Redox properties and electron paramagnetic resonance spectroscopy of the transition state complex of *Azotobacter vinelandii* nitrogenase

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Abstract Nitrogenase is a two-component metalloenzyme that catalyzes a MgATP hydrolysis driven reduction of substrates. Aluminum fluoride plus MgADP inhibits nitrogenase by stabilizing an intermediate of the on-enzyme MgATP hydrolysis reaction. We report here the redox properties and electron paramagnetic resonance (EPR) signals of the aluminum fluoride-MgADP stabilized nitrogenase complex of *Azotobacter vinelandii*. Complex formation lowers the midpoint potential of the [4Fe-4S] cluster in the Fe protein. Also, the two-electron reaction of the unique [8Fe-7S] cluster in the MoFe protein is split in two one-electron reactions both with lower midpoint potentials. Furthermore, a change in spin-state of the two-electron oxidized [8Fe-7S] cluster is observed. The implications of these findings for the mechanism of MgATP hydrolysis driven electron transport within the nitrogenase protein complex are discussed.

Key words: Nitrogenase (*Azotobacter vinelandii*); Transition state complex; Redox potential; Electron paramagnetic resonance

1. Introduction

Nitrogenase (EC 1.18.6.1) catalyzes the biological reduction of N_2 to NH_3. The enzyme complex consists of two dissociable metalloproteins: the molybdenum-iron (MoFe) protein, an $\alpha_2\beta_2$ tetramer which contains two unique [8Fe-7S] clusters (P-cluster) [1], and two molybdenum-iron-sulfur-homocitrate clusters (FeMoco); the Fe protein is a γ_2 dimer that contains a single [4Fe-4S] cluster. Substrate binding, activation, and reduction take place on the MoFe protein, presumably on FeMoco. The Fe protein provides the MoFe protein with the required electrons and couples ATP-hydrolysis to the substrate reduction [2]. A close structural similarity between the Fe protein from *A. vinelandii* and the molecular switch proto-oncogenic *ras* protein p21 [3] has been observed. In addition, nitrogenase and the proto-oncogenic *ras* protein only bind aluminum fluoride (AlF) in combination with the nucleotide diphosphate and their physiological partner (the MoFe protein or the guanosine triphosphatase activating protein, respectively) [4–6]. The structures of both AlF-nucleotide diphosphate stabilized protein-protein complexes have been published [7,8]. The structure around the nucleotide diphosphate and AlF is consistent with the transition state (or intermediate) for the s_n2 on-enzyme A(G)TPase reaction, also proposed for myosin [9] and the heterotrimeric G proteins [10,11]. Several altered Fe proteins, made by site-directed mutagenesis, are catalytically inactive but still form a complex with MoFe protein [12,13]. The altered Fe protein, L127Δ, that resembles the Fe protein in the MgATP-bound conformation without MgATP bound [14], forms a non-dissociable complex with MoFe protein. Recently the midpoint potentials of the three metal-sulfur clusters in L127Δ Fe protein-MoFe protein complex were determined [15]. Since the L127Δ Fe protein is not active in the overall catalysis and it is marginally active in the pre-steady-state electron transfer reaction, it is likely that the L127Δ Fe protein-MoFe protein complex represents a non-physiological conformation of nitrogenase. In the present paper we report on the redox properties and electron paramagnetic resonance (EPR) signals of the putative transition state conformation of the on-enzyme ATPase reaction of wild-type nitrogenase. The mechanistic significance of our data will be discussed.

2. Materials and methods

The *Azotobacter vinelandii* ATCC strain 478 nitrogenase proteins were used to prepare the AlF-MgADP inhibited nitrogenase complex as described by Duyvis et al. [4]. Dye mediated redox titrations and EPR spectroscopy were performed as described earlier [16] with the addition of 2,2′-bipyridinium-*N*,*N*′-di(propylsulfonate) (40 μM) to the mediator mixture. The EPR samples contained 56 μM AlF-MgADP stabilized transition state complex (based on the MoFe protein concentration in the complex) in 50 mM Tris-HCl buffer, pH 8.0.

3. Results and discussion

3.1. Characterization of the redox state of the [4Fe-4S] cluster in the AlF-MgADP stabilized transition state complex

The redox state of the metal centers in the AlF-MgADP stabilized transition state complex were determined by EPR spectroscopy. The [4Fe-4S] cluster of the Fe protein is a one-electron donor that operates between the +1 and +2 oxidation levels. The cluster is diamagnetic in the oxidized state, and is reduced by dithionite in a one-electron process. The E_m of this redox reaction is −473 mV for the Fe protein with MgADP bound or −435 mV for the Fe protein with MgATP bound [17]. Reduced by dithionite, the cluster exhibits EPR-spectra from a physical mixture of $S = 3/2$ and $S = 1/2$ spins [18]. In the purified AlF-MgADP stabilized transition state complex no significant $S = 3/2$ or $S = 1/2$ EPR-signals with g-values characteristic for the Fe protein could be detected at redox potentials between −550 and +100 mV. We also tested reduc-

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Abbreviations: MoFe protein, molybdenum-iron protein of nitrogenase; Fe protein, iron protein of nitrogenase; FeMoco, the iron-molybdenum-sulfur-homocitrate cofactor of nitrogenase; $FeMoco^N$, $FeMoco^{superred}$, $FeMoco^{ox}$, the dithionite reduced, super-reduced and one electron oxidized FeMoco; P-cluster, the [8Fe-7S] cluster of nitrogenase; P^N, P^{semiox}, P^{ox}, the dithionite reduced, one- and two-electron oxidized P-cluster; NHE, normal hydrogen electrode; AlF, all forms of aluminum(III) with an F^-, H_2O and OH^- coordination

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tion of the AlF-MgADP stabilized transition state complex by dithionite and titanium(III) citrate at higher pH values. At pH 10, redox centra with midpoint potentials around −650 mV will be reduced by these reductants [19,20]. None of the measured EPR signals changed intensities when the proteins were incubated with 5 mM dithionite or 1 mM titanium(III) citrate at pH 8, 9 or 10. The very weak $S = 1/2$ signal that is observed (Fig. 4, trace A), is probably caused by a small amount of free Fe protein. All the data suggest that the Fe protein in the AlF-MgADP stabilized transition state complex is diamagnetic, and therefore oxidized, within the potential range of −550 to +100 mV. This is also the case for the [4Fe-4S] cluster in the L127Δ Fe protein. Its potential also shifts to more negative values as a result of binding to MoFe protein [15]. We draw the conclusion that the specific conformation of the transition state complex lowers the midpoint potential of the +1 and +2 oxidation states of the [4Fe-4S] cluster of Fe protein to a value significantly less than −550 mV.

3.2. Characterization of the redox state of FeMoco in the AlF-MgADP stabilized transition state complex

Previous studies have shown that the purified, dithionite-reduced MoFe protein exhibits only one EPR signal: the $S = 3/2$ EPR signal that is assigned to the iron-molybdenum-sulfur-homocitrate cofactor ($FeMoco^{N}$) [21]. Under turnover conditions, e.g. in the presence of excess reduced Fe protein and MgATP, the $S = 3/2$ FeMoco signal almost completely disappears, presumably because of so-called super-reduction of the cofactor: the Fe protein transfers at least one electron to the dithionite-reduced FeMoco ($FeMoco^{superred}$) [22,23]. The dithionite-reduced FeMoco can also be oxidized to a diamagnetic redox state ($FeMoco^{ox}$) [24]. For this one-electron process, we measured a midpoint potential of −42 mV [16]. A similar redox behavior of the EPR signal at $g = 3.7$ present in the AlF-MgADP stabilized transition state complex was observed (see Fig. 1). The calculated midpoint potential ($E_m = -90$ mV) is slightly more negative compared to that of the isolated MoFe protein. Super-reduction of FeMoco was not observed within the given potential range (−550 to +100 mV). In conclusion, redox properties and EPR-spectra of the FeMoco in the AlF-MgADP stabilized transition state complex are not significantly different from those observed for the isolated MoFe protein.

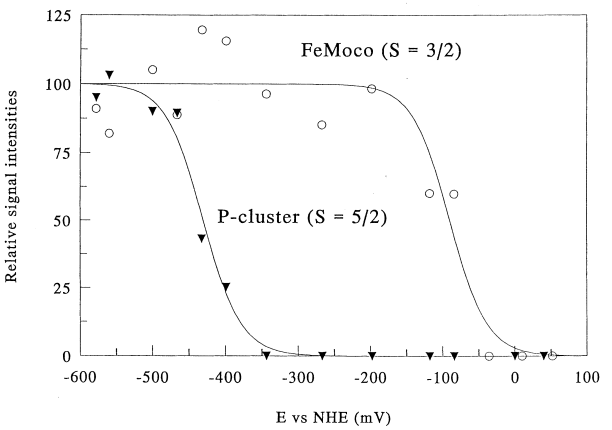

Fig. 1. Redox titration of the AlF-MgADP stabilized transition state complex. The solid lines are least-squares fits to the $n = 1$ Nernst equation with $E_m = -90$ mV for FeMoco ($S = 3/2$) and −430 mV for the P-cluster ($S = 5/2$).

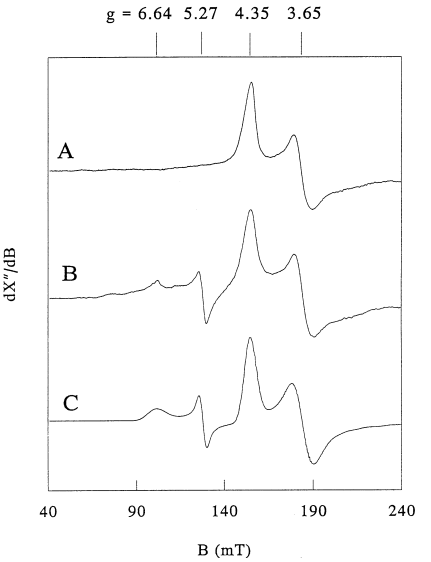

Fig. 2. Low-field EPR spectra of the AlF-MgADP stabilized transition state complex poised at different redox potentials. Trace A: complex poised at −287 mV. Trace B: complex poised at −462 mV. Trace C: simulation of trace B (see Table 1). EPR conditions: microwave frequency, 9.41 GHz; microwave power, 200 mW; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; temperature 23 K.

3.3. Characterization of the redox state of the P-cluster in the AlF-MgADP stabilized transition state complex

Different from FeMoco, the P-cluster is diamagnetic in the presence of dithionite [16,21,25]. This redox state has been proposed by Münck et al. [21] to correspond to the all-ferrous, uncharged cluster, which is therefore designated P^{N}. Upon oxidation of P^{N}, in a two-electron process with $E_m = -307$ mV, an $S = 3$ signal appears in the EPR spectrum [16]. However, the existence of a one-electron oxidized P-cluster, P^{semiox}, has also been shown and it was suggested that this redox state was associated with a physical, i.e. non-interacting, mixture of $S = 5/2$ and $S = 1/2$ signals ([25], A.J. Pierik, personal communication). The midpoint-potential for the abstraction of the first electron, $P^{N} \rightarrow P^{semiox}$, is believed to be close to that for the abstraction of the second electron, $P^{semiox} \rightarrow P^{ox}$. In the transition state complex a signal in the $g = 5$–8 region was found. Detection of the $S = 5/2$ signal is not straightforward because the coefficient D of the zero-field interaction is negative: the $S = 5/2$ multiplet is inverted and the EPR-detected $|\pm 1/2>$ doublet is an excited state. At low temperatures the signals are too weak for detection by depopulation of the $|\pm 1/2>$ doublet; at higher temperatures the signals are broadened by spin-lattice relaxation. Fig. 2, trace B ($E_h = -462$ mV), shows the $S = 5/2$ P-cluster signal at 23 K. At low temperature (4.2 K) the $S = 5/2$ signal disappears and the transition state complex and purified MoFe protein give identical FeMoco $S = 3/2$ spectra (not shown). Fig. 3 shows the dependence of the $S = 5/2$ signal intensity on temperature, both experimental and theoretical. The intensity of the $S = 5/2$ signal at different redox potentials is shown in Fig. 1. The signal appears (oxidation of P^{N} to P^{semiox}) at potentials < -550 mV, out of the range of these experiments. The mid-

Table 1
Parameters used for the simulation of EPR spectra

	Low-field spectra (cf. Fig. 2)		High-field spectra (cf. Fig. 4)	
	$S=3/2$	$S=5/2$	$S=1/2$ (I)	$S=1/2$ (II)
g_x	4.35	6.64	1.808	1.887
g_y	3.65	5.27	1.941	1.887
g_z	2	2	2.016	2.050
Δ_{xx}	0.11	0.4	0.010	0.012
Δ_{yy}	0.11	0.08	0.007	0.012
Δ_{zz}	0.11	0.1	0.009	0.007
Ratio	1	0.14	1	1.00

point potential associated with the disappearance of the $S=5/2$ signal at increasing redox potentials, e.g. the oxidation of P^{semiox} to P^{ox}, is −430 mV. Since in the purified MoFe protein this value is about −310 mV [16], the midpoint potential for the abstraction of the second electron from the P-cluster is lowered in the transition state complex. Surprisingly, P^{ox} in the AlF-MgADP stabilized transition state complex does not exhibit an $S=3$ signal at $g=12$ in parallel-mode EPR as does P^{ox} in the purified MoFe protein [16,25]. The weak $S=3$ signal that was detected at $E_h=-250$ mV may be caused by a small amount of free MoFe protein in the sample (not shown). The P^{ox} in the AlF-MgADP stabilized transition state complex was not detectable; possibly $S=0$. Whatever the spin-state of P^{ox} in the transition state complex may be, it is different from that found for the purified protein. Our data differ significantly from the data reported for the P-cluster in the L127Δ Fe protein-MoFe protein complex. In this complex the $P^{2+/N}$ couple shifts 80 mV, and there is no indication that the redox couples $P^{1+/N}$ separates from the $P^{2+/1+}$ as observed for the AlF-MgADP stabilized transition state complex. The redox behavior of the P-cluster in the L127Δ Fe protein-MoFe protein complex is more like that of the P-cluster in the MoFe protein alone. Apparently, the exchange-coupling is different in the AlF-MgADP stabilized transition state complex compared with MoFe protein alone or complexed with L127Δ Fe protein, which points to a different conformation of the P-cluster. That redox dependent structural changes in the P-cluster do occur has been shown by Peters et al. [1]. They have shown that the ligation of the P-cluster in purified MoFe protein alters when the protein changes from the P^N/$FeMoco^N$ to the P^{ox}/$FeMoco^{ox}$ redox state. This change of ligation was not observed in the crystal structure of the AlF-MgADP stabilized transition state complex [7]. It is therefore apparent that more subtle changes in the environment of the P-cluster are responsible for the observed changes in redox properties.

In the simulation of trace C, Fig. 2, the ratio of $S=3/2$ (FeMoco) over $S=5/2$ (P^{semiox}) is 1:0.14. In Fig. 3 it is seen that the population of the measured doublet is 0.085 at $T=23$ K. For FeMoco the population of the measured doublet is 0.65 at this temperature. This can be calculated from $D\approx 5$ cm^{-1} [21]. Thus for equal amounts of $S=3/2$ (FeMoco) and $S=5/2$ (P^{semiox}), the ratio should be 0.085:0.65 = 0.13. The value of 0.14 found in the simulation is very close to this, so the two signals are essentially stoichiometric, i.e. the $S=5/2$ signal accounts for 100% of the P-clusters in the sample. Consequently, this is not a matter of a physical mixture of $S=5/2$ and $S=1/2$ spins, as was previously observed for the purified MoFe protein [25].

Fig. 4 shows the high field $S=1/2$ spectra from the AlF-MgADP stabilized transition state complex. At low potentials, where the $S=5/2$ signal is maximal only a weak signal from the Fe protein is detected (Fig. 4, trace A, $E_h=-462$ mV). At higher potentials, where the $S=5/2$ signal is absent (Fig. 2, trace A, $E_h=-287$ mV), an $S=1/2$ signal is observed which consists of two $S=1/2$ species present in equal amounts (Fig. 4, trace B, $E_h=-287$ mV). The two signals are labeled I and II, see simulation parameters in Table 1. The intensity of the signal shown in Fig. 4B is sub-stoichiometric, 0.1 spin per FeMoco. One peak (the second one from low field) is de-

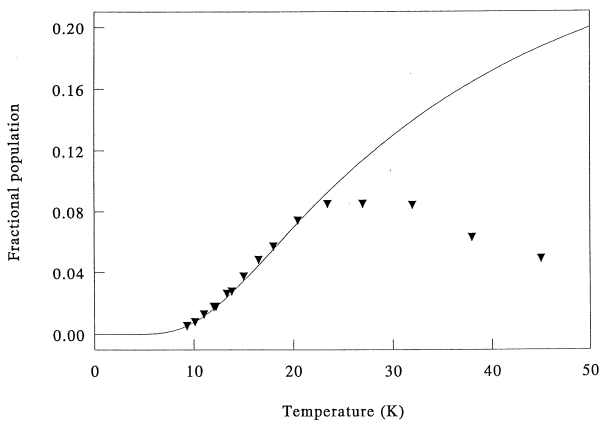

Fig. 3. Thermal (de)population of the highest doublet ($m_S=\pm 1/2$) of the inverted ($D<0$) $S=5/2$ multiplet from the P^{semiox} clusters in the AlF-MgADP stabilized transition state complex. The data points are the amplitude at $g=5.27$ multiplied by the detection temperature (i.e. corrected for Curie-law temperature dependence). The solid line is a fit of the data to a Boltzmann distribution over the sub-levels of an $S=5/2$ system with $D=-8.11$ cm^{-1}. The deviation above $T\approx 25$ K reflects lifetime broadening.

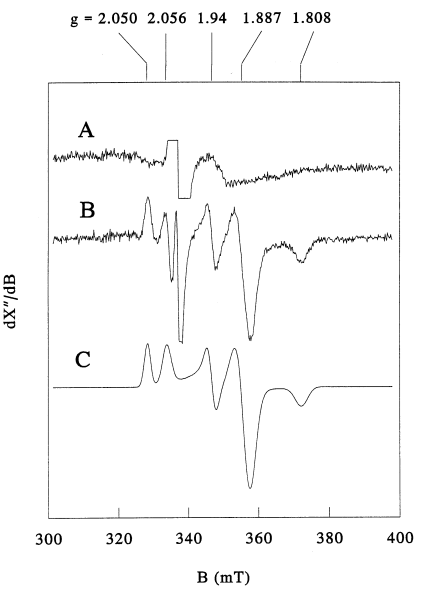

Fig. 4. $S=1/2$ spectra of the AlF-MgADP stabilized transition state complex poised at different redox potentials. Trace A: complex at −462 mV. Trace B: complex at −287 mV. Trace C: simulation of trace B (see Table 1). The (partially) truncated radical signal in traces A and B is from the redox mediators. EPR conditions: microwave frequency, 9.416 GHz; microwave power, 0.8 mW; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; temperature, 13 K.

formed by the radical and the negative g_z peak ($g = 2.0$) of the $S = 3/2$ signal from FeMoco. The $S = 1/2$ and $S = 5/2$ signals therefore do not have a parallel redox behavior, which is in contradiction with what was found previously for the purified MoFe protein [25], but it does agree with the observation above that the $S = 5/2$ signal accounts for all the P-clusters in the sample. The nature of these two sub-stoichiometric $S = 1/2$ signals present in the AlF-MgADP stabilized transition state complex and in the purified MoFe protein, remains to be established.

3.4. Mechanistic implications

Our data suggest a possible mechanism for the electron-transfer from the Fe protein to the FeMoco in the MoFe protein, the putative substrate binding site of nitrogenase. Nitrogenase is fully active at a redox potential of −500 mV [17]. At this potential, the [4Fe-4S] cluster of free Fe protein and the P-cluster of free MoFe protein are both reduced. After complex formation and on-enzyme MgATP hydrolysis, the protein-protein complex changes its conformation to the MgADP-bound conformation. The AlF-MgADP stabilized protein-protein complex is suggested to be part of this reaction coordinate. In this transition state the redox potentials of the [4Fe-4S] cluster of the Fe protein and of the P-cluster of the MoFe protein are lowered and both clusters become one-electron oxidized at the ambient redox potential. It is tempting to suggest that FeMoco is the acceptor of the electrons, but our data show that FeMoco is in its dithionite reduced state and not super-reduced as observed during turn-over conditions. There are several possibilities to explain this. The rate of formation of the AlF-MgADP stabilized transition state complex is relatively slow compared to the turnover of nitrogenase. It is therefore possible that the reducing equivalents present on FeMoco have reacted with protons or with the redox mediators present in the solution. Our data also suggest that despite the fact that Fe protein is a one-electron donor, the combination of Fe protein and the P-cluster can act as a two-electron donor for substrate reduction at FeMoco. When or if the two clusters are re-reduced in the catalytic cycle needs further investigation.

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