MgATP-independent hydrogen evolution catalysed by nitrogenase: an explanation for the missing electron(s) in the MgADP–AlF₄ transition-state complex

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INTRODUCTION

Nitrogenase, the enzyme responsible for biological nitrogen fixation, is a two-component system. Mo-containing nitrogenases (for review see [1,2]) are comprised of an MoFe protein containing the substrate-binding site, and an Fe protein which functions as an MgATP-dependent electron donor to the MoFe protein to catalyse the reaction:

\[
\text{N}_2 + 8e + 8H^+ + 16\text{MgATP} \rightarrow 2\text{NH}_3 + H_2 + 16\text{MgADP} + 16\text{P}_i
\]  

X-ray crystal structures of the two proteins from a number of bacterial species (see [1]) and a putative transition-state complex of Azotobacter vinelandii (Av) nitrogenase [3] have been published. The Fe protein structure shows that this dimeric protein contains a single [Fe₇S₆] cluster located at the interface of the γ₂ dimer, some 20 Å away from two nucleotide-binding sites. A close structural similarity of the Azotobacter vinelandii Fe protein (Av2) with the p21ras molecular switch protein was noted [4]. The X-ray structures of the MoFe proteins show that it is an α₃β₃ tetramer containing two types of metal centre. The likely catalytic site present in each α subunit is a FeMoco centre with a composition Mo:Fe₇S₆:homocitrate. In addition to FeMoco centres, the MoFe proteins also contain two unique Fe₇S₆ centres, called P-clusters, located at the interface of the α and β subunits. The latter clusters are believed to be the primary electron-accepting sites of the MoFe protein, since the structure of the Av complex shows them to be equidistant between the Fe protein [Fe₇S₆] cluster and an FeMoco centre. The P-clusters readily undergo a two-electron oxidation, which is associated with structural changes of the cluster [5].

In addition to the reduction of N₂ to NH₃, the enzyme is also capable of reducing a number of small unsaturated molecules such as CH₃H₂, N₂-, N₂O or HCN. When no reducible substrate is added, for example under an atmosphere of argon, protons are reduced to H₂. Nitrogenase activity is routinely assayed as an acetylene reductase or as an MgATP-dependent hydrogenase due to the simplicity and sensitivity of the assay procedures.

Central to the catalytic function of the enzyme is formation of a transient electron-transfer complex between the two components [2]. The consensus view is that MgATP hydrolysis is essential for electron transfer and substrate reduction, although the mechanism of the coupling is not clear. The binding of MgATP or MgADP to the Fe protein results in a conformational change which has been proposed to be important in regulating the affinity of the Fe protein for the MoFe protein to form an electron-transfer complex. The MgATP-bound conformation promotes complex formation, and following electron transfer to

Abbreviations used: the nitrogenase metalloproteins are abbreviated according to the genus and species of the diazotrophs from which they were isolated, e.g. the Fe protein from Klebsiella pneumoniae is Kp2, the Azotobacter vinelandii Fe protein is Av2 and the Clostridium pasteurianum protein Cp2; the MoFe protein from K. pneumoniae is Kp1, the MoFe protein from A. vinelandii is Av1.

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the MoFe protein, the MgADP-bound conformation of the Fe protein destabilizes the complex [2].

A recent advance in our understanding of the role of nucleotides in nitrogenase function has come from the study of AlF$_4^-$-MgADP-stabilized putative transition-state analogue complexes of Av [6,7] and Klebsiella pneumoniae (Kp) nitrogenases [8]. Structural evidence for conformational changes in the Fe protein has been provided from small angle X-ray scattering studies of the Kp [8] and X-ray structure determination of the Av nitrogenase transition-state complexes [3]. In addition, a kinetic analysis of the rate of complex formation between the MoFe (Kp1) and Fe (Kp2) component proteins of K. pneumoniae nitrogenase revealed that catalytically active molecules of the complex, and also the MgATP-independent H$_2$ complex of Av nitrogenase [10].

The most recent study has also shown that the redox potential of the Fe protein is lower than $-500$ mV in the complex of Av nitrogenase [10].

We report here the detection of H$_2$ formed during the formation of the complex, and also the MgATP-independent H$_2$ formation by nitrogenase of K. pneumoniae. These findings explain for the first time the origin of the oxidized Fe proteins in the transition-state complex and give new insight into the roles for MgATP in nitrogenase turnover.

**MATERIALS AND METHODS**

The enzymic assay of the native nitrogenase, and the separate components, Kp1 and Kp2, was conducted as described previously [11]. Sodium dithionite (BDH), MgCl$_2$ and AlF$_4$H$_2$O (Aldrich), KF and KADP (Sigma) were used as supplied. Kp1 and Kp2 were purified by published procedures [11,12]. The Kp1 used contained 1.09 Mo per $\alpha_1\beta_2$ tetramer and had a specific activity of $1222$ nmol of acetylene reduced·min$^{-1}$·mg$^{-1}$ of protein. The specific activity of Kp2 used was $1732$ nmol of acetylene reduced·min$^{-1}$·mg$^{-1}$ of protein. Metal contents were determined using inductively coupled plasma analysis. The amount of H$_2$ evolved in incubation mixtures was measured as described in [11].

**Formation of the transition-state complex**

The Kp1–AlF$_4^-$–Kp2 complex was prepared in $50$ mM Heps (pH $7.4$) by the same procedure as described in [9]. The final volume was $20$ ml and the concentrations of MgCl$_2$, KADP, Na$_2$S$_2$O$_4$ and AlF$_4^-$ in the reaction mixture were $7.5$ mM, $2.0$ mM, $20$ mM and $~5$ mM respectively. [Kp1] was $3.8 \times 10^{-4}$ M while [Kp2] was $(4-4.3) \times 10^{-4}$ M, resulting in a [Kp2]/[Kp1] of 1 or 1.1. The ratio of [Kp2]/[Kp1] was kept at approx. one because the Kp1 used contained only 1.1 Mo per molecule. The reaction mixtures were incubated at room temperature for at least $20$ h in a $25$ ml flask. Before the addition of reductant, the flask was flushed with argon and then sealed with a rubber closure. After the incubation period the gas phase was analysed for hydrogen gas.

**MgADP-dependent hydrogen evolution**

Assays were carried out in $2$ ml glass vessels with a total volume of assay mixture of $1$ ml. The glass vessels were sealed with Suba Seals (Wm. Freedman, Barnsley, U.K.) and thoroughly flushed with argon before they were charged with the assay mixture containing $50$ mM Heps buffer (pH $7.5$), $7.5$ mM MgCl$_2$, $20$ mM Na$_2$S$_2$O$_4$ and variable amounts of KADP as shown in Table 1. The incubation period was $20$ h and assays were started by the addition of $0.44$ mg of Kp1 ($1.93 \mu$M) followed by $0.133$ mg of Kp2 ($2.08 \mu$M). These vessels were then inverted in a glass beaker.

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RESULTS AND DISCUSSION
Proton reduction during the ADP-dependent formation of the transition-state complex of nitrogenase

Although under some conditions electron transfer from the Fe protein to the MoFe protein of nitrogenase has been demonstrated in the absence of MgATP, the enzyme is generally considered to have an essential requirement for MgATP in order for substrate reduction to occur. In addition, reduction of H⁺ to dihydrogen, the simplest 2 electron reduction catalysed by nitrogenase, requires a minimum of two association–dissociation reactions of the component proteins in the Lowe–Thorneley scheme for nitrogenase function (see [2]). For these reasons, substrate reduction during the formation of the stable Fe protein–MgADP–AlF₃⁻–MoFe protein transition-state complex of nitrogenase, prepared by incubation in the presence of MgADP and AlF₃⁻ [6], would not be expected and has not previously been reported. Once formed, the MgADP–AlF₃⁻ complex is inactive in steady-state assays, even in the presence of MgATP. In another study [7] the inhibited complex was prepared by turnover with MgATP in the presence of AlF₃⁻, conditions where H₂ evolution would occur from enzyme turnover before formation of the inhibited complex takes place.

In the course of our studies of the Kp1–MgADP–AlF₃⁻–Kp2 complex, prepared using the ADP incubation method, we investigated the gas phase above the incubation reaction mixture after the formation of the complex and detected a significant amount of H₂ (see Table 1, experiment 1). This finding was unexpected, since no MgATP was present in the incubation mixture, and many studies have shown that MgADP is a potent competitive inhibitor of electron transfer and substrate reduction.

To assess the second possibility we assayed the enzyme in the presence of MgADP but in the absence of AlF₃⁻. The results in Table 1 (experiments 2–5) clearly indicate that MgADP-dependent H₂ evolution by nitrogenase occurs in the absence of AlF₃⁻, and thus the activity is not a property of the putative transition-state complex. This activity showed a dependence on the Kp2 : Kp1 ratio, showing saturation kinetics as the amount of Kp2 was increased at a constant concentration of Kp1 (Figure 1). This dependence shows that the activity is nitrogenase related and therefore does not arise from a contaminating hydrogenase activity. At saturating [Kp2] the total number of electrons resulting in H₂ production after 20 h incubation (~1.9 nmol) compares well with the amount of Kp1 (1.93 nmol) present in the assay mixture, consistent with the electron transferred on oxidation of Kp2 resulting in the reduction of H⁺ ions. At the lower Kp2 : Kp1 ratios, where the amount of H₂ formed was lower at 20 h, subsequent H₂ evolution at a linear rate was observed up to 50 h incubation. These results are consistent with a single turnover of the FeMoco centre of Kp1 under these conditions. Experiments 6 and 7 (Table 1) are control experiments, which show that in the presence of MgADP a very small amount of H₂ is evolved by both Kp1 and Kp2. The amount evolved is very close to the limit of detection and probably arises from a low level of cross-contamination of Kp1 and Kp2 in the purified proteins. MgATP-dependent residual activity of the separate components is commonly encountered in nitrogenase research.

Requirement for ADP for H₂ formation

To assess the second possibility we assayed the enzyme in the presence of increasing amounts of MgADP. In this case, if MgATP is present as an impurity at low concentrations, then [MgATP] should increase at a constant ratio with [MgADP] and the activity should increase with [MgADP]. In conventional MgATP-dependent nitrogenase assays MgADP is a potent competitive inhibitor of nitrogenase activity (see [2]). A mathematical analysis assuming Michaelis–Menten kinetics, shows
that $V_{\text{max}}$ for competitive inhibition does not change, and if the [substrate]/[inhibitor] ratio is kept constant the initial velocity also remains constant as the concentration of the [substrate]/ [inhibitor] is increased, provided [I] and [S] are 10-fold higher than $K_i$ and $K_p$ respectively. In the present case for *K. pneumoniae* nitrogenase, the $K_i$ for MgADP is 20 $\mu$M [15] and [MgADP] was varied from 1 to 4 mM. Using a value of 400 $\mu$M as the apparent $K_i$ for MgATP [16] and allowing a potential contamination of 5% of MgATP in the given concentrations of MgADP, the mathematical analysis predicts a less than 1.5% increase in the reaction rate. Thus if our observed activity is due to MgATP contamination and MgADP is acting as a competitive inhibitor, then by varying [MgATP] from 1 to 4 mM any change in the activity would not be significant. Comparison of the results obtained in experiments 2–5 of Table 1 show the opposite trend, with highest activity at 1 mM MgADP, the lowest concentration tested. We therefore conclude that the activity we detect is not due to low levels of contaminating MgATP in the MgADP. At the [MgADP] resulting in the highest amount of $H_2$ evolved (1 mM), the amount, 2.09 nmol, corresponded well with the amount of Kp1 (1.93 nmol) or Kp2 (2.08 nmol) in the assay. Experiment 8 shows that reaction mixtures lacking added Mg$^{2+}$ still evolve $H_2$, but in lower amounts than when Mg$^{2+}$ is added (experiment 9).

Thus we conclude that the third possibility, where MgADP replaces MgATP, is the most likely explanation for the observed activity. The stimulation of $H_2$ evolution by low concentrations of MgADP and the inhibition observed at higher concentrations may arise from two distinct binding sites for MgADP. Binding at one site leads to activation of the enzyme, whereas binding at the other site induces inhibition. Av2 has also been reported to exhibit an ADP-dependent electron transfer to Av1 [17], although the authors attributed this to contamination of the ADP with low levels of ATP; however, from the kinetic analysis presented above we consider this to be an unlikely explanation.

Under optimum conditions, the amount of $H_2$ evolved corresponds to a single turnover of Kp1 (Figure 1), even in the presence of excess reductant. Since this is observed in the presence of excess Kp2 it seems probable that a stable complex of Kp1Kp2(−) (MgADP) is formed and stabilized by the excess MgADP, thus preventing further reaction. It has been shown that this complex is formed to some extent under normal turnover conditions where [MgADP] is low. Here, a transient association of the nitrogenase components occurs, and visible and EPR spectroscopies show that a significant proportion of the complexed Fe protein with ADP bound remains oxidized after electron transfer to the MoFe protein [18,19]. Assuming that under our conditions, reduced Kp2 provides one of the two electrons required for $H_2$ evolution and a reduced P-centre a second electron, then dissociation of the complex would allow re-reduction of the P-centres and Kp2(−) and continued activity. This is not observed, and although reduction of oxidized Kp1 by dithionite ion [20] is slow (∼1 h), over the 20 h incubation period of our experiments more $H_2$ would be expected. Thus the formation of a stabilized complex under our conditions is consistent with the absence of continued $H_2$ production that we observe.

An alternative explanation involves the slow reactions of MgADP with both Kp1 [21] and the Fe proteins Kp2 [22] and Av2 [4], which results in an AMP-modified and a slowly dissociating protein-bound ADP species respectively. These reactions may account for the inhibition by MgADP and lack of enzyme turnover that we observe, since if either of these species was inactive in the MgADP-dependent hydrogen-formation reaction, and high concentrations of MgADP favoured the formation of such an inactive entity, then the inhibition would be accounted for. Further work is required to distinguish between these possibilities.

In the absence of MgADP, very low levels of $H_2$ evolution from mixtures of Kp1 and Kp2 were detected, which under our conditions corresponded to half the Kp2 present initially (Table 1). This surprising finding is consistent with the recently reported nucleotide-independent electron transfer which has been reported to occur in the catalytically inactive and long-lived Cp2/Av1 heterologous nitrogenase complex [19] (Cp2 is the *Clostridium pasteurianum* protein). In this case, electron transfer, measured from the rate of oxidation of the Fe protein, occurred in the absence of nucleotide at a rate of 0.007 s$^{-1}$, and was increased 4-
fold in the presence of MgADP and 20000-fold by MgATP. No substrate reduction was reported in that study.

Implications for nitrogenase function

MgATP is generally considered to be essential for the catalytic function of nitrogenase (see [2]), and although electron transfer from Fe protein to MoFe protein has been reported under some conditions in the absence of MgATP [6,7,10,13,23], substrate reduction has not. The consensus view is that MgATP binds to the Fe protein to induce a conformation which is required for the formation of an effective nitrogenase complex, and that MgATP hydrolysis facilitates electron transfer to the MoFe protein [2,24]. After electron transfer, the oxidized Fe protein with MgADP hydrolysis facilitates electron transfer to the MoFe protein [2,24].

In the nucleotide-bound species, the redox potential of the Fe protein has at least four different conformations, that of the free protein, MgADP-bound, MgATP-bound (see [2]) and the structurally characterized conformation in the transition-state complex [3]. In the nucleotide-bound species, the redox potential of the Fe protein is in equilibrium with the MgADP and MgATP-conformations respectively [25].

Our new finding, that low levels of H\textsuperscript{+} reduction can be detected in the absence of nucleotides and is stimulated by MgADP, indicates that low rates of electron transfer and H\textsubscript{2} formation can occur in the absence of MgATP. This observation suggests that either the various conformations of the Fe protein are in equilibrium in the absence of nucleotides, and that the ATP’ conformation is functional, or that the different species react at different rates. Recent work has revealed that within the Fe-ADP–MoFe protein complex [10], and in the tight complex of Av1–Av2 (Leu-127 deletion mutant) [13,14], more negative potentials (\sim –620 mV) are achieved by the Fe\textsubscript{3}S\textsubscript{4} centre of the Fe protein in this pseudo-MgATP-bound conformation. At least in the case of the Av2-Leu-127 deletion protein, complex formation between Av2 and Av1 results in a further decrease in the redox potential of Av2 relative to that in the ATP-bound conformation [14]. One explanation for MgATP-independent electron transfer and the H\textsubscript{2} evolution reported here is that the complex formation between Kp2 with Kp1 decreases the mid-point potential of nucleotide-free or MgADP-bound Kp2 sufficiently to allow electron transfer and substrate reduction under conditions where a long-lived nitrogenase complex is formed.

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REFERENCES