Nitrogenase bioelectrocatalysis: heterogeneous ammonia and hydrogen production by MoFe protein

Ross D. Milton

Sofiene Abdellaoui

Nimesh Khadka
Utah State University

Dennis R. Dean

Donal Leech

Lance C. Seefeldt
Utah State University

See next page for additional authors

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Nitrogenase is the only enzyme known to catalyze the reduction of \( \text{N}_2 \) to \( 2\text{NH}_3 \). In vivo, the MoFe protein component of nitrogenase is exclusively reduced by the ATP-hydrolyzing Fe protein in a series of transient association/dissociation steps that are linked to the hydrolysis of two ATP for each electron transferred. We report MoFe protein immobilized at an electrode surface, where cobaltocene (as an electron mediator that can be observed in real time at a carbon electrode) is used to reduce the MoFe protein (independent of the Fe protein and of ATP hydrolysis) and support the bioelectrocatalytic reduction of protons to dihydrogen, azide to ammonia, and nitrite to ammonia. Bulk bioelectrosynthetic \( \text{N}_2 \) or \( \text{NO}_2^- \) reduction (50 mM) for 30 minutes yielded 70 ± 9 nmol \( \text{NH}_3 \) and 234 ± 62 nmol \( \text{NH}_3 \), with \( \text{NO}_2^- \) reduction operating at high faradaic efficiency.

The reduction of effectively-inert dinitrogen (\( \text{N}_2 \)), the major constituent of the Earth’s atmosphere (79%), to more biologically and industrially useful forms of nitrogen (i.e. \( \text{NH}_3 \)) is a key step in the global biogeochemical N cycle. From a biological standpoint, a select group of diazotrophic microorganisms (limited to bacteria and archaea) are able to reduce atmospheric \( \text{N}_2 \) to \( \text{NH}_3 \) under both aerobic and anaerobic growth conditions by way of a single enzyme, nitrogenase. Industrially, the renowned Haber–Bosch process focuses on Mo-dependent nitrogenase. In addition to the ability of nitrogenase to reduce \( \text{N}_2 \) to \( \text{NH}_3 \) (along with concomitant \( \text{H}_2 \) production) and it is able to do so at room temperature, neutral pH and ambient pressure although a large input of chemical energy is also required (16 ATP per \( \text{N}_2 \)). We have bypassed the reducing- and ATP-hydrolyzing properties of the Fe protein of nitrogenase by immobilizing the catalytic protein of nitrogenase (MoFe protein) at a carbon electrode surface whereby a suitable electron mediator (cobaltocene) can be used to support the bioelectrocatalytic reduction of \( 2\text{H}^+ \) to \( \text{H}_2 \), \( \text{N}_2 \) to \( \text{NH}_3 \) and \( \text{NO}_2^- \) to \( \text{NH}_3 \) under mild conditions (room temperature, neutral pH and ambient pressure).

Nitrogenase is a protein that is able to reduce \( \text{N}_2 \) to \( \text{NH}_3 \) at the expense of ATP hydrolysis and a reductant (typically ferredoxin or flavodoxin in vivo), of which three major classes are characterized by their Mo-, V- or Fe-dependent catalytic cofactors (FeMo-co, VFe-co, FeFe-co respectively); this study focuses on Mo-dependent nitrogenase. In addition to the ability of nitrogenase to reduce \( \text{N}_2 \), other interesting substrates include \( \text{H}^+, \text{C}_2\text{H}_2, \text{N}_3^-, \text{HCN}, \text{NO}_2^-, \text{N}_2\text{H}_4, \text{CO}_2, \text{CO}, \text{R-CN} \) and \( \text{R-NC} \).

Mo-dependent nitrogenase consists of two protein components that are highly sensitive to \( \text{O}_2 \); a Fe protein and a MoFe protein. The Fe protein (a ~ 66 kDa homodimer) is responsible for reducing the MoFe protein (a ~ 240 kDa dimer of dimers), in a series of individual electron transfer events that are coupled to the hydrolysis of two MgATP molecules to MgADP (Fig. 1). The Fe protein bound to two MgATP molecules transiently associates with the MoFe protein, where a single electron is transferred from the FeS\(_4\) cluster of the Fe protein to the MoFe protein in a process that is coupled to the hydrolysis of two MgATP to two MgADP. These events are followed by the dissociation of the Fe protein from the MoFe protein.
Within the MoFe protein, electrons are shuttled from the P cluster to the active site FeMo-cofactor where N₂ is reduced to 2NH₃ with the concomitant reduction of 2H⁺ to H₂ (eqn (1)).

\[
16\text{MgATP} + 8e^- + 8H^+ + N_2 \rightarrow 16\text{MgADP} + 2\text{NH}_3 + H_2 + 16P_i
\]  

(1)

Some key events of this electron transfer cycle occur as follows: (i) electron transfer between the Fe protein and the MoFe protein, (ii) hydrolysis of 2MgATP to 2MgADP, (iii) Pᵢ release and (iv) Fe protein dissociation from the MoFe protein. The order of electron transfer (ET) events appears to be: (i) transfer of a single electron from the P cluster to FeMo-co followed by (ii) transfer of an electron from the Fe protein Fe₄S₄ cluster to the oxidized P cluster of the MoFe protein, in a process that has been termed “deficit spending.”

To achieve sustainable substrate reduction by nitrogenase without the need for ATP, there is considerable interest in delivering electrons directly to the MoFe protein. Toward this end, alternative in vitro reducing agents have been demonstrated to be able to reduce the MoFe protein and support substrate reduction; however, success is commonly limited to the reduction of substrates other than N₂, such as N₂H₄, H⁺, HCN and N₃⁻ and at very low rates. Recently, light-dependent reduction of the MoFe protein by CdS nanorods has been shown to be able to support N₂ reduction to 2NH₃ with a high turnover number and quantum efficiency.

To date multiple enzymes (such as glucose oxidase, bilirubin oxidase, hydrogenase and carbon monoxide dehydrogenase, to name a few) have been immobilized at electrode surfaces where heterogeneous ET to/from the enzyme supports enzymatic substrate reduction or oxidation, respectively. ET takes place either directly (DET) or through the use of an electron mediator (MET), whereby the ability to directly observe electron transfer in real time to/from an enzyme provides a powerful tool for the evaluation of enzymatic mechanisms and kinetics, as well as the ability to create devices that are able to produce electrical energy from alternative energy dense fuels (enzymatic fuel cells, EFCs). Additionally, the ability to bioelectro-synthetically produce an important chemical at the expense of electrical energy could circumvent the necessity of harsh reaction conditions or expensive catalysts.

Here we report the immobilization of the MoFe protein of wild-type nitrogenase (purified from Azotobacter vinelandii, an aerobic diazotroph) at an electrode surface, where the use of an unnatural electron mediator facilitates the real-time electrochemically-observable reduction of H⁺ to H₂ and azide (N₃⁻) and nitrite (NO₂⁻) to NH₃. Importantly, observed bioelectrocatalysis is obtained in the absence of the Fe protein for wild-type MoFe protein, negating the requirement of all mechanistic steps associated with the Fe protein cycle, including the rate-limiting step for overall catalysis. We therefore anticipate that this approach will be valuable in revealing mechanistic details of nitrogenase as well as provide a technological basis to establish a bioelectro-synthetic technique for the production of hydrogen, ammonia and hydrocarbons (using CO or CO₂ as substrate) at room temperature and neutral pH.
Initially, nitrogenase MoFe protein was immobilized within a polymer at the surface of a glassy carbon electrode and preliminary cyclic voltammetry studies were performed against a series of metal-containing inorganic/organometallic redox active complexes in an attempt to screen for compatible electron mediators (data not shown). MoFe protein was immobilized at the electrode surface under a chemically-crosslinked poly(vinylamine) support to effectively increase the concentration of the MoFe protein at the electrode surface in an attempt to achieve greater sensitivities for the detection of apparent MoFe protein activity. In addition, a nitrogenase MoFe protein with a single amino acid substitution, β-98Tyr→His, was utilized that has previously been demonstrated to improve the ability of the MoFe protein to accept electrons from unnatural reducing agents.13,25

While many metallocene complexes exist, ferrocene is well established in the bioelectrochemical field following the discovery that the ferrocene/ferrocinium couple can efficiently mediate electron transfer between glucose oxidase and an electrode surface, resulting in a system that is able to bioelectrocatalytically oxidize glucose.26 In order to drive a bioelectrocatalytic reductive reaction with nitrogenase, however, the formal potential of ferrocene is expected to be too positive. Thus, its structural analogue cobaltocene (Cc+, bis(cyclopentadienyl)cobalt(III)) was investigated as a more suitable electron mediator (Fig. 2a), a reductive catalytic wave is observed that is attributed to the reduction of 2H+ to H2 by the MoFe protein bioelectrode, with an onset potential of approximately −1.04 V (vs. SCE). Following the addition of 50 mM N2− (Fig. 2b) the reduction current increases, which is attributed to the reduction of NO2− to NH3 and N2; there are three major proposed pathways for the reduction of NO2− or HN3 (eqn (2) and (3)).28 The addition of 50 mM NO2− results in a catalytic reductive wave assigned to the 6e−/Co3+ at a scan rate of 10 mm/s.27

Fig. 2 presents cyclic voltammograms for the resulting MoFe protein bioelectrodes in a solution containing Cc+, where a single electron redox couple can be observed between the oxidized cobaltocenium cationic complex and its reduced cobaltocene complex (Cc+/Cc). In the presence of enzymatically active MoFe protein and Cc+ (Fig. 2a), a reductive catalytic wave is observed that is attributed to the reduction of 2H+ to H2 by the MoFe protein bioelectrode, with an onset potential of approximately −1.04 V (vs. SCE). Following the addition of 50 mM N2− (Fig. 2b) the reduction current increases, which is attributed to the reduction of NO2− to NH3 and N2; there are three major proposed pathways for the reduction of NO2− or HN3 (eqn (2) and (3)).28 The addition of 50 mM NO2− results in a catalytic reductive wave assigned to the 6e−/Co3+ at a scan rate of 10 mm/s.27

In all cases, MoFe protein bioelectrodes prepared with the β-98Tyr→His modified MoFe protein result in improved catalytic currents; this is in agreement with previous reports whereby this single amino acid substitution improves the ability of the MoFe protein to accept electrons from unnatural electron donors.13 Control bioelectrodes consisted of either an equivalent loading of BSA (bovine serum albumin, by mass) in place of the MoFe protein, or apo-MoFe protein (obtained from a nifB-deficient strain of A. vinelandii yielding a MoFe protein lacking FeMo-co). Under non-steady state conditions, minimal contributions are observed for bioelectrodes prepared with apo-MoFe protein for H+ and N2− reduction. An increased background contribution is observed for apo-MoFe protein bioelectrodes and BSA control bioelectrodes in the presence of NO3−; increased apparent catalytic reductive current for NO2− at apo-MoFe protein bioelectrodes suggests the P cluster...
may be able to catalytically reduce $\text{NO}_2^-$.

Table S1 (ESI†) reports the catalytic current densities obtained for each substrate, corrected to apo-MoFe protein bioelectrodes.

In addition to cyclic voltammetry, steady-state amperometric analyses were also performed to validate the catalytic turnover of $\text{N}_3^-$ by the wild-type and $\beta$-98Tyr$^{+}$His MoFe protein bioelectrodes (Fig. 3). To facilitate bioelectrosynthetic reduction of the MoFe protein and thus $\text{N}_3^-$, a potential of $-1.25$ V (vs. SCE) was applied and substrate injections resulted in an increasing catalytic current. Prior to $\text{N}_3^-$ injections, the steady-state current is due to the reduction of $2\text{H}^+$ to $\text{H}_2$. Following additions of $\text{N}_3^-$ into the bulk solution, the reduction current increases as a function of the bioelectrocatalytic reduction of $\text{N}_3^-$ by the MoFe protein, whereby electrons are delivered to the protein by the $\text{Cc}/\text{Cc}^+$ electron mediator (as in Fig. 1c).

The change in the catalytic current following each addition of $\text{N}_3^-$ was analyzed against the Michaelis-Menten kinetic model by plotting the corrected current density against the corresponding $\text{N}_3^-$ concentration. Apparent Michaelis constants ($K_M$) for $\text{N}_3^-$ were calculated by nonlinear regression to be $146 \pm 15$ mM $\text{N}_3^-$ for the wild-type MoFe protein bioelectrodes, with an increase to $196 \pm 14$ mM $\text{N}_3^-$ observed for the $\beta$-98Tyr$^{+}$His MoFe protein bioelectrode. Interestingly, the apparent maximum current density ($J_{\text{max}}$) for the wild-type MoFe protein bioelectrode was calculated to be $370 \pm 14$ mA cm$^{-2}$, which almost doubled for the $\beta$-98Tyr$^{+}$His MoFe protein bioelectrodes to $725 \pm 22$ mA cm$^{-2}$. An increased $J_{\text{max}}$ as well as a similar $K_M$ provide further support for bioelectrocatalysis by intact MoFe protein at the electrode surface (and not by unfolded protein or dissociated cofactor), whereby substrate affinity remains largely unchanged although improved ET to FeMo-co result in enhanced catalytic currents (since the single $\beta$-98Tyr$^{+}$His substitution has previously been demonstrated to improve substrate turnover by the MoFe protein in the absence of the Fe protein).$^{13,25}$

Control experiments were performed using apo-MoFe protein bioelectrodes; under these steady-state conditions, negligible bioelectrocatalytic currents were observed. Additionally, apparent kinetics were not determined for $\text{NO}_2^-$ reduction under steady-state conditions at this stage; the background reductive wave for $\text{NO}_2^-$ at control bioelectrodes in the absence and presence of $\text{Cc}^+$ results in a low signal to noise ratio. Apparent kinetics for $\text{NO}_3^-$ reduction will be evaluated using an alternative bioelectrode architecture and/or MET platform.

Finally, bulk bioelectrosynthesis (using $\beta$-98Tyr$^{+}$His MoFe protein bioelectrodes) was performed at $-1.25$ V (vs. SCE) following an injection of either $\text{N}_3^-$ or $\text{NO}_2^-$ (50 mM, in the presence of $\text{Cc}^+$) to confirm $\text{NH}_3$ production; $\text{NH}_3$ was quantified using a fluorimetric assay whereby $\text{NH}_3$ forms a fluorescent complex with ortho-phthalaldehyde (ESI† Fig. S3 and S4).$^{2,23}$ Bioelectrosynthetic reduction of $\text{N}_3^-$ for 30 min yielded $70 \pm 9$ nmol of $\text{NH}_3$ where the theoretical quantity of $\text{NH}_3$ expected to be produced (as a function of the charge passed during the experiment) varied depending on the pathway of $\text{N}_3^-$ reduction (Table 1, eqn (2) and (3)). Bulk bioelectrosynthetic $\text{NO}_3^-$ reduction (performed under the same conditions) yielded $234 \pm 62$ nmol $\text{NH}_3$ where the theoretical quantity of $\text{NH}_3$ expected was $231 \pm 65$ nmol $\text{NH}_3$, corresponding to a faradaic efficiency of $101 \pm 39\%$ (ESI† Fig. S4). The high faradaic efficiency suggests that the reduced MoFe protein (reduced by Cc) largely favors the reduction of $\text{NO}_3^-$ as opposed to $2\text{H}^+$, when in the presence of $\text{NO}_2^-$.

**Conclusions**

In conclusion, we report the heterogeneous reduction of immobilized nitrogenase wild-type and $\beta$-98Tyr$^{+}$His MoFe protein, where the resulting reduced MoFe protein bioelectrode is able to support the reduction of $2\text{H}^+$ to $\text{H}_2$, $\text{N}_3^-$ to $\text{NH}_3$, and $\text{NO}_2^-$ to $\text{NH}_3$. Such bioelectrodes could be used in various applications, such as biofuel production and pollutant remediation.
NH₃ using Ce/Ce⁺ as an electron mediator. Since reduced Ce efficiently reduces MoFe protein at the electrode surface a mediated reductive catalytic response is observed, providing an alternative methodology to investigate kinetics, substrate interactions, inhibitory effects and ET pathways of the MoFe protein. Additionally, this methodology presents a novel bioselectrosynthetic system for the production of NH₃ and H₂ under mild conditions (such as room temperature and neutral pH). Future work will investigate the possibility of immobilizing the MoFe protein within a suitable redox polymer (controlling the concentration of localized electron mediator) with an aim to increase the electron flux through MoFe protein and achieve N₂ reduction. In addition to immobilizing the protein within an appropriately-designed redox polymer, improved understanding of the role of ATP in vivo would help achieve N₂ reduction at this bioelectrode surface.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SCE</td>
<td>Saturated calomel electrode</td>
</tr>
<tr>
<td>MoFe protein</td>
<td>Molybdenum iron protein of nitrogenase</td>
</tr>
<tr>
<td>Fe protein</td>
<td>Iron protein of nitrogenase</td>
</tr>
<tr>
<td>Cc/Cc⁺</td>
<td>Cobaltocenecobaltocenium</td>
</tr>
<tr>
<td>FeMo-co</td>
<td>Molybdenum iron cofactor of nitrogenase</td>
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**Notes and references**