Novel EPR signals associated with FeMoco centres of MoFe protein in MgADP-inhibited turnover of nitrogenase

Silvana Maritano, Shirley A. Fairhurst, Robert R. Eady*

Department of Biological Chemistry, John Innes Centre, Colney, Norwich NR4 7UH, UK

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Abstract Two novel electron paramagnetic resonance (EPR) signals arising from the [1Mo–7Fe–9S-homocitrate] (FeMoco) centres of MoFe protein of *Klebsiella pneumoniae* nitrogenase (Kp1) were observed following turnover under MgATP-limited conditions. The combination of the nitrogenase Fe protein of *Clostridium pasteurianum* showed similar signals. The accumulation of MgADP under these conditions causes the normal EPR signal of dithionite-reduced Kp1 (with $g = 4.3, 3.6, 2.01$) to be slowly converted to novel signals with $g = 4.74, 3.32, 2.00$ and $g = 4.58, 3.50, 1.99$. These signals do not form in incubation of protein mixtures containing only MgADP, thus they may be associated with trapped intermediates of the catalytic cycle. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Heterologous nitrogenase; Tight complex; Electron paramagnetic resonance; MgADP-inhibited turnover; *Clostridium pasteurianum*; *Klebsiella pneumoniae*

1. Introduction

Mo-containing nitrogenases are two-component enzyme systems which catalyse the reduction of N_2 to NH_3. They are comprised of two oxygen-sensitive components, an iron-containing (Fe) protein and a molybdenum–iron-containing (MoFe) protein. The MoFe protein contains the catalytic centre, a unique [7Fe–Mo–9S-homocitrate] cluster (FeMoco). Electron transfer from the Fe protein to the MoFe protein, and subsequent reduction of substrates, are coupled to the hydrolysis of MgATP to MgADP [1–4]. It has been shown that MgADP is a potent competitive inhibitor of electron transfer, and a recent X-ray crystal structure study showed that MgADP binds to the nucleotide-binding site of the Fe protein [5].

As isolated, the MoFe protein of *Klebsiella pneumoniae* nitrogenase (Kp1) displays a rhombic $S = 3/2$ electron paramagnetic resonance (EPR) signal ($g = 4.3, 3.6, 2.0$) originating from the dithionite-reduced form of FeMoco. During enzyme turnover, the intensity of this signal is diminished as successive electron transfers from Fe protein occur, and a number of steady-state intermediates of the proposed Lowe–Thorneley scheme for nitrogenase function accumulate [2,3]. The consensus view is that the $S = 3/2$ EPR signal arises only from MoFe proteins in the resting dithionite-reduced state. However, a recent pre-steady-state study of *Azotobacter vinelandii* nitrogenase in turnover, has shown that two transient FeMoco signals with different g values can be assigned to more reduced states of MoFe protein in different protonation states [6]. This report was notable in that it established that species of MoFe protein generated by further reduction of the dithionite-reduced protein could give rise to EPR signals associated with an altered environment of the FeMoco centres.

In the present paper, we show for the first time that when turnover of nitrogenase is inhibited due to the accumulation of MgADP, approximately 50% of the FeMoco centres of Kp1 give rise to a new form of the $S = 3/2$ EPR spectrum. We initially observed these signals when the Fe protein from *Clostridium pasteurianum* (Cp2) was used as electron donor to Kp1, but similar results were obtained with homologous Kp-nitrogenase components. These observations are discussed in the context of the potential for trapping intermediate species of MoFe proteins normally present in turnover in low concentrations.

2. Materials and methods

2.1. Purification of the nitrogenase components Kp1 and Cp2

Kp1 (the MoFe protein; specific activity 1800 nmol of C_2H_2 reduced min^{-1} mg^{-1}) and Kp2 (the Fe protein; specific activity 1082 nmol of C_2H_2 reduced min^{-1} mg^{-1}) were purified from *K. pneumoniae* as described previously [7,8]. Cp2 was purified from *C. pasteurianum* using a modification of a previously reported method [9]. The specific activity of Cp2 determined at pH 7.4 was 1500 nmol C_2H_2 reduced min^{-1} mg^{-1}. The Cp2 and Kp1 preparations were free of contaminating Cp1 or Kp2, since when assayed alone, no nitrogenase activity was detected. ^{57}Fe-Kp1 (specific activity 1200 nmol of C_2H_2 reduced min^{-1} mg^{-1}) was purified from cells grown on medium enriched in ^{57}Fe.

2.2. Activity assays

Nitrogenase activity of Cp2 and Kp-nitrogenase components was determined using standard methods [10], by measuring the rate of H_2 evolution under an argon atmosphere. Activity at the protein concentrations used for the EPR measurements was measured in the absence of an ATP-regenerating system in the reaction mixture. Mixtures of Kp1 (200 μM), Cp2 (400 μM), dithionite (10 mM) and MgATP (9 mM) in 25 mM HEPES, pH 7.4, were incubated at 30°C in a rubber capped vial under an argon atmosphere. Gas samples (0.5 ml) were removed by syringe at different times and analysed for hydrogen by gas chromatography.

*Corresponding author. Fax: (44)-1603-450018.
E-mail address: robert.eady@bbsrc.ac.uk (R.R. Eady).

Abbreviations: The nitrogenase metalloproteins are abbreviated according to the genus and species of the diazotrophs from which they were isolated, e.g. KP2 is Fe protein from *Klebsiella pneumoniae*, Cp2 is *Clostridium pasteurianum* protein, Kp1 is MoFe protein from *K. pneumoniae*, etc.; FeMoco, [1Mo–7Fe–9S-homocitrate] cofactor of nitrogenase

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2.3. Hydrazine estimation

Hydrazine was estimated using *p*-dimethylaminobenzaldehyde as described previously [11]. A mixture of Kp1 (100 μM), Cp2 (200 μM), dithionite (10 mM) and MgATP (9 mM) in 25 mM HEPES, pH 7.4, was incubated anaerobically for 1 h. EPR spectroscopy was used to establish that the new signals were developed, and aliquots containing 5 nmol of Kp1 were assayed for the presence of hydrazine.

2.4. ATP/ADP determination

Reaction mixtures were incubated for 1 h before samples (1, 5 and 10 μl) were removed and analysed for ATP and ADP, using previously published methods [11,12], using a Symmetry Shield high-performance liquid chromatography column (100 Å pore size, 5 μm particle size; Waters Ltd., Watford, UK) connected with a Waters 626 LC and a 996 photodiode array detector.

2.5. Electron transfer monitored by EPR spectroscopy

EPR spectra were recorded on a Bruker ER200 D-SRC spectrometer, operating at X-band (9 GHz) and equipped with an ER 900 helium cryostat. All other parameters are noted in the figure legends. Samples were prepared and manipulated using standard anaerobic techniques and contained Kp1 (200 μM), Cp2 (400 μM), dithionite (10 mM) and MgATP (9 mM) in 25 mM HEPES, pH 7.4. In experiments using ^{57}Fe-labelled Kp1 the protein concentrations were half of the above mentioned. The experimental spectra were simulated using SimFonia, Bruker Analytik GmbH, 1997.

3. Results and discussion

3.1. Novel S = 3/2 EPR signals of Kp1 in MgADP-inhibited turnover samples of Cp2/Kp1

The heterologous nitrogenase formed between Cp2 and Kp1 exhibits an unusual time-dependent pattern of substrate reduction. When turnover is initiated, protons are reduced without a lag, C_2H_2 is not reduced initially, but becomes an effective substrate after 10 min, [13], and a 30 min lag occurs before N_2 is reduced to NH_3 [14]. There is currently no explanation for this behaviour, but recently we demonstrated that, unlike homologous Kp-nitrogenase components, Cp2 and Kp1 form a stable 1:1 complex which can be isolated by gel filtration, both in the presence and absence of MgATP [15]. This stable complex is unusual, since although the Cp2 is bound to Kp1, it is reduced as indicated by the presence of a characteristic $S = 1/2$ EPR spectrum of Cp2 in the $[4Fe–4S]^{1+}$ oxidation level[16].

During an investigation of the changes in EPR signals of this system undergoing turnover, we observed a time-dependent change in the shape of the $S = 3/2$ EPR spectrum of the FeMoco centre of Kp1. Fig. 1 shows the 10 K EPR spectra of Kp1 and those observed in samples frozen at different times during turnover from a mixture Cp2Kp1 at a 2:1 molar ratio. After 30 s reaction, the $g = 4.3$, 3.7, 2.0 signal of Kp1 (Fig. 1, trace 2) shows a decrease in intensity of 50% compared with the signal of isolated Kp1 (Fig. 1, trace 1). This change is followed by a slower small decrease in intensity over the following 20 min. After 5 min reaction time, a change in the shape of the $S = 3/2$ EPR spectrum of the FeMoco centre of Kp1 is apparent. A new signal with apparent $g = 4.61$, 3.44, 2.0 values becomes visible and becomes progressively more

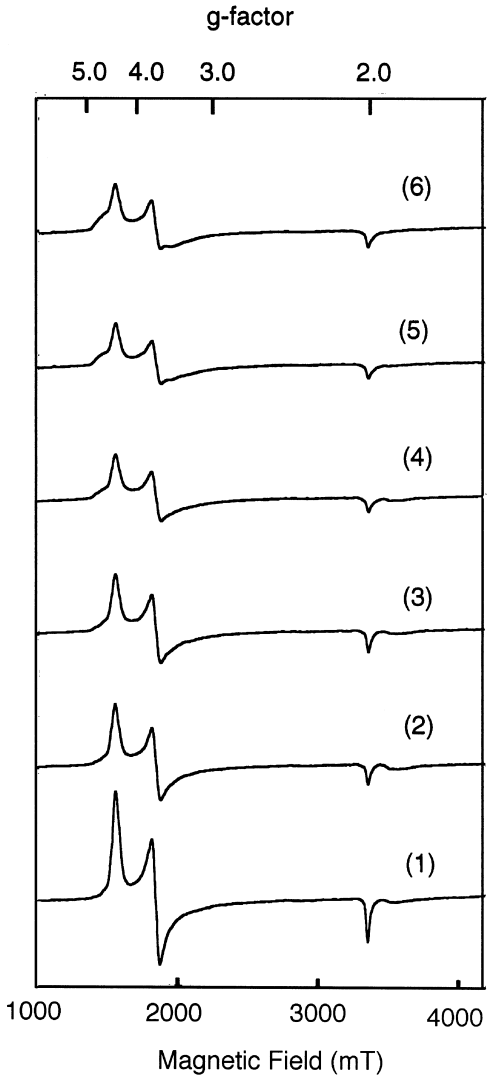


Fig. 1. Electron transfer from the Cp2 [4Fe–4S] cluster to Kp1, monitored by EPR spectroscopy. Cp2 (400 μM) was mixed with Kp1 (200 μM) in the presence of MgATP (9 mM) and sodium dithionite (10 mM) in 25 mM HEPES buffer, pH 7.4. EPR spectra are shown for the as-isolated state of Kp1 (trace 1), and the mixture of Cp2 and Kp1 30 s (trace 2), 2 min (trace 3), 5 min (trace 4), 20 min (trace 5) and 1 h (trace 6) after incubation. All EPR spectra were recorded at 10 K, with a 9.42 GHz microwave frequency, a 100 mW microwave power, a 100 kHz modulation frequency and a modulation amplitude of 0.3 mT.

Table 1
EPR parameters for the resting-state dithionite-reduced Kp1 (A) and the two novel EPR signals formed in MgADP-inhibited turnover (B and C)

Species A		Species B		Species C	
g factor	line width (mT)	*g* factor	line width (mT)	*g* factor	line width (mT)
4.33	6.5	4.74	12.0	4.58	8.5
3.66	6.5	3.32	10.0	3.50	8.5
2.00	5.0	2.00	9.0	1.99	6.5

The parameters for signals B and C were obtained by computer simulation of the experimental spectrum. The values listed are those used for the simulation shown in Fig. 3.

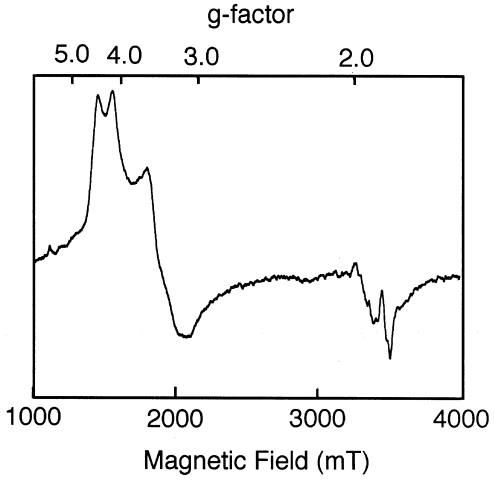


Fig. 2. EPR spectrum showing the $g = 4.61$, 3.44, 2.0 signal developed 1 h after the mixing of Cp2 and Kp1 with MgATP. This spectrum was recorded at 18 K, with a 9.42 GHz microwave frequency, a 10 mW microwave power, a 100 kHz modulation frequency and a modulation amplitude of 0.3 mT.

marked after 1 h incubation (Fig. 1, trace 6). This signal has a different temperature-dependence from the normal $S = 3/2$ EPR spectrum of the resting state of Kp1, and Fig. 2 shows that at 18 K, this new signal is better resolved.

Activity measurements at the high protein concentrations required for these experiments (200 μM Kp1), and in the absence of an ATP-regenerating system, showed H_2 evolution to cease after 5 min reaction. Thus, the changes occurring in the EPR spectrum of the FeMoco centre of Kp1 at longer times are not associated with the formation of a reduced product by nitrogenase. However, the appearance of the signals requires turnover to have occurred, since they were not observed in samples incubated with MgADP or in reaction mixtures containing ratios of MgATP/MgADP such that turnover was inhibited from the outset. This suggests that they are associated with a species of Kp1 which accumulates as the turning-over enzyme is inhibited due to the formation of MgADP.

3.2. Changes of Cp2 EPR signals during turnover

Consistent with the activity data, the $g = 2.05$, 1.94, 1.86 signals of the dithionite-reduced $S = 1/2$ state of Cp2 in turnover samples is still detectable after 30 s (Fig. 1, trace 2), but after 2 min is almost bleached (trace 3), and is absent after 5 min (trace 4). Cp2 remains oxidised at prolonged reaction times, even in the presence of excess dithionite. It has previously been shown that two Cp2 molecules are tightly complexed to Kp1 under these conditions and that the predominant species during enzyme turnover is $(Cp2_{ADPox})_2Kp1$, but no time dependence of the EPR were reported [15]. The absence of the characteristic EPR signal of Cp2 after 1 h incubation (Fig. 1 trace 6) shows that the complex is sufficiently stable so as to prevent the reduction of Cp2 by dithionite. Analysis of the incubation mixture showed that complete hydrolysis of MgATP to MgADP had occurred during this time suggesting that the complex has ATPase activity and that dissociation of the two proteins is not a pre-requisite for this reaction.

3.3. Turnover of homologous Kp-nitrogenase leads to the formation of the novel $S = 3/2$ EPR signals

The formation of these novel signals does not arise from the unusual properties of Cp2Kp1-nitrogenase in forming a stable 1:1 complex [15], since they are also developed by turnover of homologous Kp-nitrogenase. Fig. 3, trace 1 shows the EPR spectrum obtained after 1 h incubation of a 2:1 ratio of Kp2:Kp1 in the presence of 9 mM MgATP. Spectral simulation (Fig. 3, trace 2) of the overlapping spectra of the $S = 3/2$ species was performed using the EPR parameters listed in Table 1. The simulation reveals that the novel signal with apparent g values of 4.61, 3.44, 2.0 is in reality the sum of two different $S = 3/2$ components, one with $g = 4.74$, 3.32, 2.00 and the second with $g = 4.58$, 3.50, 1.99. These signals clearly differ from the two transient signals with g values of 4.21, 3.76 and 4.69, recently demonstrated in rapid-freeze experiments with Av-nitrogenase during turnover [6].

The data presented above show that the novel signal with apparent $g = 4.61$, 3.44, 2.0 values is observed only in nitrogenase samples which have undergone a normal catalytic turnover for some time, before being inhibited by the accumulation of MgADP. It was of interest to determine if this signal was associated with the accumulation of some intermediate species of Kp1 more reduced than the resting state to which the $S = 3/2$ EPR signal with $g = 4.3$, 3.6, 2.0 is normally assigned. One intermediate species of the Lowe–Thorneley scheme (see [2]) is an enzyme-bound dinitrogen hydride intermediate that can be detected as hydrazine on acid or alkali quenching of the nitrogenase under turnover [10]. A mixture of Kp1 (100 μM) and Cp2 (200 μM), was incubated with MgATP (9 μM) under a nitrogen atmosphere for 1 h, before the EPR spectrum was recorded to confirm that the novel species was formed. The mixture was then quenched with acid and samples containing 5 nmol of Kp1 were analysed for hydrazine. Using a method that would easily have

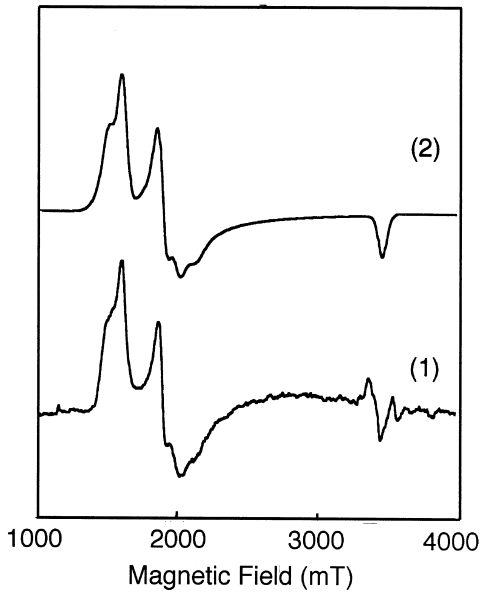
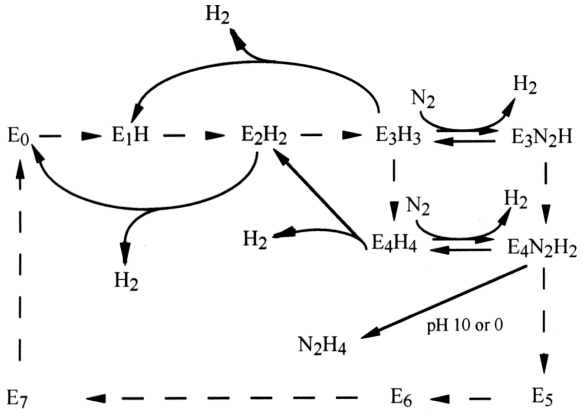


Fig. 3. EPR spectrum developed 1 h after the incubation of Kp1 (200 μM) and Kp2 (400 μM) in the presence of MgATP (9 mM) and sodium dithionite (10 mM) in 25 mM HEPES buffer, pH 7.4 (trace 1) and the relative simulation (trace 2). The EPR spectrum was recorded under the conditions of Fig. 1.



Scheme 1. The MoFe protein cycle of nitrogenase turnover. Each dotted arrow represents a reaction in which one electron is transferred from the Fe protein to the MoFe protein. The X-ray crystal structure of E_0 has been determined, an oxidation state which does not bind N_2. Under conditions of low electron flux sates, E_0 to E_4 predominate leading to H_2 evolution (see [2]).

measured 0.5 nmol of hydrazine, none was detected, indicating that the putative dinitrogen hydride intermediate [10] is not the source of the new signal. Under conditions of low electron flux, such as that which occurs in the MgADP-inhibited turnover of our experiments, the predominant reaction catalysed by nitrogenase is the reduction of protons to dihydrogen (see [2]). This reaction involves the species E_0 to E_4 of Scheme 1, in which E_0 is assigned to MoFe protein in the dithionite-reduced oxidation level and E_1 to E_4 to more reduced forms. Our data strongly suggest that E_0 and E_4 are not the species responsible for the new EPR signals we observe, and implicate E_2 or E_3 as the source.

In an attempt to unambiguously assign these signals to MoFe protein, the EPR experiments were repeated using ^{57}Fe-Kp1, both with the heterologous and with the homologous systems. We expected a broadening of the signals due to the interaction of the unpaired electron with the ^{57}Fe nuclei of the FeMoco centres of Kp1. The spectra obtained with ^{57}Fe-Kp1 were simulated as a sum of the same two components using the parameters of Table 1, but the hyperfine coupling constant could not be determined by EPR. However, the parameters of the strong EPR signal we observe are clearly different from the EPR signals which arise from the $S = 3/2$ spin-state of Fe proteins [17–19], thus we assign the $S = 3/2$ EPR components to Kp1.

In summary, in this paper we report conditions that lead to the development of two novel EPR signals arising from the MoFe protein of nitrogenase. The signals with $g = 4.74$, 3.32, 2.00 and $g = 4.58$, 3.50, 1.99, are detected in turnover samples after MgATP exhaustion. They appear to be associated with a form of Kp1 in which the FeMoco centres are reduced, but in a different state from the dithionite-reduced protein. We have shown that they are not associated with the enzyme-bound dinitrogen hydride intermediate formed during the dinitrogen reduction.

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