recorded. The height of the signals of molybdoferredoxin and azoferredoxin were measured and plotted against time. The results (Figs 4, 5) show that the first component to respond, azoferredoxin, reacted with MgATP (Fig. 4B) in a manner similar to that seen in Fig. 1B (loss of "doublet"). After this change (less than 27 ms) the total signal height decreased (Fig. 4C) suggesting a partial oxidation. After this initial change (Figs 4B, 4C and 5) the azoferredoxin EPR signal increased in magnitude. The half time for the initial change of azoferredoxin to its oxidized state is estimated from Fig. 5 to be less than 40 ms.

After azoferredoxin responded, then molybdoferredoxin began to change, *i.e.* its EPR signal(s) decreased and reached maximum loss after about 500 ms. Under

these conditions the estimated half time (based on initial rate) for change of molybdoferredoxin was less than 70 ms. The molybdoferredoxin EPR signals decreased in amplitude about 60%, *i.e.* in all experiments with nitrogenase composed of a molar ratio of 1 molybdoferredoxin to 2 azoferredoxin and with MgATP added the steady state was reached at 40% EPR-positive and 60% EPR-negative species.

Since ATP and not a generating-system was used for this experiment the observed kinetics of the spectral changes are additionally complicated by the accumulation of the inhibitor, ADP, and because of this the half times calculated above were estimated from initial rates. The use of an ATP-generating system in these

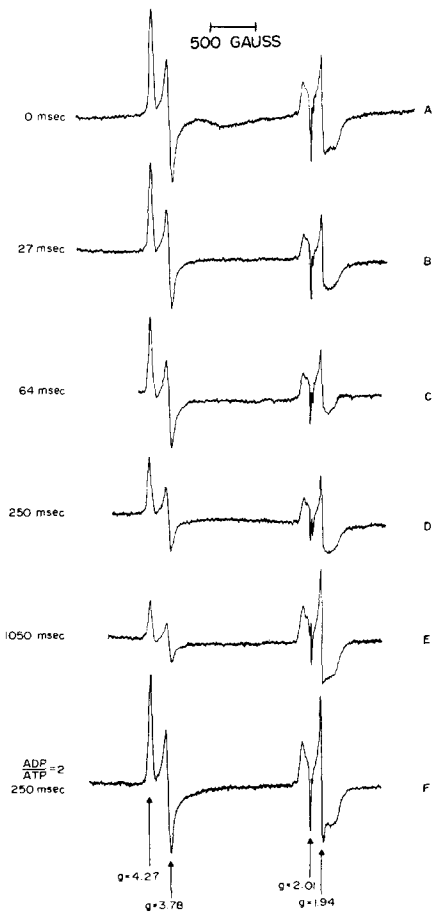

Fig. 4. Stop freeze measurement of the reaction of ATP with nitrogenase. A. A solution containing nitrogenase, 22 nmoles molybdoferredoxin and 44 nmoles azoferredoxin, and 2.5 μmoles $S_2O_4^{2-}$ in 0.25 ml was mixed for 10 ms with 0.05 ml of 0.05 M Tris buffer. The mixture was frozen in liquid N_2 and the EPR spectrum recorded at 23 °K. B. Nitrogenase *plus* MgATP, 27 ms. A nitrogenase mixture like (A) was mixed with 0.5 ml of 6 mM MgATP. C. Same as (B), 64 ms incubation. D. Same as (B), 250 ms incubation. E. Same as (B), 1050 ms incubation. F. Same as (A), *plus* 0.05 ml containing 300 nmoles MgATP and 600 nmoles MgADP, 250 ms incubation. Instrument settings different from Fig. 2 were: gain 100; and modulation amplitude 10.5 G.

stop freeze studies should yield more information and corresponding experiments are in progress. With an ATP-generating system added (Fig. 3) instead of MgATP in manual mixing experiments only traces of the molybdoferredoxin species with the EPR signal were present at the "steady state". This suggests that the ADP produced from ATP during the reaction, accumulated and slowed the reaction rate.

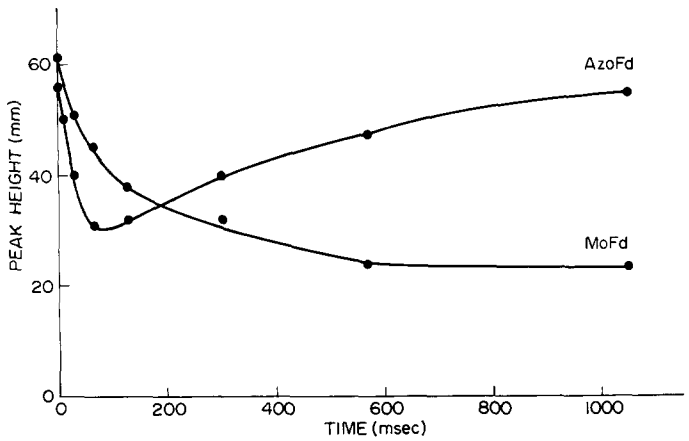

Fig. 5. Time course of the reaction of ATP with nitrogenase measured by EPR changes. The peak heights of the $g=4.27$ component of molybdoferredoxin and the $g=1.94$ component of azoferredoxin were measured and plotted as a function of time. The g-values, 4.27, 3.78 and 2.01, all decreased proportionately. For details see Fig. 4.

One can calculate that with a specific activity of 2600 nmoles acetylene reduced to ethylene per min per mg protein (molybdoferredoxin), 9.5 μmoles of electron paris would turn over per s per μmole of nitrogenase. Under optimal conditions one would expect it to turn over once in about 100 ms. The experimentally measured rate was only 10% of this probably because azoferredoxin was not in excess and ADP accumulated.

Effect of ADP on magnesium ATP-mediated electron transfer from azoferredoxin to molybdoferredoxin

When MgADP and dithionite were added to nitrogenase (a 1:2 molar mixture of molybdoferredoxin and azoferredoxin) a change in the azoferredoxin spectrum was seen similar to the effect of MgATP on azoferredoxin alone (Figs 6A, 6B). When ATP was added to this mixture so that the final ratio was 3 ADP to 4 ATP, a ratio that is about 70% inhibitory to N_2 fixation[14], the large decrease in the molybdoferredoxin signal still occurred (Fig. 6C). If an ADP to ATP ratio of 2:1 was added at zero time (Fig. 4F), a ratio that completely inhibits N_2 fixation[14], and the mixture was allowed to incubate for 250 ms, only a slight change in the azoferredoxin "doublet" occurred.

Adenosine diphosphate accumulated during the functioning of nitrogenase when MgATP without an ATP-generating system was added. When the ratio of ADP to ATP approached 2 and no reduction by nitrogenase occurred, the azoferredoxin EPR signal decreased slightly and the molybdoferredoxin EPR signal(s)

returned to its full amplitude (Fig. 1F). ADP prevented the loss of the signal of molybdoferredoxin. This suggests that electrons can not be transferred from azoferredoxin to molybdoferredoxin unless the MgATP–reduced azoferredoxin complex is available; the ADP–reduced azoferredoxin complex is inactive. This conclusion is supported by the finding that when more ATP was added (Fig. 1G), the molybdoferredoxin signal again decreased.

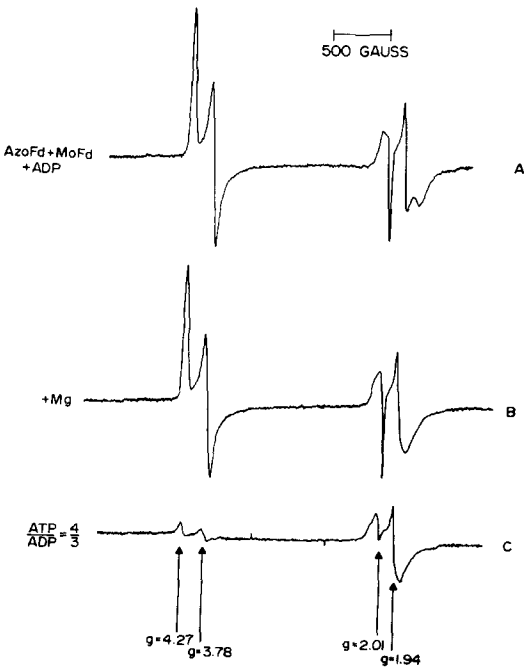

Fig. 6. Effect of ADP on the EPR spectrum of nitrogenase. A. Nitrogenase with a molar ratio of one molybdoferredoxin to 2 azoferredoxin (0.2 ml of a 62 mg/ml solution of molybdoferredoxin mixed with 0.17 ml of a 36 mg/ml solution of azoferredoxin) was mixed with 30 μl of a 0.1 M solution of ADP and frozen. B. The mixture in A was thawed and 30 μl of 0.1 M $MgCl_2$ was added anaerobically. The contents were mixed for 30 s and refrozen in liquid N_2. The amplitude should be increased by a factor of 1.1 to correct for dilution .C. The mixture in B was thawed and 40 μl of a 0.1 M solution of MgATP was added anaerobically so that the ratio of ADP/ATP was 0.75. After mixing for 30 s it was refrozen and the spectrum recorded. The amplitude should be multiplied by a factor of 1.23 to correct for dilution. The settings for the instrument were: gain 40; sweep rate 1000 G/s; time constant 0.3 s; modulation amplitude 8.9 G; temperature 23 °K.

Mechanism

A mechanism for the function of molybdoferredoxin and azoferredoxin in substrate reductions must be consistent with the following observations:

(a) When an ATP-generating system and dithionite are added to mixtures of molybdoferredoxin and azoferredoxin of molar ratios from 1:14 to 1:0.2, in all cases the EPR-positive species of molybdoferredoxin almost completely disappears whereas the EPR species of azoferredoxin decreases little or none. (b) When the above system (a) exhausts the dithionite but not the ATP source, the EPR-positive

species of molybdoferredoxin is completely restored whereas the EPR-positive species of azoferredoxin almost completely disappeared. This suggests that the azoferredoxin in (a) was turning over by an ATP-mediated transfer of electrons to molybdoferredoxin but the oxidized azoferredoxin produced was immediately rereduced by the dithionite. When the dithionite is exhausted, azoferredoxin remains oxidized and can not transfer electrons to molybdoferredoxin. The molybdoferredoxin in turn transfers electrons to substrate and returns to its original EPR-positive state.

(c) When the above system (a) exhausts the ATP but dithionite is still present, the EPR-positive species of molybdoferredoxin again is completely restored and the azoferredoxin is seen primarily in the EPR-positive state. Electrons cannot be transferred from azoferredoxin to molybdoferredoxin without ATP. In addition it is possible that dithionite reduces azoferredoxin more slowly when complexed to ADP.

(d) In the stop-freeze experiment with a 1:2 molar ratio of molybdoferredoxin to azoferredoxin, the EPR-positive species of azoferredoxin first complexed with MgATP and then decreased presumably by an ATP-mediated transfer of its electrons to molybdoferredoxin. Then the molybdoferredoxin EPR-positive species decreased and the azoferredoxin returned to its full EPR-positive state.

(e) In spectrophotometric experiments the absorption of molybdoferredoxin between 300 and 500 nm increased when oxidized by oxygen (short time), phenazine methosulfate or methylene blue[12]. If 3 nmoles of reduced azoferredoxin were added to 11 nmoles of reduced molybdoferredoxin, no change in spectrum occurred. When 36 nmoles of MgATP were added to this mixture, the molybdoferredoxin spectrum changed similar to dye oxidation. In this experiment both reductant (electrons of azoferredoxin and molybdoferredoxin) and ATP were consumed and molybdoferredoxin could not return to its original (reduced) state (Walker, M. and Mortenson, L. E., unpublished).

(f) ADP (ATP/ADP ratio of $^1/_2$) does not allow electrons to flow from molybdoferredoxin to substrate. Thus, when ATP is converted to ADP (no ATP-generating system) azoferredoxin and molybdoferredoxin remain in their EPR-positive states.

(g) Dyes like methylene blue and phenazine methosulfate oxidize both molybdoferredoxin and azoferredoxin to their EPR-negative species.

When the nitrogenase reaction is started with the EPR-positive species of both azoferredoxin and molybdoferredoxin, the mechanism in Fig. 7 is proposed. In Reaction 1 reduced azoferredoxin complexes rapidly with MgATP to activate azoferredoxin. This species then complexes rapidly with reduced molybdoferredoxin to produce a complex, $M \cdot A \cdot 2MgATP$, capable of reducing substrates. In a fast reaction, substrate (in this example, $2H^+$) is reduced (Reaction 3) and oxidized molybdoferredoxin with no EPR signal is produced. This complex then can react in two ways. The first (Reaction 4) is an exchange reaction where either the electrons of reduced molybdoferredoxin not complexed to azoferredoxin, are exchanged with bound M^{2+} or free M exchanges with M^{2+} on the $M^{2+} \cdot A \cdot 2MgATP$ complex. The latter situation only would occur when excess of molybdoferredoxin over azoferredoxin exists. In the second reaction the electrons of azoferredoxin (Reaction 5) are transferred to oxidized molybdoferredoxin. This reaction is the rate-limiting reaction and ATP is converted to ADP and inorganic phosphate. Reactions 6 and

7 involve the exchange of ATP for ADP on the complex, $M \cdot A^{2+} \cdot 2MgADP$, and the reduction of the azoferredoxin of the complex by dithionite. These reactions are fast reactions and the order is suggested by the observation that when ATP is exhausted the EPR-positive species of azoferredoxin is not completely restored.

This mechanism explains all of our findings (Table I) and argues that the rate-limiting reaction is the ATP-mediated transfer of electrons from azoferredoxin to molybdoferredoxin. Other possible mechanisms can be suggested. One could argue, for example, that molybdoferredoxin loses its signal by reduction rather than oxidation. The reduction would require an ATP-mediated transfer of electrons from azoferredoxin to molybdoferredoxin and the product would be the reductant for substrates. The rate-limiting reaction for such a mechanism would be the reduction

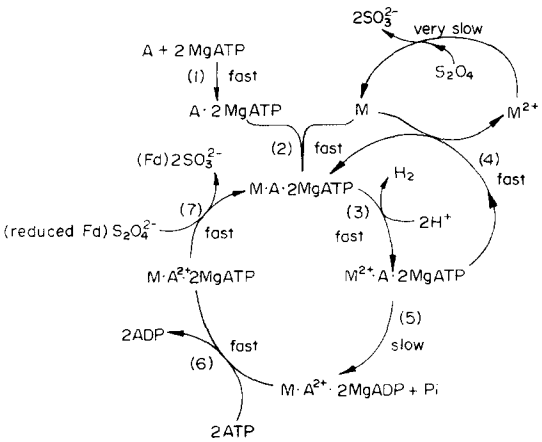

Fig. 7. Proposed mechanism of nitrogenase (where A is the EPR-positive species of azoferredoxin; A^{2+} is the EPR-negative species of azoferredoxin; M is the EPR-positive species of molybdoferredoxin; and M^{2+} is the EPR-negative species of molybdoferredoxin).

TABLE I

Conditions	*Proposed species**	*Spectra present*
1. Excess $S_2O_4^{2-}$, excess ATP	$M^{2+} \cdot A \cdot 2MgATP$ M^{2+} (varies with ratio of components)	EPR of azoferredoxin only
2. $S_2O_4^{2-}$ exhausted, excess ATP (ratios of 1:14 and 1:2, others not tested)	$M \cdot A^{2+} \cdot 2MgADP$ or $M \cdot A^{2+} \cdot 2MgATP$	EPR of molybdoferredoxin only
3. Excess $S_2O_4^{2-}$, ATP exhausted (ratios of 1:14 and 1:2, others not tested)	$M \cdot A \cdot 2MgATP$	EPR of azoferredoxin and molybdoferredoxin
4. $S_2O_4^{2-}$ exhausted, ATP exhausted (1 azoferredoxin to 4 molybdoferredoxin)	$M^{2+} \cdot A^{2+} \cdot 2MgADP$	Visible spectrum, shows most of molybdoferredoxin oxidized, azoferredoxin probably oxidized

* See legend of Fig. 7.

of substrate whereas in Fig. 7 it is the ATP-mediated transfer of electrons from reduced azoferredoxin to oxidized molybdoferredoxin.

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