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Nitrogenase Bioelectrochemistry for Synthesis Applications

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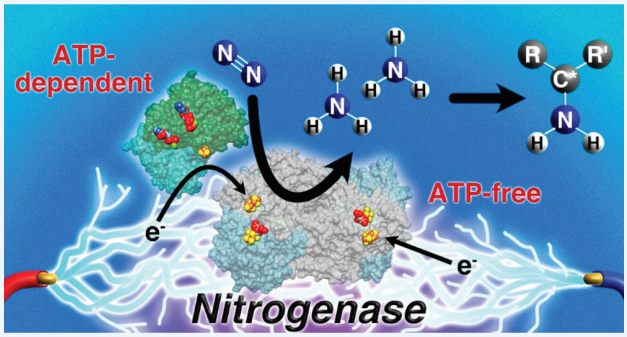
Ross D. Milton*,† and Shelley D. Minteer*,‡

†Department of Inorganic and Analytical Chemistry, University of Geneva, Sciences II, Quai Ernest-Ansermet 30, 1211 Geneva 4, Switzerland

‡NSF Center for Synthetic Organic Electrochemistry, Department of Chemistry, University of Utah, 315 S 1400 E, Salt Lake City, Utah 84112, United States

CONSPECTUS: The fixation of atmospheric dinitrogen to ammonia by industrial technologies (such as the Haber Bosch process) has revolutionized humankind. In contrast to industrial technologies, a single enzyme is known for its ability to reduce or "fix" dinitrogen: nitrogenase. Nitrogenase is a complex oxidoreductase enzymatic system that includes a catalytic protein (where dinitrogen is reduced) and an electron-transferring reductase protein (termed the Fe protein) that delivers the electrons necessary for dinitrogen fixation. The catalytic protein most commonly contains a FeMo cofactor (called the MoFe protein), but it can also contain a VFe or FeFe cofactor. Besides their ability to fix dinitrogen to ammonia, these nitrogenases can also reduce substrates such as carbon dioxide to formate. Interestingly, the VFE nitrogenase can also form carbon−carbon bonds. The vast majority of research surrounding nitrogenase employs the Fe protein to transfer electrons, which is also associated with the rate-limiting step of nitrogenase catalysis and also requires the hydrolysis of adenosine triphosphate. Thus, there is significant interest in artificially transferring electrons to the catalytic nitrogenase proteins. In this Account, we review nitrogenase electrocatalysis whereby electrons are delivered to nitrogenase from electrodes. We first describe the use of an electron mediator (cobaltocene) to transfer electrons from electrodes to the MoFe protein. The reduction of protons to molecular hydrogen was realized, in addition to azide and nitrite reduction to ammonia. Bypassing the rate-limiting step within the Fe protein, we also describe how this approach was used to interrogate the rate-limiting step of the MoFe protein: metal-hydride protonolysis at the FeMo-co. This Account next reviews the use of cobaltocene to mediate electron transfer to the VFe protein, where the reduction of carbon dioxide and the formation of carbon−carbon bonds (yielding the formation of ethene and propene) was realized. This approach also found success in mediating electron transfer to the FeFe catalytic protein, which exhibited improved carbon dioxide reduction in comparison to the MoFe protein. In the final example of mediated electron transfer to the catalytic protein, this Account also reviews recent work where the coupling of infrared spectroscopy with electrochemistry enabled the potential-dependent binding of carbon monoxide to the FeMo-co to be studied. As an alternative to mediated electron transfer, recent work that has sought to transfer electrons to the catalytic proteins in the absence of electron mediators (by direct electron transfer) is also reviewed. This approach has subsequently enabled a thermodynamic landscape to be proposed for the cofactors of the catalytic proteins. Finally, this Account also describes nitrogenase electrocatalysis whereby electrons are first transferred from an electrode to the Fe protein, before being transferred to the MoFe protein alongside the hydrolysis of adenosine triphosphate. In this way, increased quantities of ammonia can be electrocatalytically produced from dinitrogen fixation. We discuss how this has led to the further upgrade of electrocatalytically produced ammonia, in combination with additional enzymes (diaphorase, alanine dehydrogenase, and transaminase), to selective production of chiral amine intermediates for pharmaceuticals. This Account concludes by discussing current and future research challenges in the field of electrocatalytic nitrogen fixation by nitrogenase.



INTRODUCTION

Generally, we think of non-membrane-associated oxidoreductase enzymes as proteins that catalyze redox reactions in homogeneous solutions. However, oxidoreductase enzymes can be interfaced to solid electrode surfaces to promote the exchange of electrons between the enzyme and the electrode, resulting in bioelectrocatalysis. Enzymatic bioelectrocatalysis can be further divided into two categories: direct and mediated bioelectrocatalysis. Direct bioelectrocatalysis commonly involves the interaction of enzymes with electrode surfaces in a manner where their cofactors can transfer electrons to/from

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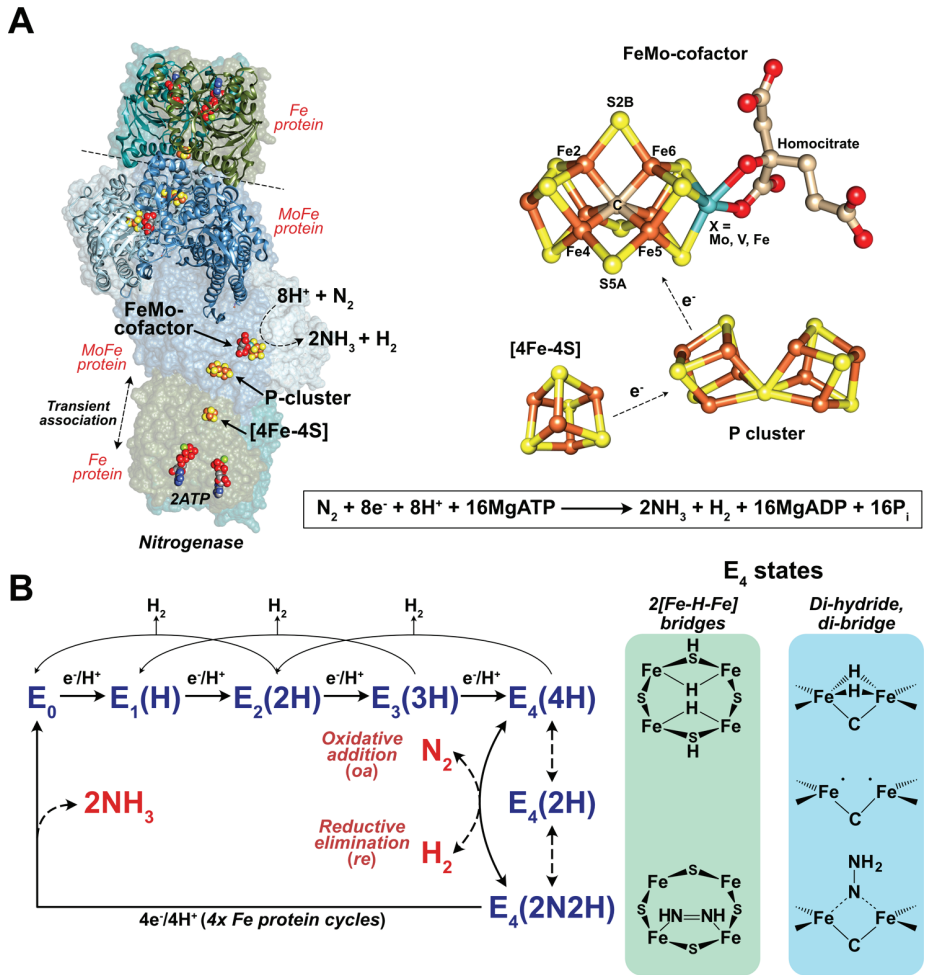

Figure 1. (A) Depiction of the Mo-dependent nitrogenase from *Azotobacter vinelandii*, adapted from the crystal structure of the Fe:MoFe protein complex formed in the presence of nonhydrolyzable ATP analogues (PDB: 4WZA). Fe = brown, S = yellow, C = beige, Mo = turquoise, and O = red. (B) Modified Lowe−Thorneley scheme for N_2 fixation by nitrogenase, showing the $E_4(4H)/E_4(2N2H)$ states as 2[Fe−H−Fe] bridges (green) or a "dihydride dibridge" (blue).

the electrode, where enzymes are typically immobilized on electrode surfaces.[1] In this situation, the electrode acts as either the source or sink of electrons. Although there are a variety of oxidoreductase enzymes (mostly metalloproteins) that can undergo direct bioelectrocatalysis, the largest class of oxidoreductase enzymes cannot undergo direct bioelectrocatalysis, because their cofactor (NAD(P)/NAD(P)H) freely dissociates and its electro-oxidation/reduction is often inefficient. Therefore, they find application in mediated bioelectrocatalysis where a redox species (i.e., small molecule or redox polymer) shuttles electrons between the enzyme/cofactor and electrode.[2] Mediators also alleviate issues associated with immobilizing an enzyme in a specific orientation for optimal electron transfer (and bioelectrocatalysis).

From an enzymology perspective, it may be unclear why you would want to "wire" an enzyme to an electrode, but this technique has great technological advantages. For instance, the commercially available glucose sensors that are used by diabetic patients to test their blood glucose levels are mediated bioelectrocatalytic systems. The enzyme provides the selectivity and the electrode provides the ability to transduce the chemical reaction into an electrical signal for readout. Bioelectrocatalysis has also been used for energy conversion and storage devices (i.e., biofuel cells and biobatteries). However, it is only recently that bioelectrocatalysis has gained popularity for electrosynthesis, because many of the applications for bioelectrosynthesis are reductive processes and reductive bioelectrocatalysis is a challenge, because most of the mediation systems for extreme reductive potentials (beyond oxygen reduction) are unstable. Nevertheless, progress has been made toward the electroenzymatic reduction of substrates such as carbon dioxide (CO_2) to "upgraded" formate ($HCOO^-$) by formate dehydrogenases in addition to the generation of NAD(P)H that is subsequently used to drive enzymatic reductions.[3−5] This Account will focus on bioelectrochemistry and electrosynthesis with the enzyme nitrogenase, because of both its ability to catalyze a variety of reductive processes (nitrogen reduction, carbon dioxide reduction, proton reduction, etc.) as well as its applicability for downstream organic electrosynthesis.

NITROGENASE

Nitrogen is an essential element of life, because reduced nitrogen in the form of ammonia, NH_3, is an important feedstock and industrial chemical commodity.[6] Since the

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advent of the Haber Bosch (HB) process in the early 1900s, NH_3 is now estimated to be produced at >150−500 million tons per year where it is thought to indirectly contribute to ∼3% of global CO_2 emissions produced annually.[6−9] While the HB process has been engineered to be highly efficient (in terms of the selectivity of dinitrogen (N_2) reduction to NH_3), high temperatures (∼300−500 °C) and high pressures (∼150−200 atm) are required. Further, the molecular hydrogen (H_2) required for the HB process is typically produced by energy intensive processes such as steam reforming of natural gas (methane, CH_4) or from other fossil fuel sources. Consequently, it is thought that, in total, the HB process consumes over 1% of the annual energy produced globally.[6,10] In contrast to this synthetic process, biological N_2 reduction (or "fixation") to NH_3 takes place at near-ambient temperature and pressure by way of a single enzyme, nitrogenase, which can achieve up to 75% efficiency in terms of selectivity for N_2 reduction while also producing one mole of H_2 for every mole of N_2 that is reduced.[11] However, biological nitrogen fixation can also be considered to be energy intensive; the hydrolysis of adenosine triphosphate (MgATP, detailed below) in vivo typically corresponds to a ΔG of −50 kJ mol^{-1},[12] and nitrogenase requires the hydrolysis of 16 MgATP for N_2 fixation.[11]

Found in select bacteria and archaea, nitrogenase is the collective term for one of three nitrogenase systems that all employ a reductase (Fe protein) and a catalytic protein that is dependent on iron and sulfur-based metallocofactors (FeFe protein), but can also incorporate molybdenum (MoFe protein) or vanadium (VFe protein) (Figure 1A).[8] Perhaps the most studied system is the Mo-dependent nitrogenase (composed of a Fe protein and MoFe protein) from *Azotobacter vinelandii*, and this will primarily be referred to herein in order to outline aspects of N_2 fixation by nitrogenase.[11,13,14] The reductase Fe protein (encoded by *nifH* in *A. vinelandii*) is a ∼66 kDa homodimer that harbors a single [4Fe-4S] cluster that bridges both monomers (ligated by two cysteine residues on each monomer in *A. vinelandii*) and can associate one ATP or similar nucleoside phosphates on each monomer. The catalytic MoFe protein is a $\alpha_2\beta_2$ tetramer (∼240 kDa in total encoded by *nifD* and *nifK* in *A. vinelandii*) where each $\alpha\beta$ unit interacts with the Fe protein in a transient association event. Each $\alpha\beta$ half contains an [8Fe-7S] cluster ("P" cluster) which bridges the $\alpha\beta$ half and is primarily ligated by cysteine residues in both NifD (α subunit) and NifK (β subunit), although additional serine and tyrosine residues located around the P cluster are thought to be important to transient states of the P cluster formed during catalysis.[15,16] The α subunit of the MoFe protein also contains a [7Fe−Mo−9S−C-homocitrate] cofactor (FeMo-co), ligated by a histidine and a cysteine residue; N_2 is reduced to NH_3 at the FeMo-co.

Decades of research has been performed in order to try to understand how nitrogenase fixes N_2.[13] While the Fe protein is known to transfer electrons to the MoFe protein (coupled to the hydrolysis of MgATP) and the MoFe protein is known to be the N_2-reducing component, research has sought to elucidate the order-of-events by which electrons are transferred between and across the Fe and MoFe proteins during their transient association. Early work by Lowe and Thorneley resulted in a kinetic model by which eight transient associations between the Fe and MoFe proteins leads to the reduction of N_2 to NH_3 by nitrogenase (Figure 1B).[11,17] The reduction of N_2 only requires six electrons (and thus six optimal transient associations and single electron transfers), and it is thought that the two additional transient associations are a necessary cost required to activate the FeMo-co for N_2 binding (and subsequent reduction); this results in the evolution of H_2, which accounts for two transient association steps (and the transfer of two electrons).[18,19]

In contrast to Mo-dependent nitrogenase, the vanadium-dependent and iron-only nitrogenases exhibit improved alternative substrate reactivities.[20] The recently determined X-ray crystal structure of the VFe protein revealed expected similarities to the MoFe protein.[21] The genes that encode for Fe protein and the catalytic VFe protein under the *vnf* operon are *vnfH* and *vnfDGK*; the VFe protein is a $\alpha_2\beta_2\gamma_2$ hexamer which, like the MoFe protein, operates as two $\alpha\beta\gamma$-halves that bind to two Fe proteins (specifically encoded for the V-dependent nitrogenase system). In addition to H^+, C_2H_2 and N_2, the VFe protein is able to utilize CO and CO_2 as substrates; excitingly, their reduction by VFe nitrogenase in solution assays (with or without the Fe protein) have yielded the production of hydrocarbons ranging from methane to butane in a biological version of Fischer−Tropsch chemistry.[22] CO/CO_2 can also be reduced by the MoFe and FeFe nitrogenases, although V-dependent nitrogenase is widely considered to be better-suited to this reactivity.

NITROGENASE BIOELECTROCHEMISTRY OF THE CATALYTIC PROTEIN OF NITROGENASE

Previous proposals that (i) electron transfer from the Fe protein takes place prior to (and independent of) MgATP hydrolysis and that (ii) the P cluster is reduced by deficit spending following electron transfer to the FeMo-co indicate that it may be possible to reduce the MoFe protein (specifically, substrates at the FeMo-co) with artificial small-molecule electron mediators in a fashion that supports substrate turnover at detectable rates and quantities.[23,24] Reduced Eu-chelates were found to be able to artificially reduce the MoFe protein and support the reduction of some nitrogenase substrates (although not N_2), such as H^+, C_2H_2, and N_2H_2.[25] In these approaches, bulk electrolysis was used to reduce Eu(III) to Eu(II) prior to the addition of various chelating molecules (generating low-potential Eu(II) complexes suitable for electron transfer to the MoFe protein) in a decoupled, "batch-mode" fashion. In this study, a β-98His variant (replacing a Tyr residue) was found to have improved catalytic rates when used alongside these Eu(II) electron donors.

Based on the reported observations that electron transfer takes place from the [4Fe-4S] cluster of the Fe protein to the P cluster prior to and independent of MgATP hydrolysis, our initial work sought to artificially transfer electrons to the MoFe protein of nitrogenase by an electrochemical approach. In this case, it was hypothesized that an electrode could reduce nitrogenase by delivering electrons to the FeMo-co via the P cluster. Typically, an enzyme can be "wired" to an electrode directly or by the use of a small molecule electron mediator/shuttle.[26] In the case of mediated electron transfer (MET), the electrode regenerates the reducing equivalents in situ and thereby permits continuous electrocatalysis as opposed to a single batch experiment. While direct electron transfer (DET) has been used to characterize other iron−sulfur metalloproteins (reporting turnover and nonturnover bioelectrochemical properties), the protein or enzyme must be oriented in such a fashion that the distance-dependence of electron

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transfer is between the electrode and metallocofactor is minimized.[27,28] Electron mediators are not well-suited to determine nonturnover bioelectrochemical properties of metalloproteins (although microcoulometry has had some limited success in this area);[29] however, they can efficiently facilitate mediated bioelectrocatalysis where electrodes can then support enzymatic turnover at physiological rates. Further, this bioelectrocatalytic approach has the added benefit of providing a readout of the number of electrons (charge) transferred to the enzyme during the experiment. Finally, sensitivity can be enhanced by immobilizing the metalloenzyme at the electrode surface (often within a polymeric matrix) such that the effective concentration of the metalloenzyme is relatively large at the surface of the electrode, while using minimal quantities of metalloenzyme.

Thus, we initially sought to immobilize the MoFe protein at the surface of electrodes in a 3D matrix in a collaborative study between the Minteer and Seefeldt groups (Figure 2A).[30] Given that electrochemistry of isolated FeMo-co had previously been reported in an aprotic solvent (which indicated that the first reduction of FeMo-co takes place at ~-550 mV vs SHE) and that electrogenerated low-potential Eu(II) chelates ($E^{0\prime}$ < −1100 mV vs SHE) had shown some promise for artificial electron transfer to the MoFe protein, we elected to investigate

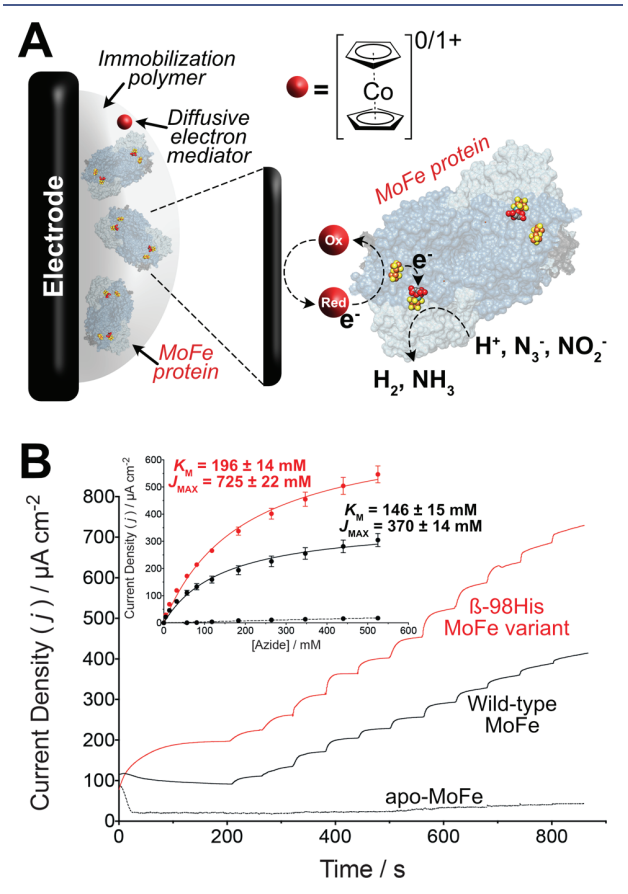

Figure 2. (A) Immobilization of the MoFe protein within a polymeric matrix at the surface of a carbon electrode. (B) Amperometric *i*–*t* curve for N_3^- reduction by the wild-type MoFe (black), the *β*-98His MoFe variant (red), and apo-MoFe (dashed black line). A reductive potential was applied to stimulate MoFe bioelectrocatalysis and N_3^- was titrated at intervals. Adapted with permission from ref 30. Copyright 2017 *Energy & Environmental Science*.

low-potential redox couples as electron mediators for MoFe protein bioelectrocatalysis.[25,31] The reduction potential of the $[4Fe\text{-}4S]^{2+/1+}$ couple of the Fe protein is approximately −430 mV vs SHE (following MgATP association), which despite the high potential of the $N_2/2NH_3$ couple (E^0 = +275 mV vs SHE) and not considering a dynamic change in the Fe or MoFe protein that may modulate the potential of reducing equivalents, indicates that the reduction potential of electron mediators to the MoFe protein should be more negative than this value (i.e., < −420 mV vs SHE).[32] Further, the reduction potential of the $P^{N/1+}$ and the $P^{1+/2+}$ states is −307 mV (vs SHE). Finally, the reduction potential for the one-electron oxidation of the resting state of the FeMo-co (M^N to M^{OX}) is approximately −42 mV vs SHE and the one-electron reduction of M^N to the M^R state is thought to have an approximate reduction potential of −465 mV vs SHE, although this has not been measured.[33,34]

Carbon electrodes were employed for electrochemical testing (due to anticipated H_2 evolution at Au or Pt electrodes in aqueous buffered electrolytes at near-neutral pH), and bis(cyclopentadienyl)cobalt(III) ($E^{0\prime}$ = −960 mV vs SHE, cobaltocene/cobaltocenium, Cc/Cc^+)[35] was found to act as an electron mediator for the MoFe protein, where H^+ reduction was initially evaluated in this communication. We also reported that this approach could support azide (N_3^-) reduction and nitrite (NO_2^-) reduction by the MoFe protein; however, we did not initially observe significant N_2 fixation. Given the complexity of nitrogenase's cofactors and the previous electrochemical investigation of the FeMo-co in aprotic solvents, we sought to confirm that the apparent bioelectrochemical approach observed in our hands was due to mediated bioelectrocatalysis of the intact MoFe protein and not a result of dissociated cofactors or partially denatured MoFe protein. To address this concern, we employed the above-mentioned *β*-98His variant which had previously been found to enhance substrate reduction when coupled with Eu(II) electron donors over the wild-type *β*-98Tyr protein.[25] By cyclic voltammetry, improved reductive catalytic currents were observed when an equivalent quantity of the *β*-98His variant was immobilized at the electrode surface in the place of the wild-type MoFe protein for H^+, N_3^-, and NO_2^- reduction. We also investigated the apparent Michaelis kinetics of the immobilized wild-type and *β*-99His variant MoFe proteins; due to the inability to easily adjust the effective $[H^+]$ without concerns surrounding changes in protein charge/folding/inactivation, we elected to employ N_3^- as the substrate for this kinetic investigation. In this experiment, N_3^- was titrated into the buffered electrolyte of the electrochemical cell and the reductive catalytic current was recorded (Figure 2B). The Michaelis constant, K_M, was calculated to be 146 ± 15 mM N_3^- for the wild-type MoFe protein, which was not too dissimilar from the value of 196 ± 14 mM N_3^- that was calculated for the *β*-98His variant. However, the maximum current density, J_{MAX} (in the place of V_{MAX}), of the *β*-98His variant was found to be almost double that of the wild-type MoFe protein (725 ± 22 vs 370 ± 14 $\mu A\ cm^{-2}$), suggesting that the *β*-98His variant improved the bioelectrocatalytic rate with an almost unchanged affinity for N_3^-; an improved catalytic rate had been previously observed when Eu(II) chelates were employed with the *β*-98His variant. Finally, additional controls were performed by employing apo-MoFe protein which is void of FeMo-co (generated by interrupting the *nifB* FeMo-co assembly gene of *A. vinelandii* with a

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kanamycin marker).[36] Taken together, the significantly diminished bioelectrocatalytic responses obtained for the apo-MoFe protein and the enhanced bioelectrocatalytic responses obtained for the β-98His variant are strong indicators that active, intact MoFe protein bioelectrocatalysis was responsible for the currents observed in this report.

To achieve substrate reduction, nitrogenase effectively accumulates electrons at the FeMo-co. Given that the Fe protein delivers electrons one at a time, one assumption is that these incoming electrons are of the same reduction potential. In order to accommodate this theory, where sequential reductions of the FeMo-co would presumably become increasingly difficult, it has been proposed that pairs of electrons accumulated at the FeMo-co are stored as metal hydrides resulting from their recombination with a proton.[37] As depicted in Figure 1B, these metal hydrides over the FeMo-co are more commonly thought to exist as two briding [Fe−H−Fe] or as a "dihydride dibridge" (two bridging hydrides between the same two Fe centers).[17] In the absence of other substrates, nitrogenase diverts 100% electron flux toward H_2 production, where protonolysis of these [Fe−H−Fe] hydrides in the E_2−E_4 states lowers the E_n state by two electrons to E_{n-2}, and further Fe protein-associated electron transfer events provide electrons for subsequent H_2 production; the rate-limiting step is associated with P_i release from the Fe protein. However, by this electrochemical approach, the rate-limiting step in the standard assays of the Fe:MoFe complex is avoided. Further, titration of the electron mediator (Cc/Cc^+) (for which the data was not presented in the communication but is typically standard practice for mediated enzymatic electrochemistry) indicated that the concentration of the electron mediator, i.e., electron transfer, was not rate-limiting. The observation that the electrocatalytic current increased in magnitude upon the addition of N_3^- or NO_2^- to the electrochemical cell indicated that the rate-limiting step may in fact be associated with H_2 evolution (by [Fe−H−Fe] protonolysis). Presumably this explains why E_4(4H) is accessible in freeze-trapped experiments (i.e., hydride protonolysis is less-favored than the "forward" reduction of a substrate such as N_2, N_3^-, or NO_2^-) and why N_2 fixation is achievable with the proposed optimal stoichiometry of H_2:2NH_3 observed and rationalized by the "reductive elimination" mechanism proposed by Hoffman et al.[13] In other words, and as recently proposed by Harris et al.,[38] our data is consistent with the hypothesis that hydride protonolysis is unproductive/undesired and that suitable electron flux to the MoFe protein favors the forward progression of FeMo-co E_n states toward the eventual reduction of alternative substrates to H^+ such as N_2 (where H^+ reduction remains necessary to enable the accumulation of electrons of constant potential at the FeMo-co from the Fe protein).[17]

Following on from this initial study, a larger collaborative study investigated the rate-limiting step of the MoFe protein, now under electrochemical control independent of the Fe protein and MgATP hydrolysis. In this study led by the Seefeldt group, a range of MoFe variants and FeMo-co-binding proteins (important for MoFe protein assembly) were electrochemically investigated for H^+ reduction and a proton inventory was performed to evaluate whether a kinetic isotope effect (KIE) was apparent under these conditions (given that N_3^- and NO_2^- addition leads to enhanced electrocatalytic currents) (Figure 3A).[39] Excitingly, a pronounced KIE was observed where increasing [D_2O] lead to a decreased

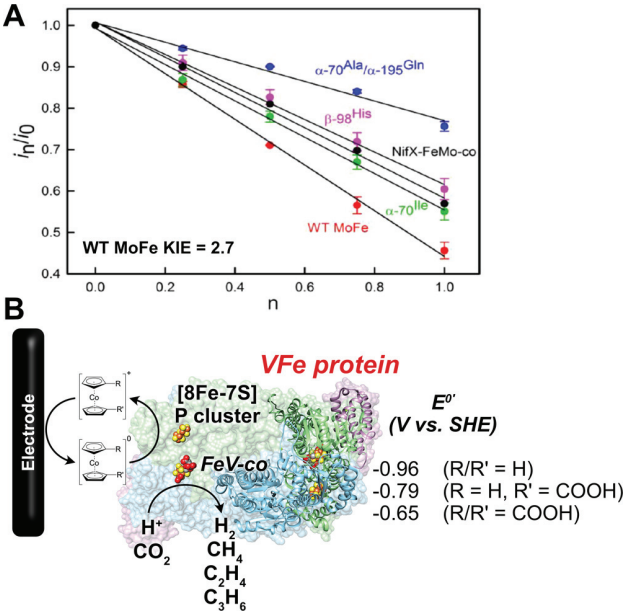

Figure 3. (A) Proton inventory plot for variant MoFe proteins and a FeMo-co-binding protein (NifX). The decrease in maximum H^+ reduction current (y axis) is reported as a function of increasing D_2O/H_2O fractions (x axis). Reprinted with permission from ref 39. Copyright 2017 American Chemical Society. (B) Cobaltocenium-mediated H^+ and CO_2 reduction by the VFe protein. Reprinted with permission from ref 40. Copyright 2018 American Chemical Society.

electrocatalytic current. First, the different KIEs observed for the MoFe protein variants and FeMo-co-binding proteins provided further evidence to support that the protein environment surrounding the FeMo-co affects electrocatalysis (i.e., electrocatalysis is simply not due to dissociated FeMo-co). Second, the linear decrease of the electrocatalytic current to increasing [D_2O] indicated that the rate-limiting step is associated with a single hydron (H^+/D^+). Computational studies reported in this work first predicted a KIE that was in close agreement with the experimentally determined KIE, and investigated the nature of the observed KIE. It was proposed that the rate-limiting step is the protonation of [Fe−H−Fe] and that the main contribution of D to the KIE is the loss of D from the S-D bond of the neighboring "belt-sulfur".

In another study, we investigated the use of Cc^+/Cc as an electron mediator for the vanadium-dependent VFe protein.[40] The VFe protein is of interest due to its exciting ability to reduce CO and CO_2 (forming upgraded C_n products). In this study, the VFe protein was evaluated in solution in order to generate large quantities of products from CO and CO_2 reduction. Interestingly, we also evaluated some Cc derivatives with more-positive potentials (1-carboxy-cobaltocenium, Cc(COOH) and 1,1′-dicarboxy-cobaltocenium, $Cc(COOH)_2$) of −788 and −648 mV vs SHE. The reductive electrocatalytic currents for these Cc derivatives decreased with a positive change in potential, presumably due to the decrease in potential difference between the electron mediator and electron-accepting P cluster ($\Delta G = -nFE$). All three Cc^+/Cc electron mediators supported H^+ reduction by the VFe protein (Figure 3B). Further, bulk electrolytic experiments confirmed that the VFe protein was able to form ethylene (C_2H_4) and propylene (C_3H_6) from CO_2 as the substrate. A similar approach was also utilized by the Seefeldt group where the Fe-

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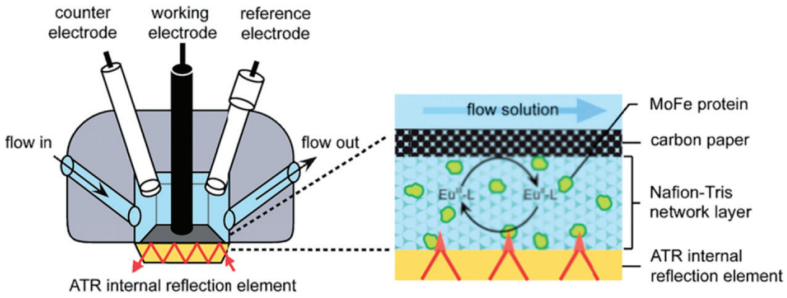

Figure 4. Spectroelectrochemistry of the MoFe protein entrapped at an electrode surface by ATR-IR. Reproduced with permission from ref 42. Copyright 2017 *Chemical Science*.

dependent FeFe protein was immobilized at electrode surfaces (also in comparison to the MoFe protein).[41] In this study, it was found that the MoFe protein was able to reduce CO_2 to $HCOO^-$; however, the FeFe protein demonstrated improved CO_2 electroreduction capability, improving on the electron flux of the MoFe protein toward $HCOO^-$ of ∼5% to ∼40% for the FeFe protein. Interestingly, ∼10% of electron flux was not accounted for and begs the question: Does electrocatalytic CO_2 reduction by the FeFe protein yield additional products (i.e., CH_4) to $HCOO^-$ and H_2?

In addition to the use of Cc^+/Cc as an electron mediator for the MoFe protein, work by Paengnakorn et al. recently demonstrated that a range of Eu-chelates could be used to mediate electrons to the MoFe, independent of MgATP hydrolysis and the Fe protein.[42] Further, the authors also immobilized the MoFe protein at the surface of a carbon paper electrode and simultaneously performed in situ infrared spectroscopy (IR) while controlling the potential of the solution and electron delivery to the MoFe protein (Figure 4). Alongside the wild-type MoFe protein, the authors also investigated apo-MoFe, the above-mentioned β-98His variant, and a further β-99His variant. Similar to our Cc^+/Cc-mediated electrochemistry, the authors also observed improved reductive catalytic currents for the variant MoFe proteins versus the wild-type MoFe protein (and very little current for the apo-MoFe protein). Interestingly, the authors also noted that a low potential (−700 to −900 mV vs SHE, an additional ∼ −250 to −450 mV beyond the H+ reduction potential under these conditions) was required to initiate catalysis by the MoFe protein under these conditions. The authors next investigated the binding of CO as an inhibitor of the MoFe protein while under electrochemical control by immobilizing the MoFe protein at a carbon electrode surface and by simultaneously employing attenuated total reflectance (ATR) IR. In comparison to the apo-MoFe protein control, the authors were able to observe the binding of CO to the FeMo-co of the MoFe protein as the applied potential decreased. While the authors observed CO binding at wavenumbers consistent with a bridging CO (as observed by X-ray crystallography),[43] the authors also observed a high wavenumber band that could resemble a Mo-bound CO (similar to that of molybdenum hexacarbonyl). In conclusion, this paper presents a promising spectroelectrochemical technique by which ligand binding to the MoFe protein can be characterized independent of the Fe protein and MgATP hydrolysis.

In the most recent work of the Minteer lab, the possibility of transferring electrons to the catalytic MoFe nitrogenase protein by DET (or, direct bioelectrochemistry) was explored.[44] In this study, a polymer (linear polyethylenimine) functionalized with pyrene moieties was reported to facilitate DET of a wide range of enzymes at electrode surfaces. A redox couple was observed by square wave voltammetry at approximately −512 mV vs SCE (saturated calomel electrode) for the MoFe protein, which was attributed to the P cluster (−307 mV vs SHE). While the reduction of H^+ and NO_2^- was reported (similarly to the cobaltocene system), the study also showed that N_2 could be reduced by nitrogenase with this bioelectrocatalytic approach to ammonia. This electrode modification can also be used to investigate the thermodynamics of VFe and FeFe proteins, as well as mutants.[45]

BIOELECTROCHEMISTRY OF THE COMPLETE NITROGENASE Fe/MoFe PROTEIN SYSTEM

With an interest in alternative biotechnologies for N_2 fixation to NH_3, we also investigated bioelectrochemical NH_3 production with the Fe protein (and associated MgATP hydrolysis), due to our inability to observe N_2 fixation by the above mediated bioelectrochemical studies at that time. Further, a larger quantity of nitrogenase can be present in the electrolyte than what is typically permissible at the surface of an electrode, and Fe:MoFe transient association is presumably inhibited by co-immobilizing both proteins at an electrode surface. The electron donor for activity assays of nitrogenase is *almost exclusively* dithionite (DT); however, a recent study indicated that the rate of the Fe protein reduction by DT (when bound to MgADP) could be improved by employing methylviologen (*N*,*N*′-dimethyl-4,4′-bipyridinium (MV), $E^{0\prime}$ $MV^{2+/+\bullet}$ = −450 mV vs SHE) as an additional electron mediator: DT reduces MV^{2+} by one electron to $MV^{+\bullet}$ which subsequently reduces the Fe protein.[46] Unlike DT, the $MV^{2+/+\bullet}$ redox couple can be efficiently oxidized or reduced at carbon electrodes (permitting control of Fe protein-dependent nitrogenase turnover) and as such, MV is a common mediator choice for hydrogenase electrochemistry (for H_2 oxidation). Thus, we sought to stimulate nitrogenase activity in the absence of DT and using an electrode to generate reduced $MV^{+\bullet}$ (Figure 5).[47] Bulk electrolysis at a mild applied potential of −610 mV vs SHE resulted in the production of NH_3 with a Faradaic efficiency of 59% at room temperature and under 1 atm of N_2 at pH 7. This Faradaic efficiency is promising for future NH_3-producing biotechnological technologies where the above-detailed reductive elimination mechanism of nitrogenase would indicate that a faradaic efficiency of 75% would be the maximum achievable value. We also demonstrated the concept of generating a small potential difference from an enzymatic fuel cell configuration; in this system, the anode employed hydrogenase for H_2 oxidation and the cathode employed the above MV-driven nitrogenase assay. MV was employed as the

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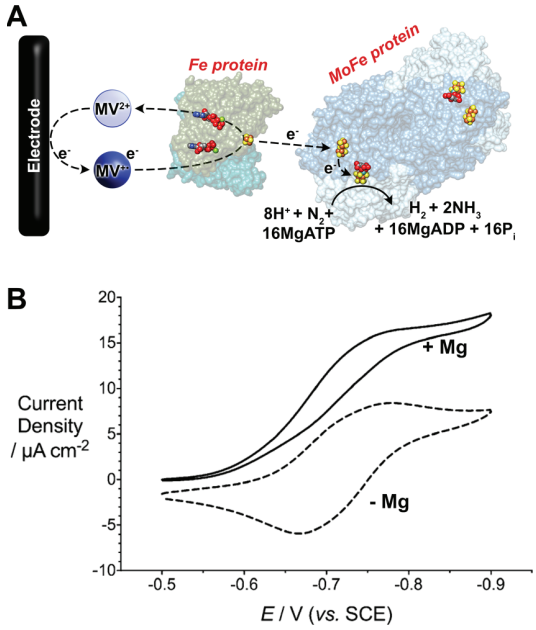

Figure 5. (A) MV-mediated bioelectrochemical N_2 fixation by Mo-dependent nitrogenase. (B) Cyclic voltammograms of bioelectrocatalytic N_2 fixation in the absence or presence of Mg (required for ATP hydrolysis by the Fe protein), pH 7 100 mM MOPS at 2 mV s^{-1}. Adapted with permission from ref 47. Copyright 2017 *Angewandte Chemie International Edition*.

anodic and cathodic mediator where a maximum open circuit potential (OCP) difference between the two compartments (separated by a Nafion membrane) of +275 mV would be expected based on the $E^{0\prime}$ values of the $2H^+/H_2$ and $N_2/2NH_3$ couples under the same conditions at equilibrium. Non-exhaustive OCP evaluation (for only 10 min) demonstrated that 228 ± 28 mV was easily achievable with the anodic chamber operating at pH 9.0 to favor H_2 oxidation by hydrogenase (equivalent to an OCP of ~ 110 mV at pH 7.0). A potential difference of 10 mV was applied to the enzymatic fuel cell for around 2 h, during which time approximately 60 mC of charge was generated alongside 286 nmol of NH_3 (mg^{-1} of MoFe protein), corresponding to a Faradaic efficiency of 26%. It is important to note, however, that this system requires a MgATP regenerating system to phosphorylate ADP with creatine phosphate and creatine phosphokinase. This system later also proved to be valuable in a study by the Seefeldt group,[48] because it is possible to monitor the quantity of charge passed (i.e., the number of electrons transferred to nitrogenase) during the experiment by this approach. The authors noted that previous nitrogenase work had reported that N_2 fixation by nitrogenase (under 1 atm of N_2) had decreased total electron flux through nitrogenase by up to 30% (in comparison to H_2 generating experiments in the absence of N_2).[49,50] With the above electrochemical approach, the authors found that electron flux through nitrogenase in fact remained constant under Ar or up to 1 atm of N_2, where the rate-limiting step was found to be 14 s^{-1}.

ELECTROSYNTHESIS OF VALUE-ADDED PRODUCTS

Although bioelectrocatalytic ammonia production is interesting, ammonia is a commodity chemical used for fertilizers, but also for the production of many value-added chemical products (i.e., pharmaceuticals and agrochemicals). Therefore, there is interest in bioelectrocatalytic production of upgraded nitrogen-based products. For instance, many pharmaceutical molecules are formed from chiral amines and the production of enantiomerically pure chiral amines is challenging to the organic chemistry community.[51−53] These products are considerably higher in value than the ammonia used to form them. Therefore, the Minteer group has developed an enzyme cascade utilizing nitrogenase, diaphorase, and alanine dehydrogenase to electrochemically drive transaminase far from its reactant favored equilibrium to produce chiral amines.[54] This system uses the same methyl viologen mediation system discussed above, where methyl viologen is mediating both nitrogenase and the cofactor regeneration by diaphorase. Figure 6 shows the enzyme cascade that combines methyl

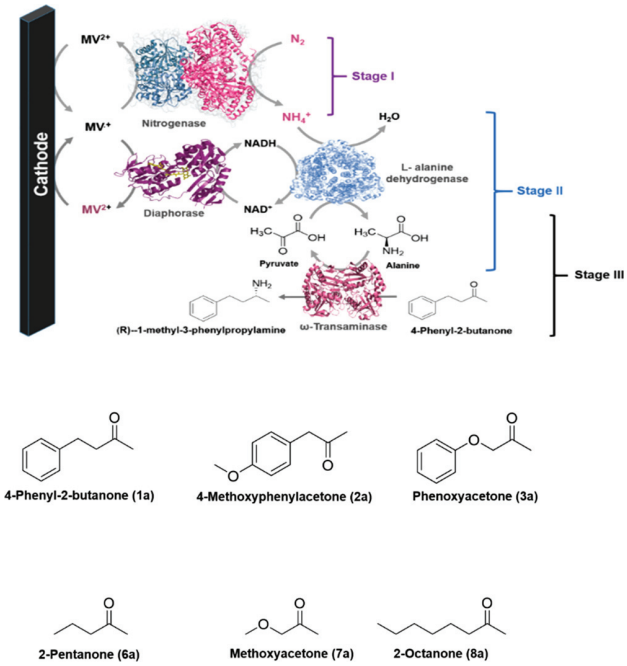

Figure 6. (Top) Schematic representation of the upgraded bioelectrocatalytic N_2 fixation system with components and the reaction process of the upgraded N_2 fixation system. (Bottom) Scope of prochiral ketones that can be used as substrates to produce chiral amines via this electrosynthesis system. Adapted with permission from ref 54. Copyright 2019 American Chemical Society.

viologen mediated ammonia bioelectrosynthesis with methyl viologen mediated cofactor regeneration for producing chiral amines via the common enzyme transaminase, as well as a sample scope of prochiral ketone substrates. This proof-of-concept work shows the ability of nitrogenase electrosynthesis to produce value-added products for the pharmaceutical or agrochemical industry.

CONCLUSIONS AND PERSPECTIVES

Nitrogenase is an interesting enzymatic system, because it catalyzes the reduction of kinetically inert dinitrogen to ammonia, but also it is promiscuous, so it can be used for a variety of electrosynthesis systems. There are fewer reductive mediators in the literature, but we have found cobaltocene/cobaltocenium mediators for mediating the catalytic protein

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and viologen mediators for mediating the Fe protein. We have shown utility for nitrite and nitrogen reduction to ammonia, carbon dioxide reduction, and even chiral amine production. It is important to note that the mediation schemes discussed above are mediators in solution. Technologically for scale-up and industrial uses, mediators need to be immobilized at the electrode surface. This is commonly done by entrapment or covalent attachment to a polymer backbone to form a redox polymer.[55] These strategies will need to be investigated in the future of bioelectrosynthesis.

Pyrene and pyrene-functionalized linear polyethylenimine have shown the ability to tailor carbon electrode surfaces for direct electron transfer between the MoFe catalytic protein and the electrode. However, there are still issues with nitrogenase electrochemistry, including its high sensitivity to oxygen, low turnover rates when immobilized, and general enzyme stability (i.e., denaturation, loss of cofactors, or being degraded by proteases). One approach to address this is to use microbial bioelectrocatalytic systems in the future. There are a couple of examples of using microbial electrochemical systems for ammonia production[56−58] and many reports of carbon dioxide reduction,[59−61] but in theory, with the use of synthetic biology, this strategy could be used for upgrading to other nitrogen-based products rather than just commodity products and biofuels.

AUTHOR INFORMATION

Corresponding Authors

*Email: ross.milton@unige.ch.
*Email: minteer@chem.utah.edu.

ORCID

Ross D. Milton: 0000-0002-2229-0243
Shelley D. Minteer: 0000-0002-5788-2249

Notes

The authors declare no competing financial interest.

Biographies

Ross D. Milton is an Assistant Professor in Inorganic Chemistry at the University of Geneva (Switzerland). He obtained a B.Sc. (2010) and Ph.D. (2014) in Chemistry from the University of Surrey (UK) where he investigated inhibition mechanisms of enzymatic fuel cells. He then held a Marie Skłodowska-Curie Postdoctoral Fellowship ("Bioelectroammonia") with Shelley Minteer (University of Utah, USA) and Dónal Leech (National University of Ireland Galway) where he studied nitrogenase electrochemistry. He undertook a second postdoctoral position with Alfred Spormann (Stanford University, USA) where he investigated metalloenzyme electron transfer mechanisms in methanogenic archaea. His research program focusses on the use of electrochemistry to study electron transfer mechanisms of enzymes, such as nitrogenase, to ultimately direct the design of bioinspired technologies.

Shelley D. Minteer is the Dale and Susan Poulter Endowed Chair in Biological Chemistry at the University of Utah. She obtained her Ph.D. in electroanalytical chemistry at the University of Iowa in 2000 under the direction of Johna Leddy after receiving her B.S. in chemistry at Western Illinois University in 1995. She was then a faculty member in the Department of Chemistry at Saint Louis University from 2000, until she moved her research group to University of Utah in 2011. She is the Center Director for the National Science Foundation Center for Synthetic Organic Electrochemistry. Her research interests include biological and bioinspired electrochemical systems for energy storage, energy conversion, sensing, and electrosynthesis.

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