Electron Flow and Management in Living Systems: Advancing Understanding of Electron Transfer to Nitrogenase

Rhesa N. Ledbetter
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ELECTRON FLOW AND MANAGEMENT IN LIVING SYSTEMS:
ADVANCING UNDERSTANDING OF ELECTRON TRANSFER
TO NITROGENASE

by

Rhesa N. Ledbetter

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biochemistry

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Logan, Utah

2018
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ABSTRACT

Electron Flow and Management in Living Systems: Advancing Understanding of Electron Transfer to Nitrogenase

by

Rhesa N. Ledbetter, Doctor of Philosophy

Utah State University, 2018

Major Professor: Dr. Lance C. Seefeldt
Department: Biochemistry

Diazotrophic microorganisms use the enzyme nitrogenase to reduce dinitrogen (N₂) into a biologically available ammonia (NH₃) and play a critical role in the global nitrogen cycle. Although there is a wealth of knowledge on many aspects of this enzyme, we have a limited understanding of how low potential reductants, in the form of reduced flavodoxin and ferredoxin, are generated and their interaction with nitrogenase. This research provides new insight into the mechanism of electron flow to nitrogenase and introduces a bioelectrochemical system for the production of fixed nitrogen.

A new mechanism known as electron bifurcation was demonstrated to generate reduced flavodoxin for nitrogen fixation in *Azotobacter vinelandii*. The reaction, facilitated by a protein complex known as FixABCX, used NADH as the electron donor to couple the exergonic reduction of coenzyme Q to the endergonic reduction of flavodoxin without the use of ATP or an electrochemical gradient.

Further, the rate limiting step of nitrogenase catalysis was revealed to be events associated with Pi release rather than protein-protein dissociation as previously
suggested. Because nitrogenase is readily reduced by artificial chemical reductants such as dithionite, few in vitro studies investigating the nitrogenase catalytic cycle had been conducted using physiological electron donors, such as flavodoxin. By comparing the rate constants of key steps in the nitrogenase catalytic cycle using dithionite and flavodoxin, novel insights into the mechanism of biological nitrogen fixation, most relevant to how it occurs in vivo, were discovered.

Biological nitrogen fixation is an attractive option for the sustainable generation of nitrogen fertilizer. Here, it was demonstrated that phototrophic, nitrogen-fixing bacteria can grow in association with an electrode, in which electrons are supplied from water oxidation and the energy provided by infra-red light. This biohybrid approach, while in its early stages, has potential for the generation of bioavailable nitrogen for food production.

Nitrogen fixation is one of the most biologically challenging reactions. Thus, understanding the fundamental mechanisms of electron flow and management can offer fresh insight into the elegant mechanisms nature has evolved. The insight may also contribute to advances in other bio-inspired applications.

(236 pages)
PUBLIC ABSTRACT

Electron Flow and Management in Living Systems:
Advancing Understanding of Electron Transfer to Nitrogenase

Rhesa N. Ledbetter

Nitrogen is a critical nutrient for growth and reproduction in living organisms. Although the Earth’s atmosphere is composed of ~80% nitrogen gas (N$_2$), it is inaccessible to most living organisms in that form. Biological nitrogen fixation, however, can be performed by microbes that harbor the enzyme nitrogenase. This enzyme converts N$_2$ into bioavailable ammonia (NH$_3$) and accounts for at least half of the “fixed” nitrogen on the planet. The other major contributor to ammonia production is the industrial Haber-Bosch process. While the Haber-Bosch process has made significant advances in sustaining the global food supply through the generation of fertilizer, it requires high temperature and pressure and fossil fuels. This makes nitrogenase an ideal system for study, as it is capable of performing this challenging chemistry under ambient conditions and without fossil fuels.

Nitrogenase requires energy and electrons to convert N$_2$ into NH$_3$. The work presented here examined how the enzyme receives electrons to perform the reaction. It was discovered that some microbes employ a novel mechanism that adjusts the energy state of the electrons so that nitrogenase can accept them. Further, the slowest step that takes place in nitrogenase once the electrons are taken up was identified. Finally, by capitalizing on fundamental knowledge, a biohybrid system was designed to grow
nitrogen-fixing bacteria in association with electrodes for light-driven production of fixed nitrogen that has potential to be used as a fertilizer for plant growth.

Gaining an in-depth understanding of nitrogenase provides insight into one of the most challenging biological reactions, and the newfound knowledge may be a catalyst in developing more efficient systems for sustainable ammonia production.
ACKNOWLEDGMENTS

Thank you to the numerous people who have been part of my Ph.D. journey. And while science isn’t a sport, it has become clear to me that it’s most definitely made up of a team.

**My head coach, Lance Seefeldt:** What a pleasure it has been working for you! There are so many qualities I admire in you—your ability to get things done without procrastinating, your gentle and kind personality, and your excellent communication skills. Above all though, I most admire your integrity. You are simply a good person that I look up to. Thank you for believing in me, even on the many days I had less than stellar data to show. Also, I will always appreciate you letting me work part-time for Utah Public Radio. Not every advisor would allow that, and I am thankful.

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scientists, who have contributed to my growth and success more than you know. But even more important to me is the friendships that have blossomed from working with you over the years.

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**My biggest fan, Tim:** It’s hard to write this, because you deserve more than my words could ever express. You have supported me in everything I have wanted to do without complaint…literally. Thank you for making me a better person, always making me laugh, and like my mom, listening to every scientific presentation I ever had to give…yes, even the one associated with this dissertation…two or three times. Now, I can’t wait to move home!

Rhesa N. Ledbetter
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CHAPTER 1
INTRODUCTION

BIOLOGICAL NITROGEN FIXATION

Nitrogen is an essential element for all living organisms as it is a constituent of proteins, nucleic acids, and other biomolecules.\textsuperscript{1,2} While the majority of Earth’s atmosphere is composed of N\textsubscript{2} gas, the triple bond between nitrogen atoms makes it chemically inert and unavailable to most living organisms. Therefore, atmospheric N\textsubscript{2} must be transformed or “fixed” into a form cells can utilize during their growth and reproduction.\textsuperscript{1}

Prokaryotes with the enzyme nitrogenase have the ability to transform N\textsubscript{2} into NH\textsubscript{3} through a process known as biological nitrogen fixation.\textsuperscript{2,3} This process is carried out by both free-living and symbiotic microorganisms known as diazotrophs.\textsuperscript{3,4} Although a relatively small number of microorganisms have the ability to reduce N\textsubscript{2}, those that do are phylogenetically and metabolically diverse, ranging from aquatic phototrophs (e.g. \textit{Rhodopseudomonas}) to soil-dwelling bacteria (e.g. \textit{Azotobacter} and \textit{Klebsiella}).\textsuperscript{4,5} Others form close symbiotic associations with plants. For example, rhizobia colonize the root system of legumes (e.g. soybeans, peas, alfalfa, and peanuts), giving plants access to fixed N in exchange for carbon from the plants.\textsuperscript{6} Biological nitrogen fixation provides over 50\% of the fixed nitrogen on Earth\textsuperscript{2,7} with 80\% of that coming from symbiotic associations and the remaining from free-living nitrogen-fixing organisms.\textsuperscript{8}
NITROGENASES

Nitrogenases are a family of enzymes found in a limited number of microorganisms that catalyze the reduction of \( N_2 \) to \( NH_3 \). The stoichiometry of the reaction under “optimal” conditions is as follows (Eq. 1-1):

\[
N_2 + 8 H^+ + 16 \text{MgATP} + 8 e^- \rightarrow 2 NH_3 + H_2 + 16 \text{MgADP} + 16 \text{Pi} \quad \text{(Eq. 1-1)}
\]

Within the nitrogenase family, there are three genetically distinct homologues, including the molybdenum (Mo), vanadium (V), and iron (Fe-only) nitrogenases.\(^9\)-\(^11\) While the basic architecture and mechanism appear to be similar among the three, the presence of Mo, V, or Fe in the active site metal cluster is one differentiating factor.\(^2\),\(^9\),\(^10\),\(^12\),\(^13\) Of the three nitrogenases, Mo nitrogenase is the best characterized and described below.

Mo nitrogenase is a two-component metalloprotein complex composed of the iron (Fe) protein and molybdenum-iron (MoFe) protein (Figure 1-1).\(^2\) The Fe protein (NifH) is an \( \alpha_2 \) homodimer whose role is to deliver electrons to the MoFe protein. The two subunits of the Fe protein homodimer are bridged by an electron-carrying [4Fe-4S] cluster, and each subunit contains an ATP-binding site.\(^14\) The MoFe (NifDK) protein is an \( \alpha_3\beta_2 \) heterotetramer with each \( \alpha\beta \) heterodimer constituting a catalytic half.\(^15\) Each half contains two metal clusters. The P-cluster [8Fe-7S] accepts electrons from the Fe protein and shuttles them to the FeMo-cofactor [7Fe-9S-Mo-C-homocitrate] where substrate reduction occurs.\(^16\) During catalysis, the Fe and MoFe protein transiently associate as the
Figure 1-1. Molybdenum nitrogenase. Top: Structural representation of Fe (green) and MoFe (α-subunit= blue; β-subunit= red) proteins of nitrogenase, including both catalytic halves (PDB ID 4WZB). Bottom: Electron flow through one catalytic half of nitrogenase.

Fe protein delivers electrons one-at-a-time to the MoFe protein. At least two ATP are hydrolyzed for each electron transferred. Following eight catalytic cycles, one N$_2$ is fully reduced to two NH$_3$ (Figure 1-1).

Although there is a wealth of knowledge on many aspects of nitrogenase, a fundamental understanding of how the low-potential reducing equivalents needed to drive nitrogen fixation are generated and their impact on the nitrogenase catalytic cycle is limited. This gap in knowledge inspired two questions addressed in this dissertation: 1) How is low-potential reductant generated for nitrogen fixation in Azotobacter vinelandii? 2) How is the rate limiting step of nitrogenase catalysis affected in the presence of a non-physiological (sodium dithionite) and physiological (Fld) reductant? The final question applied a basic understanding of biological nitrogen fixation to offer fresh insight into the
following question: 3) How can diazotrophic bacteria be harnessed to produce sustainable fixed nitrogen for food production?

**PHYSIOLOGICAL ELECTRON DONORS TO NITROGENASE**

As shown in Eq. 1-1, reducing power in the form of low reduction potential electrons is one essential element for nitrogen fixation. Small redox proteins, flavodoxin (Fld) and ferredoxin (Fd), serve as the direct electron donor to the Fe protein of nitrogenase *in vivo*.18 The redox properties of Fld and Fd are derived from bound cofactors, flavin mononucleotide (FMN) and FeS cluster(s), respectively (Figure 1-2).18

Flds are 1- or 2-electron carriers and exhibit three oxidation states: oxidized (Ox), semiquinone (Sq) and hydroquinone (Hq) forms with redox potential ranging from -50 to -250 mV for the Sq/Ox couple and -370 to -450 mV for the Hq/Sq couple.18 Because the Fe protein of nitrogenase requires electrons <-400 mV, only the Hq/Sq couple has sufficient driving force.

![Figure 1-2](image) Figure 1-2. Structural representations of NifF flavodoxin (red; FMN=yellow) and ferredoxin I (green; FeS clusters= yellow/orange) from the diazotroph *A. vinelandii* (PDB IDs 5K9B and 1FER).
Fds are distinguished by the type and number of FeS clusters they contain and have a wide range of redox potentials (0 to -645 mV) tuned by the polypeptide structure. The Fds relevant to nitrogen fixation have primarily been ascribed to those containing the [2Fe-2S] or 2[4Fe-4S] clusters.

In many cases, diazotrophs possess several Flds and Fds, and it has been demonstrated that more than one can serve as an electron donor to nitrogenase. This has presented challenges in assigning specific Flds and Fds to nitrogenase activity in the cell. An additional complexity is that these electron carriers can act in diverse metabolic processes (e.g. photosystem I, hydrogenase) and have overlapping functions. The only diazotroph in which the direct electron donor has been firmly established is *Klebsiella pneumoniae*. NifF is a flavodoxin encoded by the *nifF* gene, which is part of the nitrogenase (*nif*) gene cluster. Studies in which *nifF* was mutated rendered *K. pneumoniae* cells unable to grow under nitrogen-fixing conditions, implicating NifF as the sole electron donor for diazotrophic growth. Like *K. pneumoniae*, *A. vinelandii* harbors a homologous *nifF* gene. However, in addition to *nifF*, there is genetic evidence of at least one additional Fld and several Fds or Fd-like proteins (*Table 1-1*). NifF (Fld) and FdI (Fd) have both been demonstrated to donate electrons to Mo nitrogenase in vitro, however, their role in vivo is still being elucidated. Cells with gene disruptions in *nifF* and *fdxA* alone as well as a *nifF/fdxA* double mutant grew equally as well as wild-type cells under nitrogen-fixing conditions. These growth studies confirmed the presence of alternative electron carriers that play either a compensatory or co-functioning role. In addition to the cell growth studies, a whole-cell nitrogenase acetylene reduction assay was performed on
Table 1-1. Flavodoxins and ferredoxins in *A. vinelanda*. Adapted from Reyntjens et al.\textsuperscript{25}

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Cofactor</th>
<th>Subunit M(_r)</th>
<th>Function</th>
</tr>
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<tr>
<td><em>nifF</em></td>
<td>NifF/FldII</td>
<td>FMN</td>
<td>19,700</td>
<td>Under <em>nif</em> promoter; role in electron transfer to Mo-nitrogenase\textsuperscript{20,30}</td>
</tr>
<tr>
<td><em>fldA</em></td>
<td>FldI</td>
<td>FMN</td>
<td>19,600</td>
<td>Role in electron transfer to nitrate reductase\textsuperscript{26}</td>
</tr>
<tr>
<td><em>fdxN</em></td>
<td>FdxN</td>
<td>2 [4Fe-4S]</td>
<td>9,600</td>
<td><em>nif</em>-related; functions in M-cluster biosynthesis;\textsuperscript{32} role in electron transfer is not established</td>
</tr>
<tr>
<td><em>fixFd</em></td>
<td>FixFd</td>
<td>2 [4Fe-4S]</td>
<td>7,200</td>
<td>Unknown; in the <em>fixABCX</em> operon\textsuperscript{25}</td>
</tr>
<tr>
<td><em>vnfFd</em></td>
<td>VnfFd</td>
<td>2 [4Fe-4S]</td>
<td>6,975</td>
<td>Likely role in electron transfer to V-nitrogenase\textsuperscript{13}</td>
</tr>
<tr>
<td><em>fdxA</em></td>
<td>FdI</td>
<td>[3Fe-4S][4Fe-4S]</td>
<td>13,000</td>
<td>Unknown; not integral for nitrogen fixation\textsuperscript{20}</td>
</tr>
<tr>
<td><em>fixX</em></td>
<td>FixX</td>
<td>2 [4Fe-4S]</td>
<td>10,700</td>
<td>Part of FixABCX complex for generation of reductant for N(_2)-fixation (Chapter 2)</td>
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cells with the *nifF* disruption.\textsuperscript{30} This mutant displayed a 30% reduction in nitrogenase activity and suggested that NifF serves as a source of electrons to nitrogenase, but is not the only electron carrier in *A. vinelanda*.\textsuperscript{18,30}

Although, identifying the specific primary electron donor(s) to nitrogenase in many diazotrophs is difficult, it is clear that Flds and/or Fds play an essential role in the electron transfer process. The pathways to generate the reduced Fld or Fd, however, are just beginning to be uncovered in many diazotrophs.

**GENERATION OF REDUCTANT FOR NITROGEN FIXATION**

Four major systems capable of reducing Fld and Fd have been identified in diazotrophs thus far, including pyruvate-ferredoxin/flavodoxin oxidoreductase (PFOR),
ferredoxin-NADP$^+$ reductase, the *Rhodobacter* Nitrogen Fixation (RNF) system, and the Fix complex.

**Pyruvate ferredoxin/flavodoxin oxidoreductase (PFOR)**

PFOR catalyzes the reversible oxidation of pyruvate to acetyl CoA and CO$_2$ with Fld or Fd as the electron acceptor. In *K. pneumonia*, the *nifJ* gene encodes for a PFOR system specific for generating the reduced Fld needed for nitrogen fixation (Figure 1-3).$^{34-36}$ Like *nifF* (encodes for Fld), disruption of the *nifJ* gene in *K. pneumonia* results in non-nitrogen-fixing phenotype (Nif$^-$), implicating NifJ and NifF as the only path by which to supply the reducing equivalents for nitrogenase catalysis.

![Figure 1-3](image_url)

**Figure 1-3.** Schematic illustrating the oxidation of pyruvate to generate reduced Fld for nitrogenase in *K. pneumoniae*.
**Ferredoxin-NADP⁺ reductase**

Ferredoxin-NADP⁺ reductase is best known for its role in photosynthesis, in which Photosystem I uses Fd to catalyze the reduction of NAD(P) (Eq. 1-2).³⁷–³⁹

\[
2 \text{Fd}_{\text{red}} + \text{NADP}^+ + 2 \text{H}^+ \rightleftharpoons 2 \text{Fd}_{\text{ox}} + \text{NADPH} + \text{H}^+ \quad (\text{Eq. 1-2})
\]

However, some diazotrophic microbes, including *A. vinelandii*, *Rhodobacter capsulatus*, and nitrogen-fixing cyanobacteria, have been shown to harbor ferredoxin-NADP⁺ reductases as well. For nitrogen fixation, the reaction must run in reverse to generate reduced Fd. While this is possible, the forward reaction is generally favored, since the reduction potential of Fd is often lower than that of NADPH (\(E_{\text{m}} = -320 \text{ mV}\)). The ferredoxin-NADP⁺ reductases have been purified from *A. vinelandii* and *Rb. capsulatus* and demonstrated to reduce Fd.⁴⁰–⁴⁴ It was shown that the ferredoxin-NADP⁺ reductase from *A. vinelandii* bound specifically to FdI and that electron transfer between the two was facilitated.⁴³ To determine if this would provide a direct line for electron transfer to nitrogenase, *in vitro* acetylene reduction assays were conducted. No substrate reduction occurred, suggesting that ferredoxin-NADP⁺ reductase and FdI do not provide a route for electron transfer to nitrogenase. Interestingly, attempts to delete the ferredoxin-NADP⁺ reductase gene were lethal, indicating this protein is playing some other crucial role in the cell. Studies in cyanobacteria, on the other hand, suggest that ferredoxin-NADP⁺ reductase likely generates reductant for nitrogenase, as heterocysts (specialized nitrogen-fixing compartments) reduce acetylene when NADPH is generated.⁴⁰,⁴⁵
**Rhodobacter Nitrogen Fixation (RNF)**

The RNF complex is a putative reversible membrane-bound complex that couples Fld/Fd to the pyridine nucleotide pool.\(^{46}\) First identified in the diazotroph *Rb. capsulatus*, RNF complexes are distributed among diverse nitrogen-fixing and non-nitrogen-fixing microbes.\(^{46}\) Ten *rnf*-related genes have been identified in *Rb. capsulatus*, and while gene composition and organization can vary among organisms, a general model for generating reduced Fld/Fd for nitrogen fixation is shown in **Figure 1-4**.\(^{18,22,46}\) Initial observations of protein sequences revealed the certain RNF proteins share similar primary structure with sodium ion pumps\(^{18,47,48}\) and although there are limited biochemical studies, some have indeed demonstrated ion transport.\(^{46,49}\) Thus, a proton or sodium ion motive force generated from electron transport processes appears to be used to drive the reduction of Fld/Fd (\(E_m =<-400\) mV) using a pyridine nucleotide like NADH (\(E_m = -320\) mV) as the electron donor.\(^{46}\)

**Figure 1-4.** Model for the generation of reduced Fld/Fd for nitrogen fixation using the RNF complex.
Among diazotrophs, RNF appears to be the primary pathway for generating reductant for nitrogen fixation in \textit{Rb. capsulatus}. Disruption of genes in \textit{rnf} operon of \textit{Rb. capsulatus} resulted in a significant decrease in nitrogenase activity and abolished the ability of cells to grow under nitrogen-fixing conditions.\textsuperscript{50,51} Additionally, nitrogenase activity increased 50-100\% over wild-type upon overexpression of \textit{rnf} genes,\textsuperscript{52} supporting its nitrogenase-related function in this organism.

While most organisms only have a single RNF complex, \textit{A. vinelandii} harbors two: one that is \textit{nif}-regulated (\textit{rnf1}) and another constitutively expressed (\textit{rnf2}). Gene disruptions in \textit{rnf1} and \textit{rnf2} delayed diazotrophic growth, however, \textit{Δrnf1, Δrnf2}, and \textit{Δrnf1/rnf2} mutants could still fix nitrogen, albeit at decreased activities.\textsuperscript{53} It was suggested that the RNF complexes did not play role in electron transfer to nitrogenase, but rather served a regulatory role in expression and maturation of the enzyme in \textit{A. vinelandii}.\textsuperscript{53} However, more recent research presented in Chapter 2 has also demonstrated a role in electron transfer to nitrogenase.

\textbf{Fix complex}

Rhizobia form associations with plants and in the early 1980s, a suite of genes essential to their ability to fix nitrogen were discovered.\textsuperscript{54–56} These genes were termed \textit{fix}, and one gene cluster, known as \textit{fixABCX}, is conserved among rhizobia as well as some free-living diazotrophs, such as \textit{A. vinelandii, Rhodopseudomonas palustris}, and \textit{Rhodospirillum rubrum}.

A study in \textit{Azorhizobium caulinodans} indicated that \textit{FixABCX} may promote the formation of functional nitrogenase complexes,\textsuperscript{57} and others proposed roles in electron transfer. While \textit{FixABCX} could function in multiple capacities, in was not until 2004 that
a function in electron transport became apparent. Mutants of fix genes in Rh. rubrum had 25% of nitrogenase activity compared to wild-type, but in vitro nitrogenase assays demonstrated full functionality of the enzyme, suggesting that Fix provides an electron transfer pathway in vivo.\textsuperscript{22,58}

These genetic studies prompted further investigation into how FixABCX might generate the low-potential electrons needed for nitrogen fixation. The protein sequences encoded by fixABCX are homologous to electron-transferring flavoproteins (Etfs).\textsuperscript{59–62} Some Etfs are electron bifurcating, meaning they couple an endergonic and exergonic reaction to maximize energy conservation. By harnessing the energy from the exergonic reaction, cells can drive thermodynamically unfavorable reactions.\textsuperscript{63–65} All known electron bifurcation processes reduce or oxidize Fld/Fd; thus, it was hypothesized that FixABCX takes two electrons from NADH and sends one electron to the a high-potential donor proposed to be CoQ (based on FixCX homology to an Etf-ubiquinone oxidoreductase) and the other electron to reduce Fld/Fd (Figure 1-5).\textsuperscript{58,65}

\textbf{Figure 1-5.} Pathway of electron bifurcation by the FixABCX complex to generate reduced Fd/Fld for nitrogen fixation.
While there has been significant speculation on how FixABCX might provide a route for electron transfer to nitrogenase, **Chapter 2** provides the first biochemical evidence of an electron bifurcation pathway to generate reductant.

Multiple pathways have been identified to generate the low potential reductant needed for nitrogenase catalysis. This reductant, in the form of Fld/Fd, then provides electrons to nitrogenase for N\textsubscript{2} reduction.

**THE IRON PROTEIN CYCLE**

Reduced Fld/Fd delivers an electron (<-400 mV) to the [4Fe-4S] cluster of the Fe protein. The reduced Fe protein with two bound ATP then transiently associates with MoFe protein to deliver the electron.\textsuperscript{66} A series of events known as “the Fe protein cycle” take place in the Fe protein upon its interaction with MoFe protein.\textsuperscript{67} First, conformationally-gated electron transfer occurs ($k_{\text{ET}} = 140$ s\textsuperscript{-1}), in which the MoFe protein P-cluster transfers its electron to FeMo-cofactor. The oxidized P-cluster is quickly back-filled with the electron from the reduced Fe protein.\textsuperscript{68} This is followed by several steps, which occur in the following order: ATP hydrolysis ($k_{\text{ATP}} = 70$ s\textsuperscript{-1}), Pi release ($k_{\text{Pi}} = 16$ s\textsuperscript{-1}), dissociation of Fe protein from MoFe protein ($k_{\text{diss}} = 6$ s\textsuperscript{-1}), and finally re-oxidation of the Fe protein and nucleotide exchange (**Figure 1-6**).\textsuperscript{66,67} This completes the Fe protein cycle, and another round of electron transfer to MoFe protein can take place.

In addition to the physiological reductants, Fld/Fd, the Fe protein also readily accepts electrons from the small molecule sodium dithionite (Na-dithionite). Because of this, the majority of *in vitro* studies on nitrogenase have been conducted using this chemical reductant due to ease of use. The rate-limiting step of catalysis with Na-
Figure 1-6. The Fe protein cycle. Ovals represent ½ of the MoFe protein (P= P-cluster; M= FeMo-cofactor) and squares represent the Fe protein (Red= Reduced; Ox= Oxidized). Adapted from Patil et al.²

dithionite appeared to be dissociation of the Fe protein from MoFe protein (k<sub>diss</sub>= 6 s<sup>-1</sup>),¹,¹⁷,⁶⁷ but a recent study of the Fe protein cycle with the physiological reductant Fld revealed the rate-limiting step is Pi release (Chapter 3).

CAPITALIZING ON BIOLOGICAL NITROGEN FIXATION

Fundamental knowledge gained from studying the enzyme nitrogenase is essential as new technologies for sustainable fixed nitrogen production are considered. The availability of fixed nitrogen on Earth is often the major limiter of primary productivity.⁶⁹ In fact, biological nitrogen fixation on its own no longer meets the world’s needs due to population growth and increased food demands over the past century.² This has prompted the development of additional processes, including Haber-Bosch. Developed in the early 1900s, Haber Bosch manufactures nearly all of the chemically-fixed nitrogen (primarily
used in fertilizer) using an iron catalyst to make \( \text{NH}_3 \) from \( \text{N}_2 \) and \( \text{H}_2 \).\textsuperscript{7,70,71} While this process has revolutionized the global food industry, it requires high temperature and pressure, consumes about 2% of the world’s annual energy production, burns fossil fuels, and emits greenhouse gasses.\textsuperscript{72} Due to the environmental impacts and unsustainable nature of Haber Bosch, there is significant interest in greener approaches to generate fixed nitrogen on a large scale.\textsuperscript{73,74} Further, with plans for human space travel to Mars, an alternative to Haber Bosch for fertilizer production will be a necessity for sustainable food production.

Nitrogenase functions optimally under mild conditions and processes developed to capitalize on it would help eliminate or minimize fossil fuel use.\textsuperscript{74} The challenge of biological nitrogen fixation is not necessarily generating the fixed nitrogen, it is accomplishing it at a scale and price that is not only practical, but competitive with industry.\textsuperscript{75} While many scientists might argue that “alternative” approaches have a long way to go, they agree that continued efforts in this area are needed.\textsuperscript{74,76,77}

Recent advances in biotechnological applications of biological nitrogen fixation have primarily focused on two approaches: 1) engineering plants so they can associate with nitrogen-fixing microbes and 2) engineering plants to harbor a functional nitrogenase.\textsuperscript{78} However, another approach is emerging, in which the potential of photosynthetic diazotrophs attached to electrodes is being explored.\textsuperscript{79} An electrode offers economical source of electrons and bypasses the need for organic acids or thiosulfate typically required by the microbes, and recent work using \textit{R. palustris} established its ability to accept electrons from an electrode using \text{CO}_2 as the sole carbon source.\textsuperscript{79}
Figure 1-7. Essential steps in a phototrophic, nitrogen-fixing microbe, illustrating how these diazotrophs can use the energy from light and electrons from various sources to power nitrogenase for the production of fixed nitrogen and organic carbon. An approach like this could provide insights into applications for sustainable fixed nitrogen production.

Further, the phototrophic diazotrophs offer several key features ideal for biological nitrogen fixation on a larger-scale. They can achieve light-driven nitrogen fixation, in addition to fixing CO\textsubscript{2} (Figure 1-7). Overall, this approach capitalizes on the light-driven synthesis of ATP with electrons provided electrochemically to produce fixed carbon and nitrogen. Initial studies as well as continued directions aimed at demonstrating CO\textsubscript{2} and N\textsubscript{2} reduction via microbial electrosynthesis are addressed in Chapter 4.

CONCLUSION

Nitrogen fixation is arguably one of the most important processes on the planet, and it is hoped that the new findings presented in the following chapters provide a catalyst for further discoveries into one of the most intricate, yet, powerful biological enzymes on Earth.
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CHAPTER 2

THE ELECTRON BIFURCATING FIXABCX PROTEIN COMPLEX FROM
AZOTOBACTER VINELANDII: GENERATION OF LOW-POTENTIAL REDUCING
EQUIVALENTS FOR NITROGENASE CATALYSIS

ABSTRACT

The biological reduction of dinitrogen (N$_2$) to ammonia (NH$_3$) by nitrogenase is an energetically demanding reaction that requires low-potential electrons and ATP; however, pathways used to deliver the electrons from central metabolism to the reductants of nitrogenase, ferredoxin or flavodoxin, remain unknown for many diazotrophic microbes. The FixABCX protein complex has been proposed to reduce flavodoxin or ferredoxin using NADH as the electron donor in a process known as electron bifurcation. Herein, the FixABCX complex from Azotobacter vinelandii was purified and demonstrated to catalyze an electron bifurcation reaction: oxidation of NADH ($E_m$ = -320 mV) coupled to reduction of flavodoxin semiquinone ($E_m$ = -460 mV) and reduction of coenzyme Q ($E_m$ = +10 mV). Knocking out fix genes rendered Δrnf $A$. vinelandii cells unable to fix dinitrogen, confirming that the FixABCX system provides another electron delivery route to nitrogenase. Characterization of the purified FixABCX complex revealed the presence of flavin and iron-sulfur cofactors confirmed by native mass spectrometry, electron paramagnetic resonance spectroscopy, and transient

absorption spectroscopy. Transient absorption spectroscopy further established the presence of a short-lived flavin semiquinone radical, suggesting that a thermodynamically unstable flavin semiquinone may participate as an intermediate in electron transfer to flavodoxin. A structural model of FixABCX, generated using chemical cross-linking in conjunction with homology modeling, revealed plausible electron transfer pathways to both high- and low-potential acceptors. Overall, this study informs on a mechanism for electron bifurcation, offering insight into a unique method for delivery of low-potential electrons required for energy-intensive biochemical conversions.

INTRODUCTION

Biological dinitrogen ($N_2$) fixation is performed by diazotrophic microbes, which harbor the enzyme nitrogenase. This enzyme converts $N_2$ into bioavailable ammonia ($NH_3$) (Eq. 2-1) and accounts for at least half of the production of fixed nitrogen on Earth.$^{1-3}$

$$N_2 + 8 H^+ + 16 MgATP + 8 e^- \rightarrow 2 NH_3 + H_2 + 16 MgADP + 16 Pi \quad \text{(Eq. 2-1)}$$

As summarized by Eq. 2-1, the biological reduction of $N_2$ is an energy-demanding reaction, requiring both ATP and low reduction-potential electrons. These electrons are provided by small redox proteins, ferredoxin (Fd) and flavodoxin (Fld), which serve as direct electron donors to the iron (Fe) protein of nitrogenase.$^{4-10}$ The redox active iron-sulfur cluster(s) of Fd typically access one redox couple (Fd$^{Ox/Red}$) with midpoint reduction potentials ($E_m$) ranging from 0 to -645 mV.$^{4,11}$ Two redox couples of the flavin
in Fld are accessible, including the oxidized quinone/semiquinone (Fld$^{Ox/Sq}$) and semiquinone/hydroquinone (Fld$^{Sq/Hq}$) couples. In general, the $E_m$ of the Fld$^{Ox/Sq}$ couple ranges from $-50$ to $-250$ mV and the Fld$^{Sq/Hq}$ couple from $-370$ to $-500$ mV. Only the Fld$^{Sq/Hq}$ couple of Fld has enough driving force to donate electrons to nitrogenase. While much is known about other aspects of biological nitrogen fixation, pathways for delivery of the low-potential reducing equivalents for Fd and Fld reduction are not well understood for many diazotrophs. It has been shown that the nitrogen-fixing organism *Klebsiella pneumoniae* uses the anaerobic oxidation of pyruvate to reduce Fld, and it was proposed that other microbes likely use energy associated with the proton motive force to drive reduction of a low-potential electron donor. Recently, a new mechanism for generating reductant for nitrogen fixation was put forward. Flavin-based electron bifurcation (FBEB), considered a third fundamental form of energy conservation, couples exergonic and endergonic electron transfer reactions to limit free energy loss in biological systems. FBEB exploits a favorable electron transfer event in order to drive a thermodynamically unfavorable reaction without the use of ATP or an electrochemical gradient. Several bifurcating complexes, all of which contain a flavin as the proposed site of bifurcation, have been identified in anaerobic bacteria and archaea. While these enzymes catalyze a variety of reactions, all characterized thus far use Fld/Fd as an electron donor or acceptor. For example, the electron transferring flavoprotein/butyryl-coenzyme A (Etf-Bcd) bifurcating system uses the electron donor NADH ($E_m = -320$ mV) to reduce Fld ($E_m^{Sq/Hq} = -430$ mV) or Fd ($E_m = -405$ mV). This thermodynamically unfavorable reaction is achieved by coupling it to an exergonic one, in this case, the reduction of crotonyl-CoA ($E_m = -10$ mV).
bifurcation of electrons to both a high- and a low-potential acceptor results in an overall thermodynamically favorable reaction.\textsuperscript{17,22,23,25}

Homologs of bifurcating electron transfer flavoproteins (Etfs), known as FixAB, have been found in physiologically and phylogenetically distinct nitrogen fixing organisms such as \textit{Azotobacter vinelandii},\textsuperscript{26,27} \textit{Rhodopseudomonas palustris},\textsuperscript{28} \textit{Rhodospirillum rubrum},\textsuperscript{29} and \textit{Sinorhizobium meliloti}.\textsuperscript{30,31} Previous studies demonstrated that disrupting the Fix system in \textit{R. palustris}, \textit{Rh. rubrum}, and \textit{S. meliloti} completely abolishes or significantly impairs their ability to grow under nitrogen fixing conditions, suggesting that Fix may serve as a source of electrons to nitrogenase.\textsuperscript{28–30} Given the homology between FixAB and known bifurcating Etfs, it was hypothesized that the Fix system uses electron bifurcation to generate low-potential reducing equivalents for nitrogenase.\textsuperscript{20}

While the FixABCX complex is clearly linked to nitrogen fixation in many diazotrophs, its specific role has not been firmly established, nor has its ability to generate reductant for nitrogenase via electron bifurcation been demonstrated. Here, we report electron bifurcation by the FixABCX complex from the obligate aerobe \textit{A. vinelandii} and characterize the Fix proteins using advanced biochemical and spectroscopic tools. Further, we provide evidence that FixABCX provides electrons for nitrogenase in \textit{A. vinelandii} cells. Overall, this work establishes a new pathway for the generation of low-potential reductant required by nitrogenase and elucidates a mechanism by which biology can overcome thermodynamic barriers to accomplish a difficult reductive reaction.
MATERIALS AND METHODS

Construction of A. vinelandii Δfix mutant

A. vinelandii strain DJ was the wild type strain used for physiological studies and mutant construction. Strains UW195 (Δrnf1) and UW207 (Δrnf2) were kindly donated by Luis Rubio.32 The Δfix mutant was generated by gene disruption with an antibiotic resistance cassette as shown in Figure A1 using primers in Table A1. Briefly, two 1.2 kb DNA fragments that included 0.3 kb of fixA (Avin_10520) and 0.1 kb of fixC (Avin_10540) respectively were obtained by PCR and cloned sequentially in pT7-7 ampicillin resistant vector using Ndel/BamHI and BamHI/HindIII as restriction cloning sites. The kanamycin resistance (KmR) gene (aph), was isolated as a 1.5-kb BamHI fragment from mini-Tn533 and inserted between the 1.2 kb upstream and downstream regions previously cloned in pT7-7 vector using BamHI restriction cloning site. The final construct was transformed into A. vinelandii strain DJ as described previously.34,35 KmR transformants were screened for sensitivity to ampicillin (AmpS); AmpS derivatives were assumed to have arisen from a double crossover recombination event in which the wild-type fixABC genes were replaced by the aph-containing cassette (Figure A1). This replacement was confirmed by PCR and sequencing (Figure A1, Table A1). Burk’s media36 with no nitrogen source or supplemented with ammonium acetate (1 g/L) was used for physiological analyses of A. vinelandii DJ and Δrnf and Δfix mutants.

Homologous overexpression of FixABCX

The fix genes of A. vinelandii were homologously overexpressed by being placed under control of the nifH promoter, which is used for the transcription of genes associated with the molybdenum-dependent nitrogenase (Figure A2). The 4.3 kb fix operon, which
includes six genes (\textit{fixfd}, Avin_10510; \textit{fixA}, Avin_10520; \textit{fixB}, Avin_10530; \textit{fixC}, Avin_10540; \textit{fixX}, Avin_10550; ORF6, Avin_10560) (\textbf{Figure A2}), was PCR amplified using primers specified in \textbf{Table A2}. The PCR product was digested with \textit{XbaI} and \textit{BamHI} and inserted into a slightly modified pUC19 vector for blue-white screening.

Using \textit{NdeI} and \textit{BamHI} restriction sites, the \textit{fix} genes were then cloned into a vector built specifically to support the insertion of genes behind the \textit{nifH} promoter.\textsuperscript{37} The \textit{fix} operon was inserted between two segments of DNA from the \textit{nif}-region of \textit{A. vinelandii}. The flanking regions served as sites for homologous recombination within the chromosome, allowing replacement of \textit{nifHD} with \textit{fix} genes (\textbf{Figure A2}). The presence of a streptomycin resistance gene (Sm\textsuperscript{R}) between the flanking regions provided a selectable marker upon transformation of the plasmid into \textit{A. vinelandii}.\textsuperscript{34} The proper insertion of the \textit{fix} genes into the final plasmid and the chromosome of \textit{A. vinelandii} were confirmed by PCR and sequencing (\textbf{Table A2}).

\textbf{Growth of recombinant \textit{A. vinelandii} and purification of the \textit{FixABCX} complex}

Recombinant \textit{A. vinelandii} was grown in a 100 L fermenter (New Brunswick BioFlo 610, Hauppauge, NY) at Utah State University’ Synthetic Biomanufacturing Institute. Cells were grown in Burk’s medium supplemented with 10 mM urea as a nitrogen source.\textsuperscript{36} In the presence of urea, the \textit{nifH} promoter is repressed. When the cells reached an OD\textsubscript{600nm} of ~1.8, they were harvested in a stacked disk centrifuge (TSE 10, GEA Westfalia, Northvale, NJ) at 14,200 x \textit{g} and resuspended in Burk’s medium with no source of fixed nitrogen. Upon removal of fixed nitrogen source, the \textit{nifH} promoter is derepressed and gene expression occurs. \textit{A. vinelandii} was derepressed for 5 h to achieve optimal expression of \textit{Fix} proteins. Because \textit{nif} genes were replaced with \textit{fix} genes, Fix
rather than nitrogenase was expressed. Following the derepression, cells were harvested and stored at -80°C until further use. Fermenter conditions were maintained as follows throughout the growth and derepression: 30°C, pH 7, 20% dissolved oxygen, and agitation at ~200 rpm.

All purification steps were performed anaerobically under an argon atmosphere. 100 g of wet cell paste was resuspended in 50 mM HEPES, pH 8, 150 mM NaCl, 1 mM dithiothreitol (DTT) and 2 mg DNase at a cell to buffer ratio of 1:5. Flavin adenine dinucleotide (FAD) (0.25 mM) was also added to the lysis buffer as it increased the flavin cofactor occupancy of the Fix complex. Lysis was achieved with a French pressure cell (SLM Aminco FA-078, Aminco, Rochester, NY) at 200 MPa. The cell extract was centrifuged at 8,000 x g for 15 min to remove cell debris. A second centrifugation was then conducted at 50,000 x g for 2 h to obtain the cell membrane fraction. The membranes were solubilized for 1 h at 4°C in 50 mM HEPES, pH 8, 150 mM NaCl, 1% (w/v) n-dodecyl β-D-maltoside (DDM), and 1 mM DTT at a pellet to buffer ratio of approximately 1:6. The solubilized membrane was obtained by centrifuging at 50,000 x g for 1 h and then diluted 3-fold in Buffer A (50 mM HEPES, pH 8, 0.02% (w/v) DDM, and 1 mM DTT) to reduce the salt and detergent concentration before loading onto a 100 ml Q-Sepharose column. The Q-Sepharose column was prewashed with 2 column volumes of Buffer B (50 mM HEPES, pH 8, 1 M NaCl, 0.02% (w/v) DDM, and 1 mM DTT) and then equilibrated with 2 column volumes of Buffer A. Once the protein was loaded, unbound proteins were removed with 2 column volumes of Buffer A, followed by elution of bound proteins with a salt gradient of 15-60% (5 column volumes). FixABCX eluted between 32-36% NaCl. Fractions were pooled and diluted to less than 100 mM.
NaCl for concentration on a 15 ml Q-Sepharose column pre-washed and equilibrated as described above. After loading, the column was washed with 2 column volumes of Buffer A and bound protein eluted with 500 mM NaCl. The resultant concentrated fraction was loaded onto a Sephacryl S-200 column equilibrated with 50 mM HEPES, pH 8, 150 mM NaCl, 0.02% (w/v) DDM, and 1 mM DTT. Fractions containing FixABCX were pooled and concentrated using an Amicon (EMD Millipore, Billerica, MA) concentrator with a 100 kDa cutoff membrane and stored in liquid nitrogen. The purity of FixABCX was determined using SDS-PAGE and protein concentration was measured using the DC Protein Assay (Bio-Rad, Hercules, CA).

**Heterologous overexpression and purification of *R. palustris* FixAB**

*R. palustris* FixAB was co-expressed in *E. coli* strain NiCo21(DE3) (New England Biolabs, Ipswich, MA) transformed with plasmids based on pMCSG28 and pMCSG21 (DNASU Plasmid Repository, Arizona State University) modified to carry the *fixA* gene with a C-terminal His-tag and the *fixB* gene with an N-terminal His-tag, respectively. Cells were grown in terrific broth (TB) supplemented with riboflavin (20 mg/L) and MgSO$_4$ (2 mM) along with carbenicillin (100 μg/mL) and spectinomycin (100 μg/ml) at 37°C with shaking at 200 RPM to an OD$_{600}$ of ~2. After fully cooling the culture to 18-20°C, *fix* gene expression was induced with β-D-1-thiogalactopyranoside (IPTG) (0.1 mM) and cells were grown for an additional 12 hours at this lower temperature. Cells were harvested by centrifugation at 11,900 x g at 4°C for 6 min, and the pellet was stored at -80°C.

The cell pellet was suspended in BugBuster (80 ml) (EMD Millipore, Billerica, MA) containing 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (1
mM), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM), FAD (1 mM), benzonase nuclease (20 µL) and rLysozyme (2 µL) (EMD Millipore, Billerica, MA), and further incubated at 4°C for 2 hours with stirring. After centrifugation at 20,000 x g for 30 min at 4°C, the supernatant was filtered through a 0.22 µm syringe filter. The resulting protein solution was mixed with 3 mL pre-equilibrated Ni-NTA resin and incubated overnight at 4°C with stirring. The next day, the mixture was transferred to a column at 4°C. After collecting the flow-through, the column was washed with TPGT buffer (20 mM Tris, pH 7.8, 500 mM KCl, 10% (w/v) glycerol and 1 mM TCEP) containing 20 mM, 40 mM and then 50 mM imidazole in sequence using 20, 2 and 2 column volumes for each, respectively. Finally, the column was developed with 2 column volumes of TPGT buffer containing 100 mM imidazole and the eluate was collected in different fractions. After SDS-PAGE analysis, imidazole was removed from the pooled pure fractions by passage over a 10DG column (Bio-Rad Laboratories, Hercules, CA) equilibrated with BPGT buffer (20 mM Bis-Tris propane, pH 9.0, 200 mM KCl, 10% (w/v) glycerol and 1 mM TCEP). Any apo-flavin sites were then reconstituted by overnight incubation of the protein in 1 mM FAD at 4°C. Excess flavin was removed by gel filtration on a 10DG column (see above) prior to prompt use or flash freezing in liquid nitrogen and storage at -80°C.

**Heterologous overexpression and purification of Ro. castenholzii FixX**

*Ro. castenholzii* FixX was overexpressed in *E. coli* using a pCDFDuet-1 vector modified to include a C-terminal strep-tag. Transformed cells were grown in Luria-Bertani (LB) broth containing streptomycin (50 µg/ml) at 37°C and 250 RPM to an OD_{600} of 0.4-0.5. To induce expression of the *fixX* gene, IPTG (1.5 mM) was added to the cell
culture. Ammonium Fe(III) citrate (4 mM), L-cysteine (2 mM), and sodium fumarate (10 mM) were also added. Ammonium Fe(III) citrate and cysteine increased iron-sulfur cluster occupancy and sodium fumarate served as an electron acceptor during anaerobic metabolism. The flasks were sealed with rubber stoppers containing cannulas sparging argon into the cell suspension and suited with exhaust tubes flowing into a water trap. Cells were incubated for 4-6 h at room temperature and then anaerobically transferred to centrifugation bottles in a Coy chamber (Coy Laboratories, Grass Lake, MI) under a nitrogen atmosphere. Cells were harvested at 5,000 x g for 10 min.

All purification steps were performed anaerobically under a nitrogen or argon atmosphere. Wet cell pellets were suspended in lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM sodium dithionite (Na-dithionite), 5% glycerol, 1 μL/mL supernatant of a super saturated phenylmethylsulfonyl fluoride (PMSF) solution, 1 mg/10 mL DNAse, and 5 mg/10 mL lysozyme) at a cell to buffer ratio of 1:5. Cells were lysed using a cell bomb (Parr Bomb Instrument Co., Moline, IL) by slowly increasing the pressure to 1500 PSI and equilibrating for 30 min before collecting the lysate. This process was repeated and the supernatant collected by centrifugation at 18,000 x g for 20 min. FixX was purified using a single step affinity column purification using Strep-Tactin Superflow Plus (Qiagen, Hilden, Germany) resin pre-equilibrated with wash buffer containing 50 mM Tris-HCl, pH 8, 150 mM NaCl, and 1 mM Na-dithionite. Once the protein was loaded, the column was washed until the baseline absorbance returned. The protein was then eluted with 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM Na-dithionite, and 2.5 mM D-desthiobiotin as a brown band and anaerobically concentrated using an Amicon (EMD Millipore, Billerica, MA) centrifugal filter with a membrane molecular mass
cutoff of 10 kDa. The concentrated protein was flash frozen with liquid nitrogen for further analysis.

**Heterologous overexpression and purification of Rh. rubrum FixAB**

FixAB from *Rh. rubrum* was overexpressed and purified as described previously.\(^{26}\) Briefly, *E. coli* BL21 cells containing the plasmid pET101/d-TOPO::fixAB\(^{26}\) were cultured anaerobically in LB supplemented with 2.8 mM glucose, 17 mM KH\(_2\)PO\(_4\), 72 mM K\(_2\)HPO\(_4\) and 15 mg/L riboflavin at 20°C until early exponential growth phase. At this point gene expression was induced with the addition of 0.5 mM IPTG, and cells were cultured under the same conditions for an additional 12 h. Harvested cells were resuspended in degassed lysis buffer (20 mM Tris-Cl, pH 7.6, 10 mM imidazole, 500 mM NaCl, 5 mg/ml lysozyme, and 1 mg/ml Dnase I) and submitted to three freeze/thaw cycles. The lysate was added to a nickel-NTA column (Qiagen, Hilden, Germany) that had been equilibrated with degassed lysis buffer. The column was washed with degassed lysis buffer, and FixAB was eluted into anaerobic vials using a 10-300 mM imidazole gradient in degassed 20 mM Tris-HCl, pH 7.6, and 500 mM NaCl. Yellow fractions indicating the presence of FixAB were subjected to buffer exchange using a PD-10 column (GE-Healthcare, Little Chalfont, UK) and stored in desalting buffer (20 mM Tris-HCl, pH7.6, and 100 mM NaCl). Purified FixAB was incubated overnight at 4°C with 1 mM FAD in desalting buffer to reconstitute FAD cofactors lost during purification.\(^{38,39}\) Unbound FAD was rinsed by concentrating the samples with Amicon membrane (cutoff 30 kDa) followed by dilution with desalting buffer containing 0.5 µM FAD. Reconstituted samples were stored at -20°C. Purity and identity of the fractions were assessed with SDS-PAGE and mass spectrometry, respectively.
Dynamic light scattering

Dynamic light scattering (DLS) was performed on DynaPro NanoStar (Wyatt Technology, Santa Barbara, CA) to determine the size distribution and hydrodynamic radii of species in solution. Samples were filtered through a 0.1 μm syringe filter prior to the experiments. DLS was measured aerobically using 10 μL of buffer as the control and also 1 mg/mL enzyme using a disposable cyclic olefin copolymer cuvette at 25°C.

Protein identification, chemical cross-linking, and model construction

Protein identification from gel bands and solution digestion was performed according to standard protocols recommended by the manufacturers using a trypsin (Promega, Madison, WI) protease:complex ratio of 1:50-1:100 overnight and pepsin (Sigma, St. Louis, MO) protease:complex ratio of 1:10 for 60 s. Proteins were identified as described using a maXis Impact UHR-QTOF instrument (Bruker Daltonics, Billerica, MA) interfaced with a Dionex 3000 nano-uHPLC (Thermo-Fisher, Waltham, MA) followed by data analysis in Peptide Shaker. Chemical cross-linking was performed using 20 μg of the FixABCX complex and 1 mM bis(sulfosuccinimidyl)suberate (BS3) (Thermo-Fisher, Waltham, MA) in 50mM HEPES, pH 7.2, 150mM NaCl buffer at room temperature for 1 h. The reaction was quenched by addition of 120 mM Tris (final concentration). The resulting mixture was separated by SDS-PAGE (4-20% linear gradient gel, Bio-Rad, Hercules, CA) and stained with Coomassie Brilliant Blue (Thermo-Fisher, Waltham, MA). Protein bands of interest were excised from the gel and digested with trypsin as described above. For cross-link mapping, a Spectrum Identification Machine, SIM, was used. Precursor and fragment ion tolerances were set to ±20ppm. Intact protein analysis was performed as described previously using a Bruker
Micro-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Non-covalent mass spectrometry experiments were conducted on SYNAPT G2-Si instrument (Waters, Milford, MA) in a similar fashion as described. Briefly, the FixABCX complex sample was buffer exchanged to 200 mM ammonium acetate, pH 7 (Sigma, St. Louis, MO) using 3 kDa molecular mass cutoff spin filters (Pall Corporation, Port Washington, NY) and infused from in-house prepared gold-coated borosilicate glass capillaries to electrospray source at protein concentration of 2–3 μM and the rate around 90 nl/min. The instrument was tuned to enhance performance in the high mass-to-charge range. Settings were as follows: source temperature 30°C, capillary voltage 1.7 kV, trap bias voltage 16 V and argon flow in collision cell (trap) 7 ml/min. Transfer collision energy was held at 10 V while trap energy varied between 10–200 V. Data analysis was performed in MassLynx software version 4.1 (Waters, Milford, MA).

Protein homology models were generated by Phyre2, and energy minimized models were docked using ClusPro2 with restrictions derived from chemical cross-linking experiments. The flavin and iron sulfur cluster co-factors were added using SwissDock for individual subunits and eventually added as rigid bodies to the final FixABCX complex model. Molecular graphics were created using the UCSF Chimera package.

**Electron paramagnetic resonance spectroscopy**

Electron paramagnetic resonance (EPR) spectra were recorded with a Bruker E-500 spectrometer (X-band, 9.38 Ghz) equipped with a SHQ resonator (Bruker, Billerica, MA), an in-cavity cryogen free VT system (ColdEdge Technologies, Allentown, PA) and MercuryiTC temperature controller (Oxford Instruments, Abingdon, UK). Spin
quantifications were determined by double integration of the spectra after manual baseline subtraction in the OriginPro software package and referenced to copper-triethylamine standards (75-125 μM) measured at the same conditions. To assist with spectral deconvolution and assignment of g-factors (±0.003), computer simulations of the experimental spectra were carried out in MatLab using the EasySpin package and ‘esfit’ fitting function incorporating g-strain to replicate line broadening.

*Electron bifurcation assays*

FixABCX electron bifurcation activity was measured anaerobically in a UV-Vis spectrophotometer (Varian Cary 50 Bio, Agilent Technologies, Santa Clara, CA) using quartz cuvettes (d= 1 cm). All assays were carried out in 50 mM HEPES, pH 7.5, 10% glycerol and 0.02% DDM and contained the following: 0.8 μM FixABCX (1.7 nmol flavin/nmol FixABCX and 9.1 nmol Fe/nmol FixABCX), 85 μM Fld\textsubscript{Sq}, 200 μM NADH, and 300 μM Coenzyme Q\textsubscript{1} (CoQ\textsubscript{1}). NADH, Fld\textsubscript{Sq}, and Fld\textsubscript{Ox} were monitored at 340 nm (ε= 6.2 mM\textsuperscript{-1} cm\textsuperscript{-1}), 580 nm (ε= 5.7 mM\textsuperscript{-1} cm\textsuperscript{-1}), and 450 nm (ε= 11.3 mM\textsuperscript{-1} cm\textsuperscript{-1}), respectively\textsuperscript{12,52}. The Fld used in the assays as the low-potential electron acceptor was purified in the hydroquinone state as previously described\textsuperscript{5}. Fld\textsuperscript{Hq} was exposed to oxygen for a short period of time, upon which the majority (>80%) of the Fld\textsuperscript{Hq} converted to the semiquinone form. The protein was then degassed with argon, and the absorbance of the semiquinone species was measured at 580 nm. The concentration was then calculated using the extinction coefficient (ε= 5.7 mM\textsuperscript{-1} cm\textsuperscript{-1}).
**Thermodynamic calculations**

Standard reduction midpoint potentials of the NAD\(^+\)/NADH (\(E_m= -320\) mV), CoQ/CoQH\(_2\) (\(E_m= +10\) mV), Fld\(\text{Ox}/\text{Sq}\) (\(E_m= -180\) mV), Fld\(\text{Sq}/\text{Hq}\) (\(E_m= -460\) mV) half reactions were converted to standard Gibbs free energies (\(\Delta G^{\circ\prime}\)) with the equation,

\[
\Delta G^{\circ\prime} = -nF E^{\circ\prime}
\]

(Eq. 2-2)

where \(n\)= electrons (mol), \(F\)= Faraday constant (96,485.34 J/V·mol) (Table A2-5). The standard Gibbs free energy of reaction (\(\Delta G_{\text{rxn}}^{\circ\prime}\); J/mol) can be calculated for the reaction of interest using the equation,

\[
\Delta G_{\text{rxn}}^{\circ\prime} = \sum |v_i| \Delta G^{\circ\prime}(\text{products}) - \sum |v_i| \Delta G^{\circ\prime}(\text{reactants})
\]

(Eq. 2-3)

where \(|v_i|\) = stoichiometric coefficient, \(i\) = product or reactant, \(\Delta G^{\circ\prime}(\text{products or reactants})\) = standard Gibbs free energy of the products and reactants (J/mol) calculated from Eq. 2-2.

**Transient absorption spectroscopy**

The ultrafast (100 fs to 5.1 ns) transient absorption spectroscopy (TAS) spectrometer employed in this study uses an amplified 4W Ti:sapphire laser (Libra, Coherent, 800 nm, 1 kHz, 100 fs pulse width), and the Helios spectrometer (Ultrafast Systems LLC, Sarasota, FL). A fraction of the 800 nm Libra output was frequency-doubled in beta barium borate (BBO) to produce the desired pump wavelength (480 nm for the data described here) for sample excitation, which was then directed into the Helios. The pump pulses were passed through a depolarizer and chopped by a synchronized chopper to 500 Hz before reaching the sample. The pump pulse energy was \(1.1\) µJ per pulse at the sample. Another fraction of the 800 nm Libra output was guided
directly into the Helios for generation of the probe. Within the spectrometer, a white light continuum of wavelengths including 340–800 nm was generated using a 2 mm thick CaF$_2$ crystal. This beam was split into a probe and a reference beam. The probe beam was focused into the sample where it was overlapped with the pump beam. The transmitted probe and reference beams were then focused into optical fibers coupled to multichannel spectrometers with CMOS sensors with 1 kHz detection rates. The reference signal is used to correct the probe signal for pulse-to-pulse fluctuations in the white-light continuum. The time delay between the pump and probe pulses was controlled by a motorized delay stage. For all transient absorption measurements, the sample was made in an Mbraun glove-box (N$_2$ atmosphere), sealed in a 2 mm quartz cuvette and constantly stirred to prevent photodegradation. *Rh. rubrum* FixAB and *A. vinelandii* FixABCX concentrations were approximately 150 µM and 43 µM, respectively, and they were measured in their as-isolated state (mostly oxidized). The *Rh. rubrum* dimer contained >0.7 nmol flavin/nmol FixAB, and the *A. vinelandii* FixABCX complex contained 1.7 nmol flavin/nmol FixABCX. For the purpose of this study, light initiated the formation of the semiquinone intermediates for each flavin through generation of FAD excited state and electron donation from nearby protein residues or the other flavins. Qualitatively, this experiment shows which type of semiquinone is formed for a particular FAD site and suggests how thermodynamically stable that intermediate is, based on its lifetime. All experiments were conducted at room temperature. The change in absorbance signal (ΔA) was calculated from the intensities of signals detected after sequential probe pulses with and without the pump pulse excitation. The data collection (350 pump shots per time point) was carried out three consecutive times and then averaged. The experiment was
repeated three times for *A. vinelandii* FixABCX and once for *Rh. rubrum* FixAB. Data were corrected for spectral chirp using SurfaceXplorer (Ultrafast Systems LLC, Sarasota, FL). ASQ signals were fit in Igor Pro with a double exponential function. The 550 nm emissive feature in *Rh. rubrum* FixAB is due to stimulated emission.\(^{53}\)

**RESULTS AND DISCUSSION**

*Effect of fix and rnf deletions on nitrogen fixation in A. vinelandii*

To determine whether the Fix system is associated with diazotrophic growth in *A. vinelandii*, as in *R. palustris*, *Rh. rubrum*, and *S. meliloti*,\(^{28,29,31}\) a deletion mutant lacking *fixABC* was generated (Figure A1). This mutant, unlike those of *R. palustris*, *Rh. rubrum*, and *S. meliloti* exhibited equally robust growth on solid media with and without added fixed nitrogen (Figure 2-1). This nitrogen fixing (Nif\(^+\)) phenotype was also observed when the *A. vinelandii fix* mutant expressed the vanadium and iron-only alternative nitrogenases or when cultured under low aeration conditions (data not shown).

![Figure 2-1](image.png)

*Figure 2-1.* Phenotype of *A. vinelandii* DJ wt (A), Δfix (B), Δrnf1 (C), ΔfixΔrnf1 (D), Δrnf2 (E), and ΔfixΔrnf2 (F) strains. Wild type and mutant strains were cultivated in Burk’s medium supplemented with ammonium acetate (+N) or with no added fixed nitrogen (-N). All samples were grown aerobically.
Although the results appeared to indicate that Fix is not associated with nitrogen fixation in *A. vinelandii* and were consistent with a previous study by Wientjens,27 we also tested the possibility of redundancy,54 thinking that an alternative complex could participate in balancing the chemical energy and reductant pools in the absence of Fix, masking the effect of the ΔfixABC mutation.

Rnf (*Rhodobacter* nitrogen fixation) is a membrane-bound complex found in some diazotrophs that, like Fix, has been hypothesized to generate reductant in the form of Fd or Fld for nitrogen fixation.16,55,56 Nitrogen-fixing organisms that have genes coding for Rnf typically do not have genes coding for Fix and *vice versa*. Interestingly, *A. vinelandii* is one of the few known diazotrophs with genes coding for both complexes.57 The physiological implications of having both of Rnf and Fix are not altogether clear, but given that *A. vinelandii* fixes nitrogen under highly oxic conditions, the combination of Rnf and Fix could confer the ability to fine-tune the redox status of the cell to a high degree under a wide range of oxygen tensions.

It has been suggested that Rnf uses the energy of the proton motive force to generate reduced Fd/Fld15,16 while Fix uses electron bifurcation.18,20 Neither the Rnf nor Fix complex, however, has previously been directly implicated in electron delivery to nitrogenase. Curatti *et al.* showed that *A. vinelandii* possesses two Rnf complexes, Rnfl associated with nitrogen fixation and Rnf2, which is expressed constitutively.32 Δrnf1 mutants, although still able to grow diazotrophically (*Figure 2-1*), consistently exhibit a long lag in nif gene expression and diazotrophic growth during derepression studies.32 To test whether Rnf and Fix might be compensating for one another, we transformed Δrnf mutants of *A. vinelandii* with the Δfix construct (*Figure A1*)32 to generate double mutants
of *A. vinelandii* lacking either Rnf1 and Fix, or Rnf2 and Fix. The Δfix-Δrnf1 *A. vinelandii* strain was able to grow when fixed nitrogen was supplied, but no growth was observed under nitrogen fixing conditions (Figure 2-1). Therefore, cells lacking both Rnf1 and Fix displayed a non-nitrogen fixing (Nif-) phenotype. Our data provide support for a role of both Fix and Rnf1 in providing reducing equivalents to nitrogenase and indicate that the two complexes have compensatory activities, such that either one can replace the other to ensure electron flow. Furthermore, this is the first study to link the Fix complex to nitrogen fixation in *A. vinelandii*. The Δfix-Δrnf2 *A. vinelandii* mutant control strain was able to grow with or without added fixed nitrogen (Figure 2-1), confirming that Rnf1, but not Rnf2, can support nitrogen fixation.

**Characterization of the FixABCX complex**

To understand how Fix is able to support nitrogen fixation, we characterized the FixABCX complex and its activity *in vitro*. The fix operon of *A. vinelandii* consists of six genes, *fixFd* (annotated as *fixP* in some literature), *fixA, fixB, fixC, fixX*, and *Avin_10560* (designated ORF6) (Figure A2). FixAB is homologous to the previously characterized EtfAB, which is part of the Etf-Bcd bifurcating complex. FixCX is similar to Etf ubiquinone oxidoreductase (Etf-QO), involved in transfer of electrons to the quinone pool in the membrane. Based on the similarity to other Etf systems, it is hypothesized that the Fix complex oxidizes NADH and bifurcates one electron to the high-potential quinone pool (exergonic branch) and drives the other electron to a low-potential acceptor in the form of Fd or Fld (endergonic branch) (Figure 2-2).
Figure 2-2. (A) Proposed mechanism of FixABCX electron bifurcation in *A. vinelandii*, operating within the framework of the known oxidation-reduction potentials of NADH, Fld/Fd, and CoQ. It is hypothesized that crossed potentials of the bifurcating flavin (a-FAD) promote electron bifurcation based on the appearance of a short-lived ASQ. Once the first electron transfers into the exergonic branch, the second electron, sitting at a very low reduction potential, is thermodynamically unstable and immediately transfers into the endergonic branch composed of the low potential iron-sulfur clusters of FixX. The exergonic branch is set up to ensure electron delivery to c-FAD where electrons accumulate before being transferred to CoQ. The specific oxidation-reduction potential levels are qualitative unless otherwise noted. (B) Electron transfer pathways illustrated in FixABCX structural model generated by docking (ClusPro2) four homology models (Phyre2) (Figure A2-8).
Most microbes with fix genes only have fixABCX; homologs fixFd and ORF6 have not been investigated in fix gene clusters of any other species thus far.\textsuperscript{27,58} Although FixFd and ORF6 were not specifically addressed in this study, it is thought that FixFd, a small iron-sulfur protein,\textsuperscript{27,59} may serve as a low-potential acceptor in Fix electron bifurcation. ORF6 is described as being ferritin-like, but studies on its function have yet to be conducted.

The fix genes from \textit{A. vinelandii} were homologously overexpressed under control of the \textit{nifH} promoter (Figure A2), which regulates transcription of genes encoding molybdenum nitrogenase. This approach allows gene(s) of interest to be overexpressed upon removal of a fixed nitrogen source. Based on the hypothesis that the FixABCX complex delivers one electron per bifurcation reaction to the quinone pool (coenzyme Q, CoQ), it was anticipated that the enzyme could be associated with the membrane fraction, as observed for Etf-QO.\textsuperscript{60,61} Indeed, a previous study found that heterologously overexpressed \textit{Rh. rubrum} FixC was localized to the membrane fraction in \textit{Escherichia coli}.\textsuperscript{29} Furthermore, an N-terminal lipophilic tail and transmembrane helix were predicted on the FixC subunit based on the amino acid sequence (\textit{SACS MEMSAT2} and Phyre 2 prediction software).\textsuperscript{44,62} Purification attempts for the FixABCX complex confirmed membrane association. After trying a range of NaCl concentrations (0-1 M) and a variety of non-ionic detergents to solubilize the complex, the detergent n-dodecyl β-D-maltoside (DDM) used for many membrane proteins,\textsuperscript{61,63} was found to be most effective for the isolation of intact FixABCX in a soluble and active form.

Upon extraction of the FixABCX complex from the membrane, the DDM-solubilized fraction was subjected to anion-exchange and size-exclusion chromatography.
SDS-PAGE analysis of the purified FixABCX complex revealed four bands corresponding in size to FixA, FixB, FixC, and FixX (Figure 2-3) and was >80% homogenous (defined by percent band (Image Lab, BioRad)). Mass spectrometry confirmed the identities of the four Fix subunits (Figure A3), and densitometry of the stained gel indicated that the band intensities were consistent with a subunit stoichiometry of 1:1:1:1 for A:B:C:X.

*Dynamic light scattering*

DLS was used to determine the oligomeric state of the FixABCX complex on the basis of particle size. The complex in buffer containing 0.02% DDM displayed an average

![Figure 2-3. SDS-PAGE gel of purified (~80%) FixABCX complex from A. vinelandii (extra lanes were removed). All Fix protein bands were verified by mass spectrometry (Figure A2-3) and according to densitometry, the subunit stoichiometry of FixABCX is 1:1:1:1.](image-url)
hydrodynamic radius of $5.1 \pm 0.3$ nm, corresponding to an estimated molecular mass of 150 kDa versus the expected 129 kDa for the FixABCX heterotetramer (Figure A4). This slightly larger-than-expected complex is consistent with attachment of DDM to the protein tetramers. While there was also some contribution from larger aggregates, which are very prominent in the DLS output due to their much larger scattering efficiencies ($\approx 12$-16 nm), the aggregates comprised $<1\%$ of the population (Figure A4). Buffer containing DDM was used as a negative control and produced micelles with a radius of $\approx 4.3$ nm (Figure A4). Overall, the DLS data suggest FixABCX exists as individual heterotetramers in solution.

**FixABCX ligand composition and structural model**

Multiple electron transfer cofactors, including flavins and iron-sulfur clusters, have been identified in electron bifurcating enzyme complexes, with a single flavin proposed to serve as the site of bifurcation.\textsuperscript{17,18,20,25} Based on common sequence motifs for ligand binding, FixABCX was predicted to have 3 FAD moieties, one each in FixA, FixB, and FixC, and two $[4\text{Fe}-4\text{S}]$ clusters in FixX. Native mass spectrometry (MS) was employed to reveal the subunit stoichiometry and ligand composition of the complex.

All complex members were first identified based on fragments produced by two different proteases, trypsin and pepsin. Additionally, proteolysis reactions revealed that FixABCX and a small amount of Fld (NifF) purify together. The next step was to determine the molecular masses of the intact proteins. Reverse phase LC-MS confirmed full-length FixA and that FixB and FixC subunits did not contain N-terminal Met residues. The purified FixABCX complex, under anaerobic conditions, was analyzed using native MS conditions by direct infusion on a Synapt G2-Si. Under standard
conditions (60V collision energy), the complex dissociated into subcomplexes of FixAB and FixCX. A consistent ion signal for the tetrameric FixABCX complex could not be maintained despite the presence of all four proteins and cofactors in the sample after size-based purification. The FixAB component contained two FAD molecules and closely matched the expected size (71,414.8 Da vs predicted MW = 71,413.7 Da) (**Figure 2-4A and B**); whereas, the FixCX subcomplex containing FAD and two [4Fe-4S] (Table A2-3).

**Figure 2-4.** *A. vinelandii* FixABCX complex composition. (A) Native mass spectrum of FixAB containing two FAD cofactors (71,414.8 Da; calculated MW = 71,413.7 Da) generated during FixABCX complex activation in the gas phase. Red diamonds signify charge state envelope centered around charge 18+. The unmarked masses are the charge state envelop of the molecular chaperone DnaK. (B) Sub-complexes obtained during FixABCX complex activation. (C) SDS-PAGE gel of FixABCX complex (right) cross-linked with BS3 reagent (left). The most distinguished bands in cross-linking reaction, migrating around the 60 kDa, 70 kDa, and 88 kDa molecular weight marker, were identified as FixCX, FixAB and FixBC dimers respectively. (D) Protein-protein interaction map based on highest scoring cross-links (score 7 and higher; red and blue lines indicate intra and inter cross-links, respectively). A complete list of generated cross-links can be found in Table A2-3.
clusters was slightly heavier than expected (60,310.1 Da vs predicted MW = 59,932.9 Da: FixCX) (Figure 2-4B). The additional mass of 377.2 Da matches very closely to riboflavin (Rf) (MW = 376.4 Da), raising the intriguing possibility that it may also be associated with the complex (Figure 2-4B).

The UV-visible spectrum of FixABCX revealed a distinct peak at 428 nm, as well as oxidized flavin signatures with shoulders near 460 nm and 365 nm (Figure 2-5 (Black trace)). After denaturation of FixABCX and removal of the protein by centrifugation, the soluble fraction demonstrated clear signatures of flavins with broad maxima at 370 and 450 nm (Figure 2-5 (Red trace)).

The band at 428 nm is not a usual signature of flavins, and such a band has not been observed in FixAB from other diazotrophs that have been expressed in E. coli. Thus, it is likely that this species is associated with FixC or FixX and may be covalently attached to the protein, as it does not remain in the supernatant when denatured protein is removed. The signatures of the [4Fe-4S] clusters are not evident in as-purified FixABCX, but are not expected to be prominent because of scattering and their broad and relatively weak signals (400-420 nm, ε≈ 2-4 mM⁻¹ cm⁻¹).⁶⁴

Once we had established the subunit stoichiometry and ligand composition of the FixABCX complex, we investigated the quaternary interactions that define the active enzyme. Models for each of the Fix subunits were generated using Phyre2⁴⁴, and the figures of merit are provided in Figures A5-A8. To validate these subunit models and to elucidate protein-protein interactions within the FixABCX complex, chemical cross-linking was used. The bis(sulfosuccinimidyld)suberate (BS3) cross-linking reagent is homobifunctional, reacting with primary amines and hydroxyl groups to form covalent
Figure 2-5. UV-visible spectrum of the FixABCX complex from *A. vinelandii* demonstrating the presence of flavin. Black: as-purified FixABCX, red: cofactors released upon denaturation of FixABCX, and blue: flavins as observed in the spectrum of FixAB from *R. palustris*, vertically offset by -0.05 AU for clarity. The two spectra representing FixABCX were corrected for Raleigh scattering by fitting the baseline to the equation for scattering and then subtracting the fit from the measured spectrum to obtain a corrected spectrum. *A. vinelandii* FixABCX was prepared in 50 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.02% (w/v) DDM. *R. palustris* FixAB was prepared in 20 mM Bis-Tris propane, pH 9, 200 mM KCl, 10% (w/v) glycerol and 1 mM tris(2 carboxyethyl)phosphine (TCEP).

Cross-linked samples were separated using 1D SDS-PAGE to confirm connections between subunits and to enrich cross-linked species in samples subsequently analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Figure 2-4C and Table A3). Overall, the reaction of the FixABCX complex with BS3 reagent produced over 200 connections. For initial model building of the FixABCX complex and to confirm the subunit homology models, a reduced set of cross-links of very high confidence were selected (probability score >7, significance is >3) (Figure 2-4D).
The resulting model of the quaternary structure provides insightful hypotheses to be tested in next generation of experiments (Figures 2-2B, A8E). The MS-based model places the cofactors in locations that suggest a pair of possible paths for electron transfer, both emanating from the flavin in FixA. One provides a plausible route to the [4Fe-4S] clusters in FixX and the other can provide a path to the site at which quinone is found to be bound in the functional homolog of FixC, the Etf-QO (Figures 2-2B, A8E). While some of the distances between cofactors are longer than ideal for direct electron transfer, conformational changes could be invoked to bring them sufficiently close, but we also note that the model places conserved Trp and Tyr in locations that could provide electron transfer paths between cofactors (Figure A8E). Thus, our model provides testable hypotheses for which amino acids are expected to alter complex stability or internal electron transfer upon mutagenesis. These amino acids will be targets of experiments to elucidate fundamental elements of bifurcating activity and test attribution of bifurcating activity to FixABCX.

**EPR spectroscopy**

FixABCX was analyzed by low-temperature continuous wave X-band EPR spectroscopy to assess its flavin radical content and identity of iron-sulfur clusters. The EPR spectrum of the as-purified enzyme showed a very weak fast relaxing, broad signal that was strongest near 5 K along with a weak radical signal near \( g = 2 \) (Figure 2-6, Table A4). Upon reduction with either NADH or Na-dithionite, the broad signal almost disappeared and was replaced with multiple signals in the \( S = \frac{1}{2} \) region indicative of FeS clusters and a flavin radical. Both treatments yielded overlapping rhombic \( g = 2.07 \) (\( g = 2.072, 1.940, 1.895 \)) and axial \( g = 2.04 \) (\( g = 2.041, 1.944, 1.944 \)) signals consistent with
Figure 2-6. EPR spectra of FixABCX from A. vinelandii prepared in 50 mM HEPES pH 7.5, 150 mM NaCl, and 5% glycerol. Black: As-prepared FixABCX (100 μM); Blue: FixABCX (100 μM) reduced with NADH (1 mM); Red: FixABCX (100 μM) reduced with sodium dithionite (10 mM). Simulations of spectra of NADH- and Na-dithionite-reduced FixABCX are shown in lighter colored traces. Microwave frequency, 9.38 GHz; microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 10.0 G; sample temperature, 10 K.

the presence of two iron-sulfur clusters, in addition to an isotropic $g = 2.0$ signal consistent with a flavin radical that was dominant above 50 K (Figure A9). The temperature dependencies of the rhombic $g = 2.07$ and axial $g = 2.04$ signals were characteristic of $[4Fe-4S]$ clusters, in agreement with the sequence prediction for a pair of $[4Fe-4S]$ clusters. The optimal temperature ($T_{opt}$) for the rhombic $g = 2.07$ signal was
near 15 K with broadening above 30 K (Figure A9). The axial $g = 2.04$ signal was faster relaxing with a lower $T_{opt}$ between 5 and 10 K and broadening above 15 K. Also present for the NADH-reduced sample was a highly temperature dependent axial $g = 2.03$ signal ($g = 2.030, 2.00, 2.00$) observed at 10 K. The signal appeared to correlate with a slight loss in intensity from the axial $g = 2.04$ signal compared to the Na-dithionite treatment, suggesting that this feature could arise from the spin interaction between the faster relaxing [4Fe-4S] cluster and a flavin radical.

Further EPR analysis of the FixX subunit alone (from *Ro. castenholzii*) identified very similar rhombic $g = 2.07$ and axial $g = 2.04$ signals indicating that these signals in FixABCX originate from the FixX subunit (Figure A10). Line broadening at the high- and low-field edges of the reduced spectra was observed for both FixABCX and FixX and is indicative of spin coupling between the two [4Fe-4S] clusters.71 For FixABCX, the different relative intensities of the rhombic $g = 2.07$ and axial $g = 2.04$ signals suggest that the corresponding [4Fe-4S] clusters have different $E_m$ values. Accordingly, for FixABCX both clusters are thought to have $E_m$ values below that of Na-dithionite ($\leq$440 mV vs. SHE, pH 7.5), since the sample appeared to be only partially reduced by Na-dithionite. We hypothesize that the [4Fe-4S] cluster giving rise to the axial $g = 2.04$ signal has a lower $E_m$, due to its weaker contribution to the spectrum, based on simulations (Table A4).

Altogether, the two [4Fe-4S] cluster EPR signals show striking resemblance to the EPR signals assigned to the two [4Fe-4S] clusters in the NADH-dependent ferredoxin:NADP* oxidoreductase I (Nfn) bifurcating enzyme, as the signals share similar $g$-values, temperature dependence, and oxidation-reduction properties.25 For the
Nfn enzyme, structural and biophysical analyses showed that two [4Fe-4S] clusters in the large subunit form an electron-transfer chain from the flavin site of electron-bifurcation to an external ferredoxin redox partner. Spin-coupling between the two clusters is thought to facilitate electron-transfer between the redox cofactors. It was also found for the Nfn enzyme that one of the [4Fe-4S] clusters had an unusually low \( E_m \) that creates a thermodynamically favorable electron transfer pathway from a highly unstable semiquinone intermediate formed during electron bifurcation. An analogous model is suggested here for FixABCX where the two low-potential [4Fe-4S] clusters in the FixX subunit would form a thermodynamically favorable pathway for facile electron transfer between the site of bifurcation and Fd/Fld.

**Electron bifurcation by the FixABCX complex**

Electron bifurcation by the FixABCX complex is proposed to drive the endergonic reduction of Fld\(^{SQ} \) by coupling it to the exergonic reduction of CoQ using NADH as an electron donor (Figures 2-2, 2-7A). Electron bifurcation by the FixABCX complex was demonstrated by incubating FixABCX with the electron donor NADH, high potential acceptor coenzyme Q\(_1\) (CoQ\(_1\)), and low-potential acceptor Fld\(^{SQ} \). CoQ\(_1\) served as an amphipathic analog of the physiological acceptor coenzyme Q\(_8\) (CoQ\(_8\)) found in the electron transport chain of A. vinelandii.\(^{72,73}\) Fld\(^{SQ} \) was used as the low-potential acceptor in these assays since Fld\(^{HQ} \) has been shown to be a direct electron donor to nitrogenase.\(^5,6\) Similarly, the previously characterized electron bifurcating Etf-Bcd complex could direct electrons to either Fd or Fld,\(^{22,23}\) suggesting that Fld could likely serve as an electron acceptor from FixABCX as well.
Figure 2-7. Electron bifurcation by the FixABCX complex of *A. vinelandii* under anaerobic conditions. (A) Overview of electron bifurcation by the FixABCX complex showing the NADH bifurcating an electron to the high-potential acceptor, CoQ$_1$ and the other to the low-potential acceptor, Fld$^\text{SQ}$. Evidence of electron bifurcation was obtained using UV-visible spectrophotometry. NADH oxidation as well as Fld$^\text{SQ}$ reduction and oxidation were monitored over time as signatures of the bifurcation reaction. (B) NADH oxidation at 340 nm, (C) Fld$^\text{SQ}$ disappearance at 580 nm, and (D) formation of Fld$^\text{OX}$ at 450 nm. Final concentrations of components added to the reactions were as follows: 0.8 μM FixABCX, 85 μM Fld$^\text{SQ}$, 200 μM NADH, and 300 μM CoQ$_1$. Data were normalized and demonstrate the overall change in absorbance.

For the electron bifurcation assay, the oxidation of NADH (340 nm), disappearance of Fld$^\text{SQ}$ (580 nm), and formation of Fld$^\text{OX}$ (450 nm) were monitored over time. CoQ$_1$ reduction ($\lambda_{\text{max}}=274$ nm) was not recorded, due to significant interference at that wavelength. While the simultaneous presence of several redox active components in
the assays posed a challenge, monitoring activities at multiple wavelengths provided
evidence of electron bifurcation. Control reactions for NADH oxidation (diaphorase)
activity linked to CoQ$_1$ reduction in the absence of Fld$^{Sq}$ yielded a specific activity of 396
± 44 nmol/min/mg with a turnover frequency of 51 ± 6 min$^{-1}$ (Figure 2-7B (No Fld$^{Sq}$)).
Bifurcation reactions containing the additional component needed for bifurcation (Fld$^{Sq}$,
in addition to the control reactants FixABCX, NADH, and CoQ$_1$) exhibited 25% greater
NADH oxidation activity with a specific activity of 524 ± 21 nmol/min/mg and turnover
frequency of 68 ± 3 min$^{-1}$ (Figure 2-7B (All components)). It is important to note that the
specific activity values are not adjusted for flavin occupancy, since it could not be
confirmed how much of the protein was fully occupied (how flavins were distributed
among the flavin binding sites).

The Fld-dependent increase in NADH consumption is consistent with bifurcation,
and previous studies on the Etf-Bcd bifurcating complex also demonstrated increased
NADH consumption in a bifurcating reaction.$^{22,23}$ Omission of FixABCX or CoQ$_1$
resulted in baseline NADH oxidation activity (Figure 2-7B). To decipher whether the
enhanced consumption of NADH in the presence of Fld$^{Sq}$ could be attributed to the
endergonic reduction of Fld$^{Sq}$ to Fld$^{Hq}$ (the bifurcation reaction), disappearance of Fld$^{Sq}$
was monitored at 580 nm where optical absorption by the hydroquinone species is
negligible but absorbance by the semiquinone is strong ($\varepsilon$= 5.7 mM$^{-1}$ cm$^{-1}$). Reactions
monitoring the disappearance of Fld$^{Sq}$ were difficult to interpret on their own, due to
background activity in the absence of FixABCX (Figure 2-7C). In vivo, CoQ is in the
membrane, whereas, Fld is in the cytoplasm so direct contact between them is diminished
and spontaneous electron transfer between the two species is suppressed (Table A5). In
vitro experiments however lack this separation, and our controls revealed that Fld\textsuperscript{Sq} is able to donate electrons to CoQ\textsubscript{1} in the absence of FixABCX (Figure 2-7C). Thus, the Fld\textsuperscript{Sq} concentration decreased while an increase at 450 nm, characteristic of Fld\textsuperscript{Ox}, was observed (Figure 2-7D). This non-enzymatic reduction of CoQ\textsubscript{1} by Fld\textsuperscript{Sq} is consistent with the favorable $\Delta G^\circ$ of -36.7 kJ/mol (Table A5). However, the increase in absorbance at 450 nm produced by spontaneous Fld\textsuperscript{Sq} oxidation enabled us to account for this reaction that detracts from the apparent yield of the bifurcating reaction (Figure 2-7C (All components)). The Fld\textsuperscript{Ox} species was not detected in the bifurcating reaction assays indicating that Fld\textsuperscript{Hq}, the expected product of the bifurcation reaction, was formed instead (Figure 2-7D). However, we do not expect Fld\textsuperscript{Hq} to accumulate in the presence of CoQ\textsubscript{1} due to the favorability of electron transfer between these two that will return Fld to its semiquinone state and thus conceal the true extent of Fld\textsuperscript{Hq} production ($\Delta G^\circ$= -154 kJ/mol) (Eq. 2-4, Table A5).

$$\text{NADH} + 2 \text{Fld}^{\text{Hq}} + 2 \text{CoQ} + 3 \text{H}^+ = 2 \text{CoQH}_2 + 2 \text{Fld}^{\text{Sq}} + \text{NAD}^+ \quad \text{(Eq. 2-4)}$$

Overall, the absence of Fld\textsuperscript{Ox} in conjunction with the increased activity of the electron bifurcation reaction in comparison to diaphorase activity, suggest that the FixABCX complex can in fact perform electron bifurcation. To further investigate the flavin cofactors in FixABCX, transient absorption spectroscopy (TAS) was conducted.

*Transient absorption spectroscopy*

A comparative TAS study between FixAB from *Rh. rubrum* and FixABCX from *A. vinelandii* was performed in order to gain qualitative information on spectral
contributions from individual flavins. TAS of the incomplete Fix complex, FixAB, revealed an anionic semiquinone (ASQ) absorption at 365 nm with corresponding oxidized (Ox) flavin bleach at 447 nm. The ASQ absorption decays with two components: a short-lived component with a lifetime of tens of picoseconds and a longer short-lived component with a lifetime of 1000 picoseconds (Figure 2-8A, (Red trace), Table A6). A similar ASQ signal was also observed in the *A. vinelandii* FixABCX complex (Figure 2-8A (Black trace)). This suggests that two ASQ species can be formed in the FixAB unit, consistent with the presence of two flavins. Bifurcating flavins have been shown to exhibit crossed potentials, whereby the $E_m^{Ox/SQ}$ is at lower potential than the $E_m^{SQ/HQ}$ with the result that the high-energy SQ intermediate does not appreciably accumulate relative to Ox and HQ.

**Figure 2-8.** Transient absorption spectra of as-prepared *Rh. rubrum* FixAB (red traces) and *A. vinelandii* FixABCX (black traces). (A) Kinetic traces of ASQ signal (dots) at 365 nm. ASQ decay shows half-lives of ~15 ps and ~1000 ps when fit with a double exponential function (solid lines). (B) An NSQ absorption peak (~565-650 nm) is only observed in the *A. vinelandii* FixABCX complex.
A short-lived ASQ has been observed in the bifurcating Nfn from *Pyrococcus furiosus* and is proposed to play a central role in bifurcation.\(^{19,25}\) The current observation of relatively short-lived ASQ signals in *Rh. rubrum* FixAB and *A. vinelandii* FixABCX is consistent with the work on Nfn, and suggests that neither flavin significantly stabilizes a one-electron reduced species. Based on comparison with Etf, the flavin in FixA (a-FAD) (analogous to the β-FAD in EtfB) is the proposed site of bifurcation and likely corresponds to the tens of picosecond component in our TAS experiments.\(^{22}\) This assignment then implies that the FixB b-FAD acts as an electron transferring flavin, consistent with a slightly longer-lived ASQ, since accumulation of electrons at this site may congest electron flow and impede bifurcation. This may additionally play a role in gating electron transfer, ensuring that only one electron follows the exergonic path, and thus restricting the lower potential electron to travel down the endergonic branch, similar to the mechanism of electron bifurcation in *P. furiosus* Nfn.\(^{25}\) Due to the crossed potentials believed to characterize bifurcating flavins, the more negative potential electron of the bifurcating flavin (ASQ to Ox transition) would provide enough driving force to reduce the low-potential iron-sulfur clusters (predicted ≤ -440 mV vs. SHE, pH 7.5) in FixX directly. In the complete FixABCX complex from *A. vinelandii*, an additional peak is observed in the TAS corresponding to a neutral semiquinone (NSQ) and is assigned to the c-FAD in FixC (Figure 2-8B). We propose that the c-FAD serves to accumulate two electrons from successive bifurcations in order to permit 2-electron reduction of CoQ.
Proposed mechanism for FixABCX electron bifurcation

The data presented herein establish that the FixABCX complex from *A. vinelandii* can bifurcate electrons from NADH to CoQ\(_1\) and Fld\(^{Sq}\). The biochemical and biophysical evidence is consistent with a proposed mechanism of Fix electron bifurcation initiated at the flavin in FixA, which would accept a pair of electrons from NADH and pass one electron to the quinone pool via the flavins in FixB and FixC, and the other to Fld/Fd via the low potential [4Fe-4S] clusters in FixX, with the energetic cost being paid by favorable transfer of the former electron to the quinone pool (Figure 2-2). TAS on the FixABCX complex provides evidence by demonstrating the presence of a short-lived anionic flavin semiquinone consistent with the ability to support rapid and efficient electron transfer. The findings here provide experimental support for the mechanism proposed previously for a homologous bifurcating Etf that assigns the site of bifurcation to the flavin bound by FixA (EtfB).\(^{22}\) This assignment is further supported by the demonstration by Sato *et al.* that the flavin reduced by NADH is the one bound in FixA (EtfB), but that electron transfer to the flavin in FixB (EtfA) is favorable.\(^{39}\) Thus, upon reduction of the FixA flavin, transfer of an electron through the exergonic branch to CoQ via Fix B and FixC can leave the second electron in a very unstable, highly energetic flavin semiquinone state with a low \(E_m^{Ox/Sq}\) able to drive reduction of Fld/Fd, via FixX (Figure 2-2).

The mechanism presented here offers a means by which Fd/Fld can be reduced by NADH in a reaction that is thermodynamically favorable overall and identifies a new pathway by which low-potential reducing equivalents can be generated to drive nitrogen fixation.
ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

RNL, MHP, BMB, and LCS, FixABCX genetics; RNL, TSM and LCS, FixABCX purification and characterization; AMGC and JWP, fix and rnf mutational analyses and FixAB purification; MTL, AP, and BB, Mass spectrometry and chemical cross-linking; MTL, AP, BB, and AFM, Molecular modeling; CEL and PWK, Transient absorption spectroscopy; DWM, JHA, and PWK, Electron paramagnetic resonance; HDD, and AFM, Dynamic light scattering; JPH, RNL, and AFM, Flavin content; ZJJ, RNL, and RPC, Thermodynamics; JM, FixX purification; All authors involved in data interpretation, writing, and revisions.
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APPENDIX A

SUPPORTING INFORMATION

THE ELECTRON BIFURCATING FIXABCX PROTEIN COMPLEX FROM

AZOTOBACTER VINELANDII: GENERATION OF LOW-POTENTIAL

REDUcing EQUIVALENTS FOR NITROGENASE CATALYSIS
Table A1. Primers for Δfix mutant generation.

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Table A2. Primers for overexpression of FixABCX.

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Table A3 (starts on next page). Protein-protein interactions captured, (A) the inter-cross-links and (B) intra-cross-links within FixABCX complex during cross-linking reaction with BS3. Mapping of intra-subunit cross-links onto the homology models was consistent with the expected spanning distance of 20-25 Å between C-alpha chains. It should be noted that a subset of cross-links mapped to regions spanning 26-30 Å (for Lys-Lys) and higher. This is to be expected and has been explained by protein flexibility using known systems.¹
### Cross-linked residue Cα to Cα Distance

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- **FIXA**: `LK(156.0786)DKFGGTVTVVTMGPPMAEAALRK`
- **FIXC**: `LKDK(156.0786)FGGTVTVVTMGPPMAEAALRK`

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- **FIXC**: `LKDK(156.0786)FGGTVTVVTMGPPMAEAALRK`

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- **FIXC**: `LKDK(156.0786)FGGTVTVVTMGPPMAEAALRK`

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**Sequence: HVWVC(57.02146)IESERGVVHPVSWELLGEGRK**

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- **FIXC**: `LKDK(156.0786)FGGTVTVVTMGPPMAEAALRK`

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**Sequence: HVWVC(57.02146)IESERGVVHPVSWELLGEGRK**

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- **FIXC**: `LKDK(156.0786)FGGTVTVVTMGPPMAEAALRK`

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</tr>
<tr>
<td>FDVIVVGAGMAGNAAAYTLAKGGLKVLQIER - VVGVRTDRQGGEVRADAVILADGVNSR</td>
<td>4185.174</td>
<td>7.9</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>FDVIVVGAGMAGNAAAYTLAKGGLKVLQIERGETPGSK - VWVMDDASYVGTHYR</td>
<td>6950.565</td>
<td>23.9</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>FDVIVVGAGMAGNAAAYTLAKGGLKVLQIER - VVGVRTDRQGGEVRADAVILADGVNSR</td>
<td>4185.174</td>
<td>7.9</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>FDVIVVGAGMAGNAAAYTLAKGGLKVLQIERGETPGSK - VWVMDDASYVGTHYR</td>
<td>6950.565</td>
<td>23.9</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>FDVIVVGAGMAGNAAAYTLAKGGLKVLQIER - VVGVRTDRQGGEVRADAVILADGVNSR</td>
<td>4185.174</td>
<td>7.9</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>
Table A4. Overview of the EPR signals observed for FixABCX from *A. vinelandii*.

<table>
<thead>
<tr>
<th>Signal Name</th>
<th>g-values</th>
<th>$T_{\text{opt}}$ (K)</th>
<th>Samples present</th>
<th>Percent contribution</th>
<th>Possible source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast relaxing, broad</td>
<td>*</td>
<td>5 K</td>
<td>As-purified NADH</td>
<td>*</td>
<td>Unknown</td>
</tr>
<tr>
<td>Rhombic 2.07</td>
<td>2.07, 1.940,</td>
<td>15 K</td>
<td>NADH</td>
<td>86</td>
<td>([4\text{Fe-4S}]^{1+})</td>
</tr>
<tr>
<td></td>
<td>1.895</td>
<td></td>
<td>Na-dithionite</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Axial 2.04</td>
<td>2.041, 1.944,</td>
<td>5 – 10 K</td>
<td>NADH</td>
<td>9</td>
<td>([4\text{Fe-4S}]^{1+})</td>
</tr>
<tr>
<td></td>
<td>1.944</td>
<td></td>
<td>Na-dithionite</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Isotropic 2.005</td>
<td>2.005</td>
<td>&gt; 50 K</td>
<td>As-purified NADH</td>
<td>*</td>
<td>Flavin radical</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Na-dithionite</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Axial 2.03</td>
<td>2.030, 2.00,</td>
<td>10 K</td>
<td>NADH</td>
<td>4</td>
<td>Interacting flavin radical and ([4\text{Fe-4S}]^{1+})</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*Not determined
**Table A5.** Calculated Gibbs free energies of three possible reactions catalyzed by the Fix complex and three possible non-enzymatic reactions.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$\Delta G_{\text{rxn}}^o$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2 \text{NADH} + 2 \text{Fld}^{\text{Sq}} + \text{CoQ} = 2 \text{Fld}^{\text{Hq}} + \text{CoQH}_2 + 2 \text{NAD}^+$</td>
<td>-36.7</td>
</tr>
<tr>
<td>$\text{NADH} + \text{CoQH}_2 + 4 \text{Fld}^{\text{Sq}} = 4 \text{Fld}^{\text{Hq}} + \text{CoQ} + \text{NAD}^+ + 3 \text{H}^+$</td>
<td>118</td>
</tr>
<tr>
<td>$\text{NADH} + 2 \text{Fld}^{\text{Hq}} + 2 \text{CoQ} + 3 \text{H}^+ = 2 \text{CoQH}_2 + 2 \text{Fld}^{\text{Sq}} + \text{NAD}^+$</td>
<td>-154</td>
</tr>
<tr>
<td>$2 \text{Fld}^{\text{Sq}} + \text{CoQ} + 2 \text{H}^+ = 2 \text{Fld}^{\text{Ox}} + \text{CoQH}_2$</td>
<td>-36.7</td>
</tr>
<tr>
<td>$\text{NADH} + \text{CoQ} + \text{H}^+ = \text{CoQH}_2 + \text{NAD}^+$</td>
<td>-63.7</td>
</tr>
<tr>
<td>$\text{NADH} + 2 \text{Fld}^{\text{Ox}} = 2 \text{Fld}^{\text{Sq}} + \text{NAD}^+ + \text{H}^+$</td>
<td>-27.0</td>
</tr>
</tbody>
</table>

Fld$^{\text{Sq}} + e^- = \text{Fld}^{\text{Hq}}, E^o = -460 \text{ mV}; \text{Fld}^{\text{Ox}} + e^- = \text{Fld}^{\text{Sq}}, E^o = -180 \text{ mV}; \text{CoQ} + 2 e^- = \text{CoQH}_2, E^o = + 10 \text{ mV}; \text{NAD}^+ + 2 e^- = \text{NADH}, E^o = -320 \text{ mV}
Table A6. Fitting of the ASQ kinetic curves for *Rh. rubrum* FixAB and *A. vinelandii* FixABCX reveal a biphasic decay, with one component having a lifetime of tens of picoseconds and a slightly longer lifetime of one thousand picoseconds. Fitting was performed in Igor Pro using a double exponential fit function.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ASQ $\tau_1$</th>
<th>ASQ $\tau_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. rubrum</em> FixAB</td>
<td>14 ps</td>
<td>970 ps</td>
</tr>
<tr>
<td><em>A. vinelandii</em> FixABCX</td>
<td>14 ps</td>
<td>1140 ps</td>
</tr>
</tbody>
</table>
Figure A1. Scheme for $\Delta fix$ mutant generation.
Figure A2. Scheme for overexpression of $fix$ genes under the $nifH$ promoter in $A. vinelandii$. 
Figure A3. Protein identification within FixABCX complex purified from *A. vinelandii*. The identity of FixA (panel A), FixB (panel B), FixC (panel C) and FixX (panel D) subunits was confirmed based on 54, 22, 59 and 9 unique tryptic peptides that correspond to 79%, 63%, 85% and 73% sequence coverage (top row). Peptides and proteins are represented by blue and red spheres, respectively. Identified peptides were rendered in blue on primary sequence (middle row) and homology models of corresponding subunits (bottom row). Homology models were generated based on following protein templates: FixA, 4L2I; FixB, 3CLR, FixC, 4K2X; FixX, 2GMH.
Figure A4. Size distribution of protein complexes as revealed by dynamic light scattering, comparing buffer containing DDM (negative control) and the purified FixABCX complex. (A) Buffer containing 0.02% DDM (w/v) with a determined hydrodynamic radius of 4.32 nm. (B) 1.0 mg/mL of FixABCX complex with a determined hydrodynamic radius of 4.69 nm accounted for 99.4% of the total population. Triplicate measurements yielded an average hydrodynamic radius of 5.1 ± 0.3 nm.
**Figure A5.** Evaluation of subunit homology models. *ProQ2 quality assessment:* a support vector machine is used to predict local and global quality of the model; *Clashes:* indicates which residues lie too close to one another due to incorrect side chain and/or backbone placement; *Ramachandran analysis:* provides information on residues position (favorable/allowed/disallowed) in the model and potential problems with the backbone phi/psi angles; *Alignment confidence:* reliability of the pair-wise query-template alignment as reported by HHsearch. The confidence values are obtained from the posterior probabilities calculated in the forward-backward algorithm; *Disorder:* identifies dynamically flexible regions. This prediction has been made by the knowledge-based Disopred method (PSIPRED server). Overall, models for each subunit were obtained with the highest template confidence of 100% (with the exception of 99.8% for FixX). However, it was impossible to attain complete sequence coverage, primarily due to insufficient structural information in the database. For example, sequence coverage for FixA, FixB, FixC and FixX were 92%, 88%, 82% and 96%, respectively.
**Figure A6.** Predicted structural features in Fix subunits. *Conservation:* sequence analysis based on Jensen-Shannon divergence; *Largest pocket:* the pockets are detected by the fpocket2 program and highlighted in red. This provides a tool for tracking small molecule binding sites, pockets for molecular docking or detection of subpockets of conformational ensembles. The highest degree of conservation (from green to red color) is associated with cofactor binding sites. A helix (Ala338-Leu353) on the C-terminal domain of FixB appears to be critical for A and B subunit interactions. Further, large pockets are detected around FixA, FixB and FixX cofactor binding sites. In addition to flavin binding sites, two large cavities are present between the N-terminal and C-terminal domain of FixB (important for interactions with FixA) and near the predicted transmembrane region (Met299-Leu325) on FixC.
Figure A7. Protein interface. PI-site interface residues\textsuperscript{6} and ProtinDB interface residues provide information on prediction of protein-protein interaction sites based on information extracted from protein complexes stored in the Protein Data Bank. Interface residues are colored in red.
**Figure A8.** Structural model of FixABCX from *A. vinelandii*. Fix subunit homology models were built using Phyre2 and had the closest match to following templates from the Protein Data Bank (PDB): FixA, 4L2l; FixB, 3CLR, FixC, 4K2X; FixX, 2GMH. Chemical cross-linking data was used to produce distance constraints for generating structural models. (A) ClusPro2 docking model of FixAB subunits (visualized in Chimera). FixA in blue, FixB in tan. (B) ClusPro2 docking model of FixCX subunits (visualized in Chimera). FixC in green, FixX in red. (C) FixABCX complex model generated by docking (ClusPro2) four homology models (Phyre2). Restraints derived from the chemical cross-linking experiment. The final model was evaluated and satisfied two requirements: 1) Most of the cross-links could be explained by the model and 2) cofactor distances of <14 Å that enable electron transfer. (D) Cofactors and protein residues that could function in electron transfer. (E) Distances between cofactors taking into account the presence of conserved Trp and Tyr side chains that may convey electrons between cofactors. FixA-Tyr37 is conserved as Tyr in 44 of the 53 FixAs of which we are aware, FixB-Trp275 is conserved as Trp in 53 out of 53 FixBs of which we are aware, and FixC-Tyr277 is Tyr in 8/8 sequences examined.
Figure A9. EPR temperature profiles of FixABCX from *A. vinelandii* reduced with either Na-dithionite or NADH. (A) FixABCX (100 µM) reduced with Na-dithionite (10 mM). (B) FixABCX (100 µM) reduced with NADH (1 mM). The spectra were recorded at 5, 10, 15, 20, 30, 40, 50, 60 and 80 K. Simulations of the overall spectra at 10 K are shown in the lighter shade of blue. The individual signal components and g-values comprising the overall simulated spectra at 10 K are shown at the top (blue, axial 2.04; red, rhombic 2.07 magenta, isotropic 2.0; green, axial 2.03) and varied slightly for each treatment (Table S4). The overall signal intensities for NADH (0.40 spins/mol protein) and Na-dithionite treatments (0.45 spins/mol protein) were similar at 10 K, indicating partial reduction of the protein complex. Microwave frequency, 9.38 GHz; microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 10.0 G.
Figure A10. Comparison of EPR spectra of reduced FixABCX from *A. vinelandii* (Av) and the reduced, individual subunit FixX from *R. castenholzii* (Rc). FixABCX from *A. vinelandii* (100 μM) was reduced 10 mM Na-dithionite. FixX from *R. castenholzii* (200 μM) was reduced with 5 mM Na-dithionite. Signal intensities were normalized for sample concentration. Experimental (exp) spectra are colored black and simulated (sim) spectra are colored gray. The individual signal components and $g$-values comprising the overall simulated spectra are shown at the top (blue, axial 2.04; red, rhombic 2.07 magenta, isotropic 2.0; green, axial 2.03). Microwave frequency, 9.38 GHz; microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 10.0 G; sample temperature, 10 K.
REFERENCES


CHAPTER 3

EVIDENCE THAT THE Pi RELEASE EVENT IS THE RATE LIMITING STEP IN THE NITROGENASE CATALYTIC CYCLE

ABSTRACT

Nitrogenase reduction of dinitrogen (N_2) to ammonia (NH_3) involves a sequence of events that occur upon the transient association of the reduced Fe protein containing two ATP with the MoFe protein that includes electron transfer, ATP hydrolysis, Pi release, and dissociation of the oxidized, ADP containing Fe protein from the reduced MoFe protein. Numerous kinetic studies using the non-physiological electron donor dithionite have suggested that the rate limiting step in this reaction cycle is the dissociation of the Fe protein from the MoFe protein. Here, we have established the rate constants for each of the key steps in the catalytic cycle using the physiological reductant flavodoxin protein in its hydroquinone state. The findings indicate that with this reductant, the rate limiting step in the reaction cycle is not protein-protein dissociation or reduction of the oxidized Fe protein, but rather events associated with the Pi release step. These results allow a revision of the rate limiting step in the nitrogenase Fe protein cycle.


*Modified version focuses on contributions from Rhesa Ledbetter. The full study can be found in Appendices B and C.
INTRODUCTION

Nitrogenase is the catalyst responsible for biological nitrogen fixation, the reduction of dinitrogen (N\textsubscript{2}) to ammonia (NH\textsubscript{3}).\textsuperscript{1–3} The molybdenum-dependent nitrogenase consists of two catalytic components called the MoFe protein and the Fe protein.\textsuperscript{4} The Fe protein is a dimer of two identical subunits connected by a single [4Fe-4S] cluster and is responsible for transferring a single electron to the MoFe protein.\textsuperscript{5,6} The MoFe protein is a heterotetramer, composed of two symmetric \(\alpha\beta\) units. Each \(\alpha\beta\) unit contains two unique metal clusters, the electron carrier P-cluster [8Fe-7S] and the active site FeMo-cofactor [7Fe-9S-Mo-C-homocitrate].\textsuperscript{7–11} An Fe protein, with two bound MgATP molecules, binds transiently to each \(\alpha\beta\) unit of the MoFe protein during the electron transfer (ET) event (Figure 3-1).\textsuperscript{6,12,13} ET is initiated upon association of the Fe protein to the MoFe protein, followed by a conformationally gated one-electron transfer from the P-cluster to FeMo-cofactor.\textsuperscript{14,15} This is followed by the one-electron transfer from the reduced Fe protein (Fe\textsuperscript{red}) [4Fe-4S] cluster to the oxidized P-cluster (P\textsuperscript{1+}), in what has been called a “deficit spending” ET process.\textsuperscript{14} Following ET, the two ATP molecules are hydrolyzed to two ADP and two Pi molecules. Next, the two Pi are released from the complex, followed by the dissociation of the oxidized Fe protein (Fe\textsuperscript{ox}) with two bound MgADP (Fe\textsuperscript{ox}(ADP)\textsubscript{2}) from the MoFe protein.\textsuperscript{16} The Fe\textsuperscript{ox}(ADP)\textsubscript{2} is then reduced by a reductant and the two ADP molecules are replaced by two ATP molecules.\textsuperscript{17,18} This cycle, often referred to as the Fe protein cycle, must be repeated a sufficient number of times to accumulate the electrons necessary for substrate reduction in the MoFe protein.\textsuperscript{1–3,18–20}
Over the past five decades, most of the in vitro mechanistic studies of nitrogenase have been conducted with the non-physiological reductant dithionite (DT), largely because of ease of use.\(^1,3,18\) The kinetic parameters for each step in the Fe protein cycle using DT as reductant have been deduced and are summarized in the scheme shown in Figure 3-2.\(^16\)–\(^18,21,22\) It was concluded from a comparison of the rate constants that the overall rate limiting step in the Fe protein cycle is the dissociation of the Fe\(^{ox}\)(ADP)\(_2\) from the MoFe protein with a rate constant of ~6 s\(^{-1}\).\(^16,17\) A problem that is often overlooked in such studies is the reversible dissociation of DT (S\(_2\)O\(_4^{2-}\)) to generate the actual reductant, the radical anion SO\(_2^{\bullet-}\), with a \(K_d\) of ~1.5 nM and a slow rate constant of ~2 s\(^{-1}\).\(^17\) This leads to slow reduction of Fe\(^{ox}\), with rate constants for this reduction near the rate constant that is reported for dissociation of Fe\(^{ox}\)(ADP)\(_2\) from MoFe protein.\(^16,17,22,23\)
Figure 3-2. Fe protein cycle with first order and pseudo-first order kinetic rate constants for each step.

The physiological reductants of Fe protein are known to be the electron carrier proteins flavodoxin (Fld) and/or ferredoxin (Fd).\textsuperscript{24–30} Because nitrogenase is readily reduced by artificial electrons donors, such as DT, a limited number of kinetic studies using physiological reductants have been conducted.\textsuperscript{24,25,27,30–35} The diazotroph \textit{Azotobacter vinelandii} contains several Flds and Fds. NifF (Fld) has been implicated as an electron donor to nitrogenase\textsuperscript{26,29,30,36} and has been shown to transfer electrons to Fe protein \textit{in vitro}.\textsuperscript{24,25,27,33,34} Further, disruption in the \textit{nifF} gene results in a 30\% decrease in whole-cell acetylene reduction activity. This previous research demonstrates that Fld is a major, but not the sole, electron donor to nitrogenase.\textsuperscript{26,29} Fld has three different redox states designated as the oxidized quinone (Fld\textsuperscript{Q}), one-electron reduced semiquinone (Fld\textsuperscript{SQ}), and two-electron reduced hydroquinone (Fld\textsuperscript{HQ}).\textsuperscript{24,37,38} The pH- and temperature-
dependent midpoint redox potentials ($E_m$) for the two redox couples are estimated as -180 mV (vs the NHE) for the $\text{Fld}^\text{Q}/\text{Fld}^\text{SQ}$ couple, and -480 mV for the $\text{Fld}^\text{SQ}/\text{Fld}^\text{HQ}$ couple (pH 8.5 and 22°C). The $\text{Fld}^\text{SQ}/\text{Fld}^\text{HQ}$ redox couple has sufficient driving force for reduction of the oxidized Fe protein with a midpoint potential of -290 mV without nucleotide bound and -440 mV with ADP bound. Earlier kinetic studies using Fld as reductant have yielded results that contradict some aspects of the Fe protein cycle deduced with DT: (i) the one-electron reduction of Fe protein by $\text{Fld}^\text{HQ}$ is much faster than that by DT, and this reduction event might happen when $\text{Fe}_\text{oxygen}^\text{ox} \text{(ADP)}_2$ is still bound to the MoFe protein and (ii) in the Fe protein cycle, the dissociation step might be faster than what has been widely accepted (~6 s^-1). In the present work, kinetic studies were performed using both DT and Fld (NifF from *A. vinelandii*) as reductant to determine the key kinetic parameters in the Fe protein cycle. This work reveals that $\text{Fld}^\text{HQ}$ cannot reduce the Fe protein while it is bound to MoFe protein and the rate limiting step in the Fe protein cycle is not the dissociation of the Fe protein from the MoFe protein, but rather events associated with Pi release.

**MATERIALS AND METHODS**

**General procedures**

All chemicals, unless otherwise noted, were obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification. Hydrogen, acetylene, ethylene, argon, and dinitrogen gases were purchased from Air Liquide America Specialty Gases LLC (Plumsteadville, PA). The argon and dinitrogen gases were passed through an activated copper-catalyst to remove dioxygen contamination prior to use. *A. vinelandii* strains DJ995 (wild type MoFe protein) and DJ884 (wild type Fe protein) were grown, and
nitrogenase proteins expressed and purified as previously described. Both proteins were greater than 95% pure as confirmed by SDS-PAGE analysis using Coomassie blue staining and fully active (see results and discussion). Proteins and buffers were handled anaerobically in septum-sealed serum vials under an inert atmosphere (argon or dinitrogen), on a Schlenk vacuum line, or anaerobic glove box (Teledyne Analytical Instruments, MO-10-M, Hudson, NH). The transfer of gases and liquids were done with gastight syringes.

*Strain construction and expression, Escherichia coli growth, and NifF purification*

The *nifF* gene from *A. vinelandii* was PCR amplified and cloned into the NdeI-BamHI sites of a T7-7 plasmid containing an ampicillin resistance gene for selection. Fld was overexpressed in *Escherichia coli* (*E. coli*) BL21 DE3 cells. The cells were grown to an optical density (600 nm) of 0.6-0.8 at 37 °C before the protein expression was induced at 32 °C by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) and flavin mononucleotide (FMN) to a final concentration of 1 mM and 10 mg/L, respectively. Following a 10 hour induction, cells were harvested.

All steps during the purification of Fld were conducted anaerobically under an argon atmosphere. 100 grams of cell paste were resuspended in 50 mM Tris, pH 8 with 2 mM dithiotheritol (DTT) at a biomass to buffer ratio of 1:5 (w/v). The resuspended cells were lysed in a french pressure cell (SLM Aminco FA-078, Rochester, NY) at 200 MPa. The cell lysate was centrifuged (Sorvall Lynx 4000, ThermoScientific, Waltham, MA) at 48,000 x g for 30 minutes at 4°C. Following centrifugation, 0.5% (w/v) streptomycin sulfate was added to the supernatant to precipitate nucleic acids. The precipitation was removed via a second centrifugation as described above. The supernatant was loaded
onto a 150 ml Q-sepharose column, which was first washed with two column volumes of Buffer B (50 mM Tris, 1 M NaCl, 1 mM DTT, pH 8) and then equilibrated with two column volumes of Buffer A (50 mM Tris, 1 mM DTT, pH 8). A 15-75% salt gradient was run over 5 column volumes. Fractions containing Fld (blue color) were combined and diluted with Buffer A to a final NaCl concentration of less than 100 mM. FMN was added to a final concentration of 2 mM to increase the percentage of holo-Fld in the presence of 2 mM DT, which slowly changed the color of the protein solution from blue to yellow. The reconstitution was carried out for at least 2 hours at room temperature. The protein was then loaded onto a Q-sepharose column (~30 mL) pre-reduced and equilibrated with Buffer B and Buffer A containing 1 mM DT. After loading, the column was washed with 1 column volume of Buffer A and eluted with 100% Buffer B as a concentrated fraction for loading onto Sephacryl-200 column (~600 ml) equilibrated with an 100 mM HEPES (pH 7.8) with 150 mM NaCl, 0.5 mM DTT, and 1 mM DT. The Fld was followed as a yellow-green band. The protein was concentrated using an Amicon (EMD Millipore, Billerica, MA) concentrator with a 10,000 kDa cutoff membrane and stored in liquid nitrogen. The purity of the Fld was greater than 95% based on SDS-PAGE method described above. The FMN content was determined to be ~70% of the total protein. This was determined by measuring the absorbance of Fld\(^{Q}\) at 452 nm and using an extinction coefficient of 11.3 mM\(^{-1}\) cm\(^{-1}\).\(^{37}\) The Fld concentration used in this work refers to the concentration of the holo-Fld with FMN bound.
**Stopped-flow (SF) spectrophotometry and reduction of Fe protein by DT and Fld^{HQ}**

SF spectrophotometry was conducted using an AutoSF-120 stopped-flow instrument equipped with a data acquisition system (KinTek Corp., Snow Shoe, PA). The change in absorbance was monitored at 426 nm over time. This wavelength detects a decrease in absorbance as the Fe protein [4Fe-4S] cluster becomes reduced. 426 nm rather than 430 nm was chosen since it is the isosbestic point of Fld. Reactions were conducted at 4°C, unless otherwise stated, with a mixing ratio of 1:1. All samples were prepared in 100 mM MOPS buffer (pH 7.3). Fe protein was oxidized in an anaerobic glove box using ~3-fold molar excess of phenazine methosulfate (PMS). The protein-PMS mixture was allowed to incubate for 15 minutes prior to separation with Sephadex G-25 equilibrated with 100 mM MOPS, pH 7.3 with 150 mM NaCl. The oxidized Fe protein was contained in one drive syringe with or without nucleotide. The other drive syringe contained the electron donor, DT or Fld^{HQ}-DT mixture, with or without nucleotide. All reagents were used at the following final concentrations and kept under an argon atmosphere: 40 μM oxidized Fe protein, 10 mM DT, 300 μM Fld, 10 mM ATP or ADP, and 10 mM MgCl₂ (only used in the presence of nucleotides). For the reduction of Fe^{ox} by DT, the data were fit to a single exponential equation to obtain the pseudo-first order rate constants. Due to the presence of both Fld^{HQ} and DT in the experiments, the pseudo-first order rate constants for reduction of Fe^{ox} by Fld^{HQ} were obtained by fitting the data to a double exponential equation.

**Reduction of Fe^{ox}(ADP)₂ by different reductants in the presence of MoFe protein**

The Fe-MoFe dissociation rate constant was determined using SF as described above except that the temperature was 25°C. Fe protein was oxidized (see details above).
and MoFe protein was stripped of DT in an anaerobic glove box. DT was removed from MoFe protein using a DOWEX-Sephadex G-25 column equilibrated with 100 mM MOPS, pH 7.3 containing 150 mM NaCl. One drive syringe contained 80 μM Feox, 80 μM MoFe protein, and 9 mM MgADP. The other syringe contained the reductant mixture: 1) 20 mM DT, 2) 100 μM methyl viologen (MV)-20 mM DT mixture, or 3) 400 μM FldHQ-20 mM DT mixture. All reductants contained 9 mM MgADP. DT data were fit to a single exponential decay equation, and MV-DT and FldHQ-DT data were fit to a double exponential decay equation.

**Primary electron transfer**

Primary electron transfer from the Fe protein to the MoFe protein in the presence of DT and Fld was measured at 25°C using SF spectrophotometry as described above. All mixtures were prepared in 100 mM MOPS, pH 7.3 and were kept under an argon atmosphere. One syringe contained 80 μM Fe protein, 20 μM MoFe protein, 1 mM DT with or without 200 μM FldHQ. The other syringe was loaded with 1 mM DT and 20 mM MgATP. As turnover occurred in the [Feed(MgATP)2-MoFe] complex, the oxidation of the [4Fe-4S] cluster of the Fe protein was monitored by an increase in absorbance at 426 nm. Data were fit to a single exponential curve.

**Real-time measurement of inorganic phosphate (Pi) release**

Pi release was determined in a stopped-flow (SF) fluorometer (Auto SF-120, KinTek Corp.) using a coumarin (N-[2-(1-maleimidyl)ethyl]-7-(diethylamino) coumarin-3-carboxamide) labeled phosphate binding protein (MDCC-PBP) assay.42 Pi was quantified from binding MDCC-PBP that was monitored by an increase in fluorescence (λ-excitation = 430 nm, λ-emission >450 nm), using a standard curve generated with
KH$_2$PO$_4$ as described.$^{42}$ The experiments were carried out at 25°C in 25 mM HEPES, pH 7.4 containing 1 mM DT. The SF syringes and flow lines were treated with a Pi mop (25 mM HEPES buffer at pH 7.4 with 320 µM 7-methylguanine (7-meG), and 0.12 U/mL purine nucleoside phosphorylase (PNPase)) before each experiment for 45 minutes to remove contaminating Pi and then rinsed with DT reduced buffer. The same concentration of the Pi mop system was also added to the reaction mixtures. 1 µM MoFe and 16 µM Fe were rapidly mixed with a solution of 25 µM MDCC-PBP, 7.5 mM MgCl$_2$ and 6 mM ATP and the change in fluorescence was monitored over time. 100 µM Fld$^{HQ}$ was added to the MoFe and Fe protein mixture to monitor the effect of Fld$^{HQ}$ on Pi release.

RESULTS AND DISCUSSION

*Establishing $k_{cat}$ with DT or Fld$^{HQ}$*

In the two-component catalytic system of nitrogenase, both the Fe protein and the MoFe protein are catalysts. The rate limiting step for the overall reaction is held to be the dissociation step in the Fe protein cycle (Figure 3-2). This means that the rate constant of the rate limiting step should be the same as the $k_{cat}$ for both Fe protein and MoFe protein cycles in terms of electrons donated/accepted per active site per unit time. Earlier studies established a turnover number ($k_{cat}$) for electron flow to substrate reduction of between 5-10 s$^{-1}$ regardless of substrate.$^{31,43}$ This value is approximately the same as the first order rate constant for dissociation of the Fe$^{\alpha\gamma}$(MgADP)$_2$ from the MoFe protein of $\sim$6 s$^{-1}$, leading to the conclusion that the overall reaction rate limiting step is dissociation (Figure 3-2).$^{1,16-18,31}$ However, a few previous studies have shown that the rate of
dissociation of the Fe-MoFe protein complex using DT is slower than the $k_{\text{cat}}$ under saturating (‘high flux’) conditions.\textsuperscript{40,43}

Zhi-Yong Yang determined the $V_{\text{max}}$ for both Fe protein and MoFe protein cycles using either DT or Fld as reductant by varying the ratio of Fe protein to MoFe protein (called the “electron flux”). ‘Low-flux’ conditions, which saturate the Fe protein with MoFe (e.g. 1:1 [Fe:MoFe]) protein, resulted in a Fe protein cycle $k_{\text{cat,Fe}}$ of ~6 s\textsuperscript{-1} with DT as reductant. When Fld\textsuperscript{HQ}-DT was used as the reductant, a $k_{\text{cat,Fe}}$ of 10-11 s\textsuperscript{-1} was observed (Table 3-1). These results revealed that using Fld as reductant speeds up the overall reaction, suggesting that the rate limiting dissociation step has been accelerated by about 2-fold when Fld is the reductant compared to the dissociation rate constant when DT is the reductant.\textsuperscript{40} This enhancement effect, however, was dependent on the electron flux, with lower flux (most representative of physiological conditions) showing the largest effect. When the electron flux was increased to saturate the MoFe protein with Fe protein (‘high flux’, e.g. [Fe]:[MoFe] = 16:1) to measure the MoFe protein cycle, the $k_{\text{cat}}$ was found to be ~10-11 s\textsuperscript{-1} with both DT and Fld as reductant. The greater $k_{\text{cat,Fe}}$, observed for Fld\textsuperscript{HQ} compared to DT as reductant under ‘low flux’ conditions could be explained by an increase in the rate of dissociation of the Fe protein from MoFe protein when Fld\textsuperscript{HQ} is the reductant. Based on Yang’s steady-state data, we then determined an apparent dissociation rate constant for Fe\textsuperscript{0x}(ADP)\textsubscript{2} – MoFe protein with either DT or Fld as reductant.
**Table 3-1.** Summary of steady-state $k_{\text{cat}}$ values for Fe protein and MoFe protein with different reductants. Data provided by Zhi-Yong Yang.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reductant</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fe Protein</em></td>
<td>DT</td>
<td>5-6</td>
</tr>
<tr>
<td>'<em>Low flux'</em></td>
<td>Fld$^{\text{HQ}}$</td>
<td>10-11</td>
</tr>
<tr>
<td><em>MoFe protein</em></td>
<td>DT</td>
<td>10-11</td>
</tr>
<tr>
<td>'<em>High flux'</em></td>
<td>Fld$^{\text{HQ}}$</td>
<td>10-11</td>
</tr>
</tbody>
</table>

**Dissociation of the Fe$^{\text{ox}}$(ADP)$_2$ from the MoFe protein**

To determine the apparent dissociation constant using SF spectrophotometry, the Fe$^{\text{ox}}$(ADP)$_2$-MoFe protein complex was pre-formed in one syringe of the SF spectrophotometer and rapidly mixed against a reductant mixture. Here, such experiments were conducted with either DT or Fld$^{\text{HQ}}$ in the second syringe. The reduction of the Fe$^{\text{ox}}$ was monitored at 426 nm, an isosbestic point for reversible conversion between Fld$^{\text{SQ}}$ and Fld$^{\text{HQ}}$. As shown in Figure 3-3, the estimated first order rate constant for dissociation ($k_{\text{obs}}$) with DT as reductant was $\sim$4 s$^{-1}$. When Fld$^{\text{HQ}}$ was the reductant, the dissociation and reduction of Fe$^{\text{ox}}$(ADP)$_2$ was much more rapid, with the majority of the reduction ($\sim$65%) occurring in less than 1 ms. A $k_{\text{obs}}$ of $>760$ s$^{-1}$ was estimated from a fit of the points captured after the dead time. These findings reveal that with Fld$^{\text{HQ}}$ as reductant, the reduction of Fe$^{\text{ox}}$ is much faster than the observed $k_{\text{cat}}$, implying that dissociation of Fe$^{\text{ox}}$(ADP)$_2$ from MoFe protein is not the rate limiting step when Fld$^{\text{HQ}}$ is used as the reductant. These results are consistent with the previously reported data from similar experiments.$^{25}$ To further test the effect of reductant on the apparent rate of dissociation, another non-physiological reductant, methyl viologen was used.$^{44}$ As can be seen in Figure 3-3, this electron donor gave a $k_{\text{obs}}$ of 100 s$^{-1}$, again much faster than the $k_{\text{cat}}$. 

Figure 3-3. Reduction of Fe$^{\text{ox}}$(ADP)$_2$ protein by DT (red), MV (green), or Fld$^{\text{HQ}}$ (blue) in the presence of MoFe protein. The reduction of Fe$^{\text{ox}}$(ADP)$_2$ by different reductants was monitored as the decrease of the absorbance at 426 nm as a function of time. The data are displayed as gray dots and were fit to different equations as described in the Materials and Methods section to obtain the pseudo-first order $k_{\text{obs}}$. Syringe 1 contained 20 mM DT with 100 μM MV, or 400 μM Fld$^{\text{HQ}}$ with 20 mM DT. Syringe 2 contained 80 μM Fe$^{\text{ox}}$ and 80 μM MoFe. MgADP (9 mM) was present in both syringes.

The faster reduction of Fe$^{\text{ox}}$(ADP)$_2$ in the presence of MoFe protein by Fld$^{\text{HQ}}$ could be explained by two possible mechanisms: (i) the very rapid dissociation of the Fe$^{\text{ox}}$(ADP)$_2$ from the MoFe protein when Fld$^{\text{HQ}}$ is the reductant, pointing to a different rate limiting step,$^{40}$ or (ii) Fld$^{\text{HQ}}$ reduction of the Fe protein while still complexed to the MoFe protein,$^{45}$ a mechanism proposed earlier by Haaker et al.$^{25}$ To test the second model, the ability of Fld$^{\text{HQ}}$ to reduce the Fe$^{\text{ox}}$(ADP)$_2$ while still bound to the MoFe protein was examined.
**Effect of Fld\textsuperscript{HQ} on the primary electron transfer of nitrogenase**

The pre-steady state ET from the Fe protein to the MoFe protein offers a straightforward way to monitor the possibility of ET from Fld\textsuperscript{HQ} to Fe\textsuperscript{ox} while still bound to the MoFe protein. When the protein mixture of Fe\textsuperscript{red} and MoFe protein is mixed with MgATP in a SF spectrophotometer, an apparent first order ET event is monitored by the increase in absorbance (oxidation of the Fe protein) that can be fit to a single exponential to yield a rate constant for ET ($k_{ET}$). With DT as the reductant, a $k_{ET}$ of 173 s\textsuperscript{-1} was observed (Figure 3-4), consistent with literature values that range from 140-200 s\textsuperscript{-1}.\textsuperscript{14-16,22,25} Important for this study, the absorbance value plateaus starting at 10 ms and stays roughly flat up to 30 ms. This plateau in absorbance reflects no reduction by DT of the Fe\textsuperscript{ox} protein while still in the complex. At much later times (100 ms), the absorbance does change, reflecting a complex set of events as the Fe protein dissociates from the MoFe protein, is reduced, and rebinds to the MoFe protein. When the ET study is conducted with Fld\textsuperscript{HQ} as reductant, nearly identical primary ET kinetics are observed (Figure 3-4). Importantly, no reduction of the Fe\textsuperscript{ox} protein is observed during the 10 to 30 ms time frame. Given the earlier observation of reduction of Fe\textsuperscript{ox}(ADP)\textsubscript{2} protein-MoFe protein with Fld\textsuperscript{HQ} of greater than 760 s\textsuperscript{-1}, an on-complex reduction of the Fe protein in the ET study should have resulted in a significant (if not complete) reduction of the Fe protein before 10 ms. The lack of any observed reduction of the Fe\textsuperscript{ox} protein in the ET experiment reveals that the Fld\textsuperscript{HQ} protein reduction of the Fe\textsuperscript{ox}(ADP)\textsubscript{2} protein on complex with the MoFe protein must be very slow, as has been reported earlier.\textsuperscript{45} Thus, these studies, coupled with the dissociation studies presented in the previous section, rule out Fld\textsuperscript{HQ} reduction of the Fe protein while still bound to the MoFe protein.\textsuperscript{25} and instead
favor a rapid dissociation of Fe\textsuperscript{ox}(ADP)\textsubscript{2} protein from the MoFe protein when Fld\textsuperscript{HQ} is the reductant.

**Reduction of Fe\textsuperscript{ox} protein by DT and Fld\textsuperscript{HQ}**

The studies, to this point, favor rapid dissociation of the Fe\textsuperscript{ox}(ADP)\textsubscript{2} protein from the MoFe protein when Fld\textsuperscript{HQ} is the reductant. The observed lower rate of dissociation when DT is the reductant could indicate slow reduction of Fe\textsuperscript{ox} protein, rather than slow dissociation of the Fe protein from the MoFe protein.\textsuperscript{17,23,25} Several previous studies have illustrated low activity for nitrogenase at low concentrations of SO\textsubscript{2}\textsuperscript{•} as the reductant.\textsuperscript{17–19,22,46} To determine the rates of reduction of Fe\textsuperscript{ox} protein by different reductants, the pre-steady state studies were conducted using Fe\textsuperscript{ox} in the absence and presence of nucleotides
(ADP or ATP). Fe\textsuperscript{ox} protein in one syringe was rapidly mixed against either DT or Fld\textsuperscript{HQ} in the other syringe. Additionally, these studies were conducted at 4°C to slow the reactions down enough to observe. Consistent with previous studies, nucleotides significantly slow down the rates of reduction of Fe\textsuperscript{ox} protein by DT (by ~ 100-fold).\textsuperscript{22,23}

In contrast, with Fld\textsuperscript{HQ} as reductant, the rates of reduction remained fast and roughly unchanged with or without nucleotide present (Figure 3-5).\textsuperscript{25,27}

**Figure 3-5.** Kinetics of the reduction of Fe\textsuperscript{ox} protein by DT and Fld\textsuperscript{HQ} with DT in the presence and absence of nucleotides. ET is monitored by observing the change in absorbance at 426 nm as a function of time. Panel A: Reduction of Fe protein by DT and Fld\textsuperscript{HQ} with DT with no nucleotide. Panel B: Reduction of Fe protein by DT and Fld\textsuperscript{HQ} with DT in the presence of MgADP. Panel C: Reduction of Fe protein by DT and Fld\textsuperscript{HQ} with DT in the presence of MgATP. Syringe 1 contained 80 μM Fe\textsuperscript{ox}. Syringe 2 contained 20 mM DT or 600 μM Fld\textsuperscript{HQ} with 20 mM DT. MgADP and MgATP, when included, were present in both syringes at a final concentration of 10 mM. The $k_{\text{obs}}$ values were averaged from two independent experiments except for Fld with MgATP.
Taken together, the studies presented so far suggest that when Fld\textsuperscript{HQ} is the reductant, the Fe\textsuperscript{ox}(ADP)\textsubscript{2} dissociation from the MoFe protein is not rate limiting and does not correspond to the overall $k_{\text{cat}}$ for substrate reduction. Rather, the dissociation step when Fld\textsuperscript{HQ} is used as the reductant is much more rapid than the $k_{\text{cat}}$. What then is the rate limiting step in the overall reaction cycle?

**Kinetic analyses of ATP hydrolysis and Pi release**

In the Fe protein cycle using DT as the reductant (Figure 3-2), the ATP hydrolysis ($k_{\text{ATP}} = 50\text{--}70 \text{ s}^{-1}$) and the Pi release ($k_{\text{Pi}} = 16\text{--}22 \text{ s}^{-1}$) steps both are slower than the ET ($k_{\text{ET}} = 140\text{--}200 \text{ s}^{-1}$). Sudipta Shaw compared pre-steady state rate constants for ATP hydrolysis using DT or Fld\textsuperscript{HQ} as reductant. A 1:4 ([MoFe]:[Fe]) electron flux revealed similar values ($k_{\text{ATP,DT}}$ and $k_{\text{ATP,Fld}}$) of $40 \text{ s}^{-1}$ and $44 \text{ s}^{-1}$ (25\degree C), respectively (data not shown). These rate constants were about double the $k_{\text{cat}}$ (10\text{--}11 s\textsuperscript{-1}), which is based on the finding that 2 ATP molecules are hydrolyzed per electron (determined by Sudipta Shaw, data not shown). Overall, Shaw’s data indicate that ATP hydrolysis is not the rate-limiting step for the overall reaction. Interestingly, Shaw also discovered that the steady state ATP hydrolysis rate constant of 25\text{--}27 s\textsuperscript{-1} is about the same at that reported for the pre-steady state Pi release step (16\text{--}22 s\textsuperscript{-1}, Figure 3-2).\textsuperscript{16,18} Given that two Pi are released per electron transferred, the Pi release rate constant should be double the $k_{\text{cat}}$ value (about 20 s\textsuperscript{-1}) if it is the overall rate limiting step.

Pi release was measured using an established fluorometric method\textsuperscript{42} with either DT or Fld\textsuperscript{HQ} as reductant under high electron flux conditions ([Fe]:[MoFe]: = 16:1). The data showed an initial lag phase followed by a linear phase with both reductants, with the linear phases having rate constants for Pi release of $k_{\text{Pl,DT}} = 27 \text{ s}^{-1}$ and $k_{\text{Pl,Fld}} = 25 \text{ s}^{-1}$.
These rate constants are about double the overall reaction rate constant $k_{\text{cat}}$ of 10-11 s$^{-1}$ per electron. Two Pi are released for each electron transferred, showing that the overall rate limiting step is likely events associated with Pi release. We have no data on the ADP release event, so the position of this event in the cycle remains unknown.

*Establishing the rate-limiting step in the Fe protein cycle*

Considering all of the rate constants for the steps in the Fe protein cycle with Fld$^{\text{H}2}$ as reductant: electron transfer ($k_{\text{ET}} = 173$ s$^{-1}$), ATP hydrolysis ($k_{\text{ATP}} \approx 40-44$ s$^{-1}$), Pi release (25-27 s$^{-1}$) and re-reduction of Fe$^{\text{ox}}$(ADP)$_2$ ($k_{\text{obs}} > 1200$ s$^{-1}$), it is concluded

![Figure 3-6. Real-time measurement of Pi release during nitrogenase catalysis using DT (gray) or Fld$^{\text{H}2}$ with DT (black) as reductant at 25°C. The Pi release was monitored by a fluorescence increase caused by the binding of Pi to MDCC-PBP. The ratio of Pi/MoFe protein is plotted as a function of time. After the initial lag phase (~30 ms), the data collected from 40 to 200 ms were fitted to a linear equation (solid line), giving rate constants of $k_{\text{Pi,DT}} = 27$ s$^{-1}$ and $k_{\text{Pi,Fld}} = 25$ s$^{-1}$.](image)
that the overall rate limiting step for the reaction are events associated with the Pi release step, not complex dissociation. Our findings are consistent with other ATP hydrolyzing systems, including helicases\textsuperscript{47,48} and myofibrillar ATPases,\textsuperscript{49–52} where Pi release is the rate limiting step.

**Summary**

The Fe protein cycle can now be updated with the rate constants determined here using Fld\textsuperscript{HQ} as the reductant, as shown in Figure 3-7. The cycle begins with the rapid equilibrium docking of Fe\textsuperscript{red}(ATP)\textsubscript{2} to MoFe protein, which is followed by the conformationally gated ET events. Next occurs ATP hydrolysis, which is followed by events associated with Pi release. It is unknown if the Pi release event is conformationally gated, but it is clear that events associated with this step are rate limiting for the overall Fe protein cycle. The dissociation of the Fe\textsuperscript{ox}(ADP)\textsubscript{2} is fast, with rapid reduction by the Fld\textsuperscript{HQ}. Finally, ATP replaces ADP in the free Fe protein. The order of these last two events is not established, but the rapid reduction by Fld\textsuperscript{HQ} suggests that reduction should occur before nucleotide exchange.
Figure 3-7. Updated Fe protein cycle with Fld\textsuperscript{HQ} as reductant.

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**ACKNOWLEDGMENTS**

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**AUTHOR CONTRIBUTIONS (BASED ON MODIFIED VERSION)**

ZYY and RL, Purification of Fld; ZYY and LCS, Steady state kinetics; RL, ZYY, SS, and LCS, Pre-steady state kinetics; SS and LCS, ATP studies; NP and EA, Preparation of reagents for Pi release study; VLC and DRD, Fld genetics; All authors involved in data interpretation, writing, and revisions.
REFERENCES


CHAPTER 4

CO₂ AND N₂ FIXATION BY MICROBIAL ELECTROCATALYSIS

ABSTRACT

The biological and industrial conversions of atmospheric N₂ to bioavailable ammonium (NH₄⁺) are essential for the production of fertilizer in conventional agriculture. However, as Earth’s population continues to grow and we look toward sending humans to Mars, new approaches for the sustainable and economical production of fixed N are necessary. Here, we present a biohybrid approach, in which the phototrophic, nitrogen-fixing bacterium Rhodopseudomonas palustris was grown in association with a platinum electrode. In this system, the cells used light for energy and electrocatalytically-generated H₂ as the source of electrons. Initial observations revealed novel features of R. palustris, including its ability to grow photoautotrophically using both incandescent light and infra-red photons (847 nm) with an equivalent generation time of 4 days. Further, cells fixed CO₂ under H₂-mediated electrocatalytic conditions. The next step is to grow the cells electrocatalytically under both CO₂ and N₂ fixing conditions and optimize NH₄⁺ production. Using such an approach would offer an alternative route for the microbial electrosynthesis of NH₄⁺ that could help make food production more sustainable.

*Unpublished data with contributions from Rhesa N. Ledbetter, Mathangi Soundararajan, Zhi-Yong Yang, Paul Kusuma, Bruce Bugbee, Scott A. Ensign, Paul W. Ludden, and Lance C. Seefeldt
INTRODUCTION

Fixed nitrogen compounds are a critical nutrient for the growth of all food crops. These compounds are most often added as fertilizers (i.e. NH₃, NH₄⁺, or NO₃⁻) with the nitrogen being ultimately derived from the reduction of atmospheric dinitrogen (N₂). The reduction of N₂, which is abundant in the Earth’s lower atmosphere, to ammonia (NH₃) is achieved through a process called nitrogen fixation.

There are two major processes that accomplish nitrogen fixation: biological nitrogen fixation in diazotrophic bacteria and industrial nitrogen fixation through the Haber-Bosch process. Biological nitrogen fixation is achieved through the action of the enzyme nitrogenase. This “natural” process has many positive attributes including N₂ fixation under benign conditions, but is of limited usefulness in agriculture. In cases where diazotrophic bacteria form symbiotic relationships with plants like legumes (soybeans, alfalfa, and peanuts), biological nitrogen fixation can provide the nitrogen needs of the crop. Most crops (e.g., corn, wheat, and rice), however, do not associate with diazotrophs.

The invention of the Haber-Bosch industrial process in the early 20th century provided nitrogen fertilizer derived from N₂ and was a tremendous boon to food production, with estimates of over half of all nitrogen used coming from this process. The downside to the Haber-Bosch process is that it requires significant energy inputs derived from fossil fuels. Estimates are that the Haber-Bosch process utilizes around 2\% of global fossil fuels and is responsible for 3\% of the CO₂ production worldwide. There is growing realization that future food production must move to more sustainable methods for achieving N₂ reduction. Further, as we look toward the future of deep space
travel to Mars, development of technologies to manufacture products, such as fixed nitrogen, independent of resources from Earth is also crucial.

Several alternative approaches\textsuperscript{11} for fixed nitrogen production have been explored, including the use of transition metal catalysts,\textsuperscript{12,13} electrocatalysts,\textsuperscript{10,14} photocatalysts,\textsuperscript{15–20} diazotrophic bacteria,\textsuperscript{21–23} and purified nitrogenase.\textsuperscript{16,24} One area of particular interest is capitalizing on nitrogenase functioning inside bacteria. A challenge with using bacteria as an industrial catalyst is that they need a source of energy to sustain nitrogen fixation, which is usually provided in the form of fixed carbon, such as sugars. Providing sugars is not an economical or energy efficient way to support nitrogen fixation in bacteria for larger-scale applications. Thus, a more sustainable way to deliver electrons is the critical missing link in developing bacteria as a way to produce fertilizer at scale for non-leguminous crops.

Here, we provide a demonstration of how to couple low-energy, infra-red (IR) light to CO\textsubscript{2} fixation under ambient conditions using a biohybrid approach, in which the phototrophic bacterium \textit{Rhodopseudomonas palustris} is cultured in association with platinum electrodes. For cell growth, electrocatalytically-produced H\textsubscript{2} served as a source of electrons and the energy for the cells was derived from long-wavelength IR light that does not compete with wavelengths used by plants. Further, the cells grew electrocatalytically under CO\textsubscript{2} fixing conditions eliminating the need for an organic carbon source. The next step is take our collective knowledge and grow cells under both CO\textsubscript{2} and N\textsubscript{2} fixing conditions to validate this biohybrid approach as a potential next generation system for sustainable fixed N production for food production.
MATERIALS AND METHODS

Microbial media and R. palustris growth conditions

*R. palustris* TIE-1 was routinely cultured anaerobically under photoheterotrophic growth conditions in photosynthetic medium (PM) with 20 mM acetate as the carbon source. Photoheterotrophic growths were made anaerobic by filling the tubes completely full with PM medium and then allowing the cells to naturally achieve anaerobicity following consumption of the remaining oxygen. Growth was measured by monitoring the optical density (660 nm) of the cultures.

For photoautotrophic growths, cells were cultured in nitrogen-fixing (NF) medium. The overall composition of NF medium is the same as PM, except the nitrogen source is removed; however, for these studies thiosulfate was also eliminated as the electron donor, as H$_2$ was used instead. The NF base medium, lacking both fixed N and thiosulfate, was then supplemented with Wolfe’s vitamin solution (0.5 ml/100 ml), 1 μM NiSO$_4$, and 200 mM NaCl. The 200 mM NaCl was specifically added to increase conductivity in the electrochemical growths, but was included in all photoautotrophic cultures for consistency. N$_2$ gas was bubbled through to medium to remove any oxygen, and the headspace was equilibrated to 1 atm. An equal volume of H$_2$ gas was included in the headspace to reach a total pressure of 2 atm, and just prior to inoculation, 30 mM NaHCO$_3$ was added to the degassed medium. Cells grown photoheterotrophically served as the inoculum. To remove any residual fixed N, carbon source, and electron donor, these cells were first washed 3x in NF medium before addition to the sealed vials. Cultures were then grown with either a 60W incandescent (broad spectrum) or IR light
(847 nm) (Figure 4-2) at 25°C. Spectral traces of the incandescent light and IR LEDs were made with a spectroradiometer (Apogee Instruments, Model PS-100, Logan, UT).

**Bioelectrochemical system set-up and growths**

The bioelectrochemical system (BES) consisted of two ~600 ml chambers separated with a 20% polyacrylamide gel plug (4 x 6 cm). For electrolysis, NF medium described above served as the electrolyte solution and platinum wires (16 gauge; length= ~3 cm) acted as the working and counter electrodes. The working chamber (cathode) was degassed with N₂ and then 30 mM NaHCO₃ was added to both the cathode and anode. *R. palustris* TIE-1 cells grown photoheterotrophically served as the inoculum. Prior to inoculation, cells were washed 3x in NF medium as described above. The fixed N for cell growth was derived from NH₄⁺ production from release of amide groups in the polyacrylamide plug. The cathodic chamber was loosely covered with a stopper to maintain the atmosphere, while minimizing H₂ gas pressure build-up. The anodic chamber, however, was open to the atmosphere. Using a Gamry potentiostat (Warminster, PA), the BES was maintained under a constant current of 5.0 mA for the duration of the experiment. Each day, CO₂ was bubbled through the cathode to replenish the carbon source as well as maintain the pH of the medium at ~7.0 (in general 1 min. for each 0.1 change in pH). Following, the addition of CO₂, N₂ was also replenished by bubbling through the solution for 4 min and the headspace for 2 min. All growths were conducted at 25°C.
RESULTS AND DISCUSSION

*R. palustris* TIE-1 is a purple, non-sulfur phototrophic bacterium with a diverse metabolism, capable of fixing N\(_2\) and CO\(_2\), as well as accepting electrons from various sources (H\(_2\), Fe(II), thiosulfate, organic acids, and an electrode).\(^{29-32}\) To capitalize on these properties, a biohybrid system was proposed, in which *R. palustris* TIE-1 is grown in association with platinum electrodes (Figure 4-1), with the electrons for microbial growth derived from H\(_2\) produced from electrolysis, and the ATP required for nitrogenase and the Calvin cycle generated from light energy. This approach would replace the need for chemicals in the form of sugar required by many bacteria.

**Figure 4-1.** A microbial electrosynthesis cell for light-driven CO\(_2\) and N\(_2\) reduction by *R. palustris* (red oval).
Prior to growing the cells electrochemically, however, it was confirmed that *R. palustris* could in fact grow in vials, under CO$_2$ and N$_2$ fixing conditions with H$_2$ as the electron donor. In conjunction with this, the response of *R. palustris* growing with broad-spectrum incandescent light and narrow spectrum IR photons (847 nm) was tested. While incandescent light is generally used for growing *R. palustris* in the laboratory, it is well established that purple bacteria harbor photosynthetic pigments known as bacteriochlorophylls (Bchls) in their photosynthetic reaction centers and light-harvesting complexes that have absorption maxima in the IR range (>700-1100 nm). The photosynthetic reaction centers in *R. palustris* absorb light at ~870 nm. The Bchls are evolutionarily significant, as these organisms are able to grow beneath the plants and cyanobacteria where “leftover” IR photons penetrate. Thus, as we look toward biological solutions for NH$_4^+$ production, especially on Mars, having cells that use lower-energy wavelengths distinct from those of plants would be advantageous. Previous studies have indeed demonstrated the versatility of these cells to grow photoheterotrophically, albeit at different efficiencies, with various wavelengths of light. In this study, cells were grown under photoautotrophic, N$_2$ fixing conditions using both incandescent light and IR photons.

The incandescent light (60W) displayed a broad spectrum ranging from ~400 to >950 nm, and the IR LED presented a narrow emission band with a maximum of ~847 nm (Figure 4-2). A generation time of 4 days was observed for cells grown under photoautotrophic, nitrogen fixing conditions with both the incandescent light and the IR LED (Figure 4-3). A previous study with *R. palustris* cells cultivated under
Figure 4-2. Spectra of the (A) incandescent light (60W) and (B) IR LED (DMetric IR Illuminator, 96 LED) used in this study.

Figure 4-3. Growth curves of *R. palustris* grown under phototrophic, nitrogen fixing conditions with incandescent (blue) and IR light (red). CO₂ was the carbon source, N₂ was the nitrogen source, and H₂ was the electron donor. Vials contained a headspace of 50% N₂:50% H₂ at 2 atm of pressure. The cells had an average generation time of 4 days.
photoheterotrophic conditions grew 76% less with IR than incandescent.\textsuperscript{38} The work presented here, however, cannot be directly compared to the previous study due to different growth parameters. Further, correlations between light treatments in this study are not possible either since the number of photons varied between the light treatments. Difference in the availability of photons may, in fact, account for the incandescent growth reaching death phase before the IR growth (Figure 4-3). Even without comparison, however, the data presented here demonstrate that cells can exclusively use lower-energy IR light for growth under photoautotrophic, nitrogen-fixing conditions.

Upon revealing the effectiveness of IR light for \textit{R. palustris} growth, it was subsequently employed as the energy source when examining the growth of the cells under electrochemical conditions. To grow the cells electrochemically, platinum electrodes were connected to a potentiostat, and anodic and cathodic chambers were separated with a polyacrylamide plug. H\textsubscript{2} produced electrocatalytically served as the electron source to the bacterial cells. A current of 5 mA was applied to maintain a constant amount of H\textsubscript{2} production (2.2 mmol/H\textsubscript{2}/day) at the cathode. While further experimentation on minimal and maximal currents for growth should be conducted, it was thought that this amount of H\textsubscript{2} would be sufficient to support microbial growth as \( K_m \) values for H\textsubscript{2} uptake by hydrogenases are in the low μM range and H\textsubscript{2} saturation levels are 0.78 mM, well above the \( K_m \).\textsuperscript{39,40}

Initially, studies were designed to grow cells electrochemically with CO\textsubscript{2} as the carbon source and N\textsubscript{2} as the nitrogen source. However, it was demonstrated the acrylamide plug released NH\textsubscript{4}\textsuperscript{+} over time resulting in fixed N in the extracellular medium. This meant that the study was in fact conducted only under CO\textsubscript{2} fixing
conditions. Cells did, however, grow under these conditions and exhibited an average generation time of 6 days (Figure 4-4). Cells grown without the electrocatalytically produced H\textsubscript{2} did not grow (data not shown), suggesting H\textsubscript{2} is serving as the only source of electrons.

While there is no current evidence of N\textsubscript{2} fixation under electrochemical conditions, further studies on alternative membranes (e.g. Nafion, dialysis membranes, and agarose) that will not release NH\textsubscript{4}\textsuperscript{+} are being evaluated. It should not go unnoticed, however, that cells exhibited growth when cultivated under non-electrochemical photoautotrophic, nitrogen fixing conditions (Figure 4-3) and electrochemically under CO\textsubscript{2} fixing conditions (Figure 4-4). Collectively, these results suggest that \textit{R. palustris} has a high probability of achieving both CO\textsubscript{2} and N\textsubscript{2} fixing conditions in the electrochemical set-up. Further, H\textsubscript{2}-mediated electrocatalytic growth under CO\textsubscript{2} and N\textsubscript{2} fixation has been observed.

\textbf{Figure 4-4.} Growth of \textit{R. palustris} in a microbial electrosynthesis cell with IR LEDS. A constant current of -5 mA was applied and yielded voltages of approximately -3.2 V. The cells had an average generation time of 6 days.
fixing conditions was recently demonstrated in a study that cultivated the autotroph *Xanthobacter autotrophicus.*

As efforts continue in developing a biohybrid approach with *R. palustris*, Figure 4-5 outlines the strategy of establishing this system as an alternative for production of fixed N for plant growth. First, it will be demonstrated that *R. palustris* can grow electrochemically under both CO$_2$ and N$_2$ fixing conditions. Then, the accumulated biomass containing the fixed N will be tested as a fertilizer for plant growth (Figure 4-5).

While it is has been demonstrated that some plants can take up microbial cells directly as a source of nutrients, it is anticipated that the biomass may need to be composted to release the fixed N depending on the plant needs. Further, alternative photoautotrophic, nitrogen-fixing organisms should be investigated, as the generation time of *R. palustris* grown photoautotrophically is relatively slow.

In conclusion, we have demonstrated that a biohybrid system with *R. palustris* utilizing IR light exclusively as an energy source and electrocatalytically produced H$_2$ as an electron donor can grow under CO$_2$ fixing conditions. The next step is to establish that the cells can accomplish both CO$_2$ and N$_2$ fixation. Demonstrating this would be particularly useful when considering deep space missions where *in situ* production of NH$_4^+$ and minimal infrastructure requirements are a critical consideration for fertilizer production. Even on Earth, this could provide a novel route to fix N$_2$ at ambient temperature and pressure with a much lower dependence on fossil fuels, which the current industrial process depends on heavily. While further research is needed on this
system, this study lays some groundwork and insight for further studies focused on capitalizing on biological nitrogen fixation.

**Figure 4-5.** Development of a biohybrid system for fertilizer production, in which microbial biomass is used a source of fixed N for plant growth.
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AUTHOR CONTRIBUTIONS

RNL, MS, LCS, *R. palustris* growth experiments; RNL, MS, ZYY, Electrochemical set-up; RNL, MS, PK and BB, Incandescent light and IR LED measurements; SAE and PWL, IR experiment ideas and insight; RNL, MS, ZYY, LCS, BB, Data interpretation, writing, and revisions.

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CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

INTRODUCTION

Living cells have evolved novel mechanisms to catalyze chemical reactions. Nitrogenase is an ideal enzyme for study, as it generates a highly reduced product (NH$_3$) under mild conditions. Industry has developed processes to “mimic” biological reactions, but they tend to rely on fossil fuels and often require high temperature and pressure.$^1$ Thus, it is essential to continue building a fundamental knowledge of biological systems to provide additional perspective and inspire more sustainable applications for energy conservation and production.

The research here revealed new observations into the mechanism of electron transfer to nitrogenase, including how low reduction potential reducing equivalents are generated as well as fresh insight into the nitrogenase catalytic cycle using a physiological reductant. Further, this research presented a potential biohybrid (bacterial-electrode) system for NH$_4^+$ production.

These studies lay a groundwork in three primary areas described below: 1) Electron bifurcation by FixABCX, 2) Electron transfer to nitrogenase, and 3) Microbial electrosynthesis for NH$_4^+$ production.

ELECTRON BIFURCATION BY FIXABCX

Chapter 2 showcased the first biochemical evidence of electron bifurcation by FixABCX to generate reduced flavodoxin or ferredoxin (Fld/Fd) for nitrogen fixation.$^2$ While there have been considerable advances in uncovering processes related to the
generation of low reduction potential electrons in the context of electron bifurcation,\textsuperscript{3,4} they likely just scratch the surface of a world still largely unexplored. Below, three broad areas of research on FixABCX are proposed, including control of electron transfer through conformational changes, tuning the reduction potential of the bifurcating flavin, and determining the role of FixFd in the FixABCX catalytic cycle.

**Uncover conformational changes in FixABCX**

An important aspect of electron bifurcation is controlling electron transfer to ensure that the two electrons from NADH are partitioned into both an exergonic and endergonic pathway (overall reaction thermodynamically favorable).\textsuperscript{5} One element of control could be conformational changes that occur in the enzyme upon substrate binding or upon release of the first bifurcated electron. A recent report on the bifurcating electron transfer flavoprotein/butryl-CoA dehydrogenase provides evidence of a domain rearrangement during electron bifurcation, but a direct role in electron transfer is still ambiguous.\textsuperscript{6} Other reports on bifurcating NADH-dependent reduced ferredoxin:NADP oxidoreductase (Nfn) complexes have also revealed conformational changes, albeit more subtle.\textsuperscript{7,8}

**Figure 5-1** overlays hypothetical electron transfer paths in the FixABCX structural model presented in Chapter 2. Two possible pathways of electron bifurcation are shown. The first pathway denoted by the orange arrows illustrates electron transfer distances from cofactor to cofactor. In this pathway, distances from the a-FAD to b-FAD and b-FAD to c-FAD are >25 Å. The compatible distance for rapid electron transfer is 14 Å.\textsuperscript{9} Thus, this mode of electron transfer would rely on conformational changes during catalysis. The second pathway represented with black arrows focuses on conserved Tyr
Figure 5-1. FixABCX structural model illustrating possible electron transfer pathways. Orange arrows: Electron transfer distances from cofactor to cofactor. Black arrows: Electron transfer distances when conserved Trp and Tyr are considered in the electron transfer pathway. FixA=Blue; FixB= Tan; FixC= Green; FixX= red.

and Trp residues that appear to shorten electron transfer distances between cofactors and may also be important in the electron transfer process.

To examine the protein conformational changes in FixABCX, H/D exchange-mass spectrometry (HDX-MS) could be employed. This approach monitors the exchange of amide hydrogens with deuterium. The amount of exchange coupled to the rate can reveal substrate/ligand binding sites as well as conformational dynamics under a given set of conditions. For example, FixABCX could be analyzed in both the presence and absence of substrates to reveal changes in protein conformation. Further, the role of the Trp and Tyr residues could be probed using site-directed mutagenesis.
Once a conformational change is confirmed, additional experiments exploring the roles of conformation in electron transfer could be conducted using substrate analogs and/or chemical crosslinking. This in conjunction with transient absorption spectroscopy (TAS) could resolve rates of electron transfer, ultimately, enhancing understanding of the correlation of electron flow through FixABCX and conformational dynamics.

*Elucidate factors that tune the properties of the bifurcating flavin*

Mass spectrometry studies on FixABCX revealed the presence of three FADs, two in FixAB and one in FixCX (*Chapter 2*). However, upon release of flavin from the protein complex, only two FADs could be accounted for in the UV-vis absorption spectrum of the supernatant. The precipitate, however, retained a strong signal at ~425 nm (*Figure 5-2*). It was discovered that this signature has significant similarity to a signal in the literature identified as a covalently-bound 6-S-cysteinyll flavin.\(^\text{10}\)

![Figure 5-2. UV-vis spectrum of FixABCX with non-covalently bound cofactors removed. This spectrum was obtained by subtracting the released flavin spectrum from the spectrum of intact protein.](image-url)
Using molecular modeling, it appears that the FixABCX from *Azotobacter vinelandii* contains a conserved Cys within 5.6 Å of the C6 position of the proposed bifurcating a-FAD (Figure 5-3). Interestingly, this particular Cys residue is conserved in all diazotrophic bifurcating electron-transfer flavoproteins (ETFs).\(^1\) If FixABCX does indeed have a covalently-bound FAD in the bifurcating site, it would be the first known case. The fact that the redox potential of flavins can be modified by the protein environment has been known for a long time,\(^2\) thus, we hypothesize that a flavin derivative may tune the redox properties of the flavin to control electron transfer events at the bifurcating site.

In the absence of a crystal structure, evidence of a covalently-bound FAD could be obtained using Raman spectroscopy. Raman is chemical fingerprinting tool that probes

![Figure 5-3](image.png)

**Figure 5-3.** FixABCX structural model illustrating a conserved Cys residue on FixB (tan) within 5.6 Å of the C6 position of the proposed bifurcating FAD in FixA (blue). This Cys may contribute to the formation of a covalent bond between the cofactor and protein.
molecular vibrations, where the observed vibrational frequencies represent specific functional groups or groups of bonds. The Raman frequencies of C-S bonds are 580-704 cm\(^{-1}\). Using known Raman frequencies as a guide, a fingerprint of FixABCX could be generated and compared to known covalently-bound flavins. Evidence of the C-S bond should be absent from non-covalently bound FAD control samples.

Further, to test our hypothesis that a flavin derivative tunes the cofactor for bifurcation, the Cys could be converted to Ala using site-directed mutagenesis. Assuming the variant binds FAD non-covalently, a suite of biochemical experiments could be conducted to compare the WT and variant proteins. For example, protein activities using diaphorase and bifurcating assays would reveal if the variant is impaired in both NADH oxidation activity, bifurcation activity, or both. Additionally, TAS could be employed to assess the lifetimes of flavin intermediates. Recently, bifurcating flavin sites have been shown to display crossed-potentials, in which the \(E_{\text{m}}^{\text{SST/HST}}\) is lower than the \(E_{\text{m}}^{\text{OXT/SQ}}\) couple. This means the high-energy semiquinone species does not accumulate, therefore, exhibiting a shorter lifetime detectable by TAS. A flavin not tuned for bifurcation would be expected to have uncrossed potentials and a semiquinone with a longer-lifetime. Finally, a comparison of the reduction potentials of the non-covalently versus covalently bound flavin could be determined using spectrophotometry.

Determine the role of FixFd in the FixABCX catalytic cycle

In addition to genes \(\text{fixA}, \text{fixB}, \text{fixC},\) and \(\text{fixX},\) the \(\text{fix}\) operon of \(A.\ vinelandii\) harbors, two additional genes, \(\text{fixFd}\) (sometimes referred to as \(\text{fixP}\)) and \(\text{Avin\_10560}\) (ORF6). These genes, not commonly found in \(\text{fix}\) operons, open doors for investigation into their characterization and cellular roles.
Based on sequence analysis, ORF6 has been designated as “ferritin-like.” Although this gene product has never been examined, it may serve as an iron reserve for the iron-sulfur containing components of Fix. On the other hand, an initial characterization of FixFd was conducted over twenty years ago; however, a direct cellular role of the protein was never discovered. Reyntjens et al. determined FixFd is a 7.2 kDa, 2[4Fe-4S]-containing Fd with the reduction potential of both clusters at -440 mV vs. SHE, a potential with enough driving force to send electrons to nitrogenase.

With new understanding of the role of FixABCX in electron transfer to nitrogenase, a study focused on determining the role of FixFd seems a clear direction. The initial study by Reyntjens purified FixFd from *A. vinelandii* at its native expression levels, a monumental task as they acquired only ~3 mg FixP per 500 g cell paste. In an effort to increase protein yield, we have overexpressed *fixP* (no tag) in *E. coli*, and are currently optimizing a purification protocol and ensuring the integrity of the protein (e.g. insertion of the FeS clusters). Once purified to homogeneity, bifurcation assays with FixABCX could be conducted to determine if FixP can serve as the low-potential acceptor. Furthermore, the bifurcation assay could be coupled to nitrogenase (Figure 5-4). This would demonstrate, *in vitro*, if a direct electron transfer chain can be formed between FixABCX, FixP, and nitrogenase. This study on FixP would also offer an opportunity to compare the electron transfer kinetics using NifF Fld (from initial study) and FixP as low-potential acceptors in the bifurcation reaction.
**Figure 5-4.** Scheme for coupling the FixABCX electron bifurcation assay to nitrogenase activity.

**ELECTRON TRANSFER TO NITROGENASE**

Because artificial electron donors such as dithionite (DT) easily reduce nitrogenase, studies focused on physiological electron donors have been limited. Chapter 3 offered insight into electron transfer events taking place *in vivo* by investigating the nitrogenase catalytic cycle using the physiological reductant Fld and comparing it to DT. The study revealed the true rate-limiting step of nitrogenase catalysis as events associated with Pi release rather than those with dissociation of the Fe protein from MoFe protein as previously determined with DT. This shed light on the importance of investigating native electron donors to nitrogenase. Further, the study on FixABCX (Chapter 2) provided compelling reasons to better understand electron transfer events taking place inside the cell. Included below are two areas of continued investigation into electron transfer to nitrogenase. They include determining the Flds and Fds used for nitrogen fixation in *A. vinelandii* and investigating the nitrogenase catalytic cycle *in vitro* using Fd, rather than Fld, as an electron donor.
Identify the flavodoxins and ferredoxins critical for nitrogenase activity in *A. vinelandii*.

Among diazotrophs, there are diverse routes for supplying electrons to nitrogenase.\textsuperscript{20} *Klebsiella pneumonia* uses a single Fld electron carrier;\textsuperscript{21,22} whereas, other microbes like *A. vinelandii* possess several Flds and Fds (Table 1-1).\textsuperscript{18,20} Among the electron carrying Flds and Fds, NifF Fld is the only one that has been clearly linked to molybdenum nitrogenase activity *in vivo* in *A. vinelandii*.\textsuperscript{23} Its disruption, however, only results in a 30% decrease in nitrogenase activity suggesting that other electron donors either co-function with NifF or substitute when NifF is unavailable. In addition to NifF, there are three other Fds that seems like logical sources of investigation: 1) FdxN plays a significant role in nitrogen fixation; however, a direct role in electron transfer is not as clear. Disruption of the *fdxN* gene results in a 60-65% decrease in acetylene reduction both *in vivo* and *in vitro*.\textsuperscript{24} FdxN appears to serve a primary function in cofactor biosynthesis;\textsuperscript{24,25} however, a role in electron transfer as well cannot be precluded. 2) A role for the Fix-linked FixFd has yet to be established; although, it seems likely to be the primary electron acceptor from the Fix system, since its gene is in the same operon.\textsuperscript{18} 3) While *fdxA*, which encodes for FdI, does not seem to be integral for nitrogen-fixation processes *in vivo*,\textsuperscript{23} further studies into a possible substitution role should be considered.

While a complex array of Flds and Fds exist in *A. vinelandii*, future studies could focus on the construction and characterization of *A. vinelandii* Fld and Fd deletion strains by incorporating antibiotic resistance cassettes into the coding regions. Emphasis could be placed on generating the following strains: ΔnifFΔfdxN, ΔnifFΔfixFd, ΔnifFΔfdxNΔfixFd. Growth studies measuring the optical density (OD\textsubscript{600}) could be
conducted to determine generation times of WT vs deletion strains grown under both non-nitrogen-fixing and nitrogen-fixing conditions. Further, whole cell acetylene reduction assays using gas chromatography could be performed to measure nitrogenase activity. Additional deletions could be made with \( fdxA \) if nitrogenase is still active.

It is expected that the \( \Delta nifF \Delta fdxN \Delta fixFd \) strain would significantly decrease, if not abolish nitrogenase activity. One important aspect to recognize in a study such as this is that Fld or Fd deletions may render a significantly impaired or lethal phenotype as these small redox proteins may play multiple roles in cellular processes; however, for \( A.\) vinelandii, it has already been established that individual disruptions in \( nifF, fdxN, \) and \( fdxA \) are not lethal.\(^{23,24} \) Armed with this information, it is expected that this systematic approach will aid in deducing probable electron donors to nitrogenase in vivo. Further, this research could be expanded upon by also including how these deletions (and others) affect the alternative vanadium (V) and iron-only (Fe-only) nitrogenases.

**Investigate Fd as an electron donor to nitrogenase in vitro**

Upon establishing which Fds can serve as electron donors to nitrogenase in vivo (see section above), additional in vitro characterization of their electron transfer capabilities and interactions with Fe protein could be determined. Chapter 3 demonstrated that the observed rate constant of electron transfer is significantly greater in the presence of the physiological reductant Fld compared to DT.\(^{19} \) Although, similar results might be expected with alternative physiological electron donors, it seems plausible that they could exhibit different rates and affinities for Fe protein, especially if they only serve under conditions in which the primary electron donor is not available. Pre-steady state kinetic experiments using stopped-flow spectroscopy could be done to
determine rate constants for electron transfer. Further, interactions between Fe protein and Fd(s) could be explored using limited proteolysis in conjunction with LC/MS. A previous study, focused on the interaction between Fe protein and Fld, revealed nucleotide-induced structural changes in the Fe protein altered its interaction with Fld. A similar study with Fd would garner deeper insight into the influence of protein-protein interactions on electron transfer processes.

**MICROBIAL ELECTROSYNTHESIS FOR NH$_4^+$ PRODUCTION**

In Chapter 4, a new approach was presented for the production of fixed N, in which phototrophic, nitrogen-fixing bacteria are grown in association with electrodes. The goal of this project is to eventually achieve both CO$_2$ and N$_2$ fixation, with electrons supplied from water oxidation and the energy provided by light. Thus far, it was demonstrated that the phototrophic bacterium, *Rhodopseudomonas palustris*, can be cultivated exclusively with IR light (~850 nm) and achieve CO$_2$ reduction under electrochemical conditions. Further, it was shown that *R. palustris* can grow non-electrochemically using H$_2$ as a source of electrons, CO$_2$ as a carbon source, and N$_2$ as a nitrogen source. This proof-of-concept paves the way for achieving N$_2$ reduction in conjunction with CO$_2$ reduction under electrochemical conditions. If successful, this would offer an alternative route for the microbial electrosynthesis of “fertilizer” for sustainable food production with opportunities to optimize NH$_4^+$ production. Further, experiments directed toward bacterial uptake of electrons directly from the electrode as well as identification of other value-added products, such as hydrocarbons, also offer new avenues of study.
Optimize $\text{NH}_4^+$ production

In addition to using *R. palustris* biomass as a fertilizer, it could be worth investigating the potential for $\text{NH}_4^+$ release into the extracellular medium. Previous reports in the diazotrophs *Rhodospirillum rubrum* and *Xanthomonas autotrophicus* used small molecules to inhibit glutamine synthetase in the ammonium assimilation pathway.\textsuperscript{27,28} Under these conditions $\text{NH}_4^+$ was released from the cells. Similar inhibition studies could be applied to glutamine synthetase in *R. palustris*.\textsuperscript{29}

Another approach to consider is disrupting $\text{NH}_4^+$ transporters in the cells. Previous studies on AmtB in *A. vinelandii* and *Pseudomonas stutzeri* suggested that it likely minimizes loss of $\text{NH}_4^+$ in the environment.\textsuperscript{30,31} Further, when disrupted, the cells displayed no growth defects; thus, the homologous transporter genes in *R. palustris* likely provide a viable target.

We hypothesize that disrupting $\text{NH}_4^+$ regulatory and uptake pathways would result in the release of fixed nitrogen by *R. palustris*. A comparison between electrochemical and non-electrochemical growths as well as combining the glutamate synthetase inhibitor with the transporter disruption(s) could be done and the amount of $\text{NH}_4^+$ in the medium quantified over time. Overall, this study would target specific proteins and begin to identify optimal conditions under which to generate extracellular $\text{NH}_4^+$.

Grow cells with electrons direct from the electrode

Our initial work growing *R. palustris* electrochemically utilized mediated electron transfer (MET), in which electrons from water oxidation were used to reduce $\text{H}^+$ to $\text{H}_2$. The cells then used the $\text{H}_2$ as a source of electrons. The next step could be to use a direct
electron transfer (DET) approach, in which the electrons are supplied to the cells direct from the electrode (Figure 5-5). In comparison to MET, DET offers several advantages. For example, the electrolysis in MET results in significant pH changes in the cathode and anode, which must be adjusted regularly. Further, the MET system must be open so H₂ does not build up; thus, the electron donor is being “wasted.” DET would help eliminate these disadvantages.

A recent study by Bose et al. did in fact demonstrate the ability of *R. palustris* to fix CO₂ using electrons from an electrode. Given that success, it is expected that having cells fix both carbon and nitrogen would be feasible. Cells could be cultured using graphite electrodes with CO₂ as the carbon source, N₂ as the nitrogen source, the

![Figure 5-5. Mediated vs. direct electron transfer from an electrode to a bacterium.](image-url)
electrode as electron source, and light as energy source. If this works, it would be the first demonstration of light-driven carbon and nitrogen fixation by whole cells using electrons from an electrode. The previous work by Bose et al. poised electrodes at +100 mV vs. SHE, because it is the potential of ferrous iron (Fe(II)), which R. palustris can use as an extracellular electron donor. An extension of this could be testing a wide range of potentials and examining the effect on cellular growth. This work on extracellular electron uptake could then lead to additional studies on genetic determinants facilitating this ability as well as additional insight into bio-based applications for valuable products.

**Identify other value-added products**

It has been demonstrated that variants of nitrogenase as well as the wild-type Fe-only nitrogenase can reduce CO₂ to hydrocarbons (e.g. CH₄, formate, C2 and C3 products). This observation encourages studies into other hydrocarbon products R. palustris might produce when grown electrochemically. A profile of product formation with cells expressing Mo, V, and Fe-only nitrogenases could be generated. It would be expected that the Mo nitrogenase-expressing cells would produce NH₄⁺ and H₂, while the Fe nitrogenase-expressing cells, would produce hydrocarbons, such as CH₄ in addition to NH₄⁺ and H₂. Variant forms of nitrogenase capable of CO₂ chemistry could be explored as well. Finally, investigation into whether product distribution could be manipulated by varying the potential of electrons would be extremely valuable as we search for ways to optimize products of interest.

**CONCLUSION**

This research focused on one of the most important and elegant biological processes on the planet—nitrogen fixation. The beauty of science is that as we discover
more answers, it brings up more questions. The cycle never ends. Reflecting on the work here, my hope is that the contributions are a catalyst as scientists continue their quest for understanding and capitalizing on the intricate biological processes that shape us.

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APPENDIX B

FULL MANUSCRIPT

EVIDENCE THAT THE Pi RELEASE EVENT IS THE RATE LIMITING STEP IN THE NITROGENASE CATALYTIC CYCLE
EVIDENCE THAT THE Pi RELEASE EVENT IS THE RATE LIMITING STEP IN THE NITROGENASE CATALYTIC CYCLE

ABSTRACT

Nitrogenase reduction of dinitrogen (N$_2$) to ammonia (NH$_3$) involves a sequence of events that occur upon the transient association of the reduced Fe protein containing two ATP with the MoFe protein that includes electron transfer, ATP hydrolysis, Pi release, and dissociation of the oxidized, ADP containing Fe protein from the reduced MoFe protein. Numerous kinetic studies using the non-physiological electron donor dithionite have suggested that the rate limiting step in this reaction cycle is the dissociation of the Fe protein from the MoFe protein. Here, we have established the rate constants for each of the key steps in the catalytic cycle using the physiological reductant flavodoxin protein in its hydroquinone state. The findings indicate that with this reductant, the rate limiting step in the reaction cycle is not protein-protein dissociation or reduction of the oxidized Fe protein, but rather events associated with the Pi release step. Further, it is demonstrated that: (i) Fe protein transfers only one electron to MoFe protein in each Fe protein cycle coupled with hydrolysis of two ATP molecules; (ii) the oxidized Fe protein is not reduced when bound to MoFe protein; (iii) the Fe protein interacts with

flavodoxin using the same binding interface that is used with the MoFe protein. These findings allow a revision of the rate limiting step in the nitrogenase Fe protein cycle.

INTRODUCTION

Nitrogenase is the catalyst responsible for biological nitrogen fixation, the reduction of dinitrogen ($N_2$) to ammonia ($NH_3$). The molybdenum-dependent nitrogenase consists of two catalytic components called the MoFe protein and the Fe protein. The Fe protein is a dimer of two identical subunits connected by a single [4Fe-4S] cluster and is responsible for transferring a single electron to the MoFe protein. The MoFe protein is a heterotetramer, composed of two symmetric $\alpha\beta$ units. Each $\alpha\beta$ unit contains two unique metal clusters, the electron carrier P-cluster [8Fe-7S] and the active site FeMo-cofactor [7Fe-9S-Mo-C-homocitrate]. An Fe protein, with two bound MgATP molecules, binds transiently to each $\alpha\beta$ unit of the MoFe protein during the electron transfer (ET) event (Figure B1). ET is initiated upon association of the Fe protein to the MoFe protein, followed by a conformationally gated one-electron transfer from the P-cluster to FeMo-cofactor. This is followed by the one-electron transfer from the reduced Fe protein ($Fe^{\text{red}}$) [4Fe-4S] cluster to the oxidized P-cluster ($P^{1+}$), in what has been called a “deficit spending” ET process. Following ET, the two ATP molecules are hydrolyzed to two ADP and two Pi molecules. Next, the two Pi are released from the complex, followed by the dissociation of the oxidized Fe protein ($Fe^{\text{ox}}$) with two bound MgADP ($Fe^{\text{ox}}$(ADP)$_2$) from the MoFe protein. The $Fe^{\text{ox}}$(ADP)$_2$ is then reduced by a reductant and the two ADP molecules are replaced by two ATP molecules. This cycle, often referred to as the Fe protein cycle, must be repeated a sufficient number of times to accumulate the electrons necessary for substrate reduction in the MoFe protein.
**Figure B1.** Overview of nitrogenase catalysis with a focus on the Fe protein cycle showing dithionite (DT) and flavodoxin (Fld^{Hq}) as a non-physiological and physiological reductant, respectively.

Over the past five decades, most of the *in vitro* mechanistic studies of nitrogenase have been conducted with the non-physiological reductant dithionite (DT), largely because of ease of use.\(^1,^3,^18\) The kinetic parameters for each step in the Fe protein cycle using DT as reductant have been deduced and are summarized in the scheme shown in **Figure B2.**\(^16^-^18,^21,^22\) It was concluded from a comparison of the rate constants that the overall rate limiting step in the Fe protein cycle is the dissociation of the Fe\(^{ox}\)(ADP)\(_2\) from the MoFe protein with a rate constant of \(\sim 6\) s\(^{-1}\).\(^16,^17\) A problem that is often overlooked in such studies is the reversible dissociation of DT (S\(_2\)O\(_4^{2-}\)) to generate the actual reductant, the radical anion SO\(_2^-\), with a \(K_d\) of \(\sim 1.5\) nM and a slow rate constant of \(\sim 2\) s\(^{-1}\).\(^17\) This leads to slow reduction of Fe\(^{ox}\), with rate constants for this reduction near the rate constant that is reported for dissociation of Fe\(^{ox}\)(ADP)\(_2\) from MoFe protein.\(^16,^17,^22,^23\)
Figure B2. Fe protein cycle with pseudo-first order kinetic rate constants for each step.

The physiological reductants of Fe protein are known to be the electron carrier proteins flavodoxin (Fld) and/or ferredoxin (Fd).\textsuperscript{24–30} Because nitrogenase is readily reduced by artificial electrons donors, such as DT, a limited number of kinetic studies using physiological reductants have been conducted.\textsuperscript{24,25,27,30–35} The diazotroph, \textit{Azotobacter vinelandii}, contains several Flds and Fds. NifF (Fld) has been implicated as an electron donor to nitrogenase\textsuperscript{26,29,30,36} and has been shown to transfer electrons to Fe protein \textit{in vitro}.\textsuperscript{24,25,27,33,34} Further, disruption in the \textit{nifF} gene results in a 30\% decrease in whole-cell acetylene reduction activity. This previous research demonstrates that Fld is a major, but not the sole, electron donor to nitrogenase.\textsuperscript{26,29} Fld has three different redox states designated as the oxidized quinone (Fld\textsuperscript{Q}), one-electron reduced semiquinone (Fld\textsuperscript{SQ}), and two-electron reduced hydroquinone (Fld\textsuperscript{HQ}).\textsuperscript{24,37,38} The pH- and temperature-dependent midpoint redox potentials ($E_m$) for the two redox couples are estimated as -180
mV (vs the NHE) for the Fld\textsuperscript{Q}/Fld\textsuperscript{SQ} couple, and -480 mV for the Fld\textsuperscript{SQ}/Fld\textsuperscript{HQ} couple (pH 8.5 and 22°C).\textsuperscript{38} The Fld\textsuperscript{SQ}/Fld\textsuperscript{HQ} redox couple has sufficient driving force for reduction of the oxidized Fe protein with a midpoint potential of -290 mV without nucleotide bound and -440 mV with ADP bound.\textsuperscript{25,27,33,34,39} Earlier kinetic studies using Fld as reductant have yielded results that contradict some aspects of the Fe protein cycle deduced with DT: (i) the one-electron reduction of Fe protein by Fld\textsuperscript{HQ} is much faster than that by DT, and this reduction event might happen when Fe\textsuperscript{ox}(ADP)_2 is still bound to the MoFe protein;\textsuperscript{25} (ii) Fe protein can be reduced by two electrons by Fld\textsuperscript{HQ} to an all ferrous state, possibly allowing two electrons to be transferred per two ATP hydrolyzed (a ratio of 1ATP:1e\textsuperscript{-});\textsuperscript{33,34} and (iii) in the Fe protein cycle, the dissociation step might be faster than what has been widely accepted (~6 s\textsuperscript{-1}).\textsuperscript{25,31–33,40}

In the present work, kinetic studies were performed using both DT and Fld (NifF from \textit{A. vinelandii}) as reductant to deduce the key kinetic parameters in the Fe protein cycle. This work reveals that Fld\textsuperscript{HQ} cannot reduce the Fe protein while it is bound to MoFe protein, the ratio of ATP hydrolyzed per electron transferred remains at 2:1 for a wide range of substrates, and the rate limiting step in the Fe protein cycle is not the dissociation of the Fe protein from the MoFe protein, but rather events associated with Pi release.

**MATERIALS AND METHODS**

**General procedures**

All chemicals, unless otherwise noted, were obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification. Hydrogen, acetylene, ethylene, argon, and dinitrogen gases were purchased from Air Liquide America Specialty Gases LLC
(Plumsteadville, PA). The argon and dinitrogen gases were passed through an activated copper-catalyst to remove dioxygen contamination prior to use. *A. vinelandii* strains DJ995 (wild type MoFe protein) and DJ884 (wild type Fe protein) were grown, and nitrogenase proteins expressed and purified as previously described. Both proteins were greater than 95% pure as confirmed by SDS-PAGE analysis using Coomassie blue staining and fully active (see results and discussion). Proteins and buffers were handled anaerobically in septum-sealed serum vials under an inert atmosphere (argon or dinitrogen), on a Schlenk vacuum line, or anaerobic glove box (Teledyne Analytical Instruments, MO-10-M, Hudson, NH). The transfer of gases and liquids were done with gastight syringes.

**Strain construction and expression, *Escherichia coli* growth, and NifF purification**

The *nifF* gene from *A. vinelandii* was PCR amplified and cloned into the NdeI-BamHI sites of a T7-7 plasmid containing an ampicillin resistance gene for selection. Fld was overexpressed in *Escherichia coli* (*E. coli*) BL21 DE3 cells. The cells were grown to an optical density (600 nm) of 0.6-0.8 at 37 °C before the protein expression was induced at 32 °C by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) and flavin mononucleotide (FMN) to a final concentration of 1 mM and 10 mg/L, respectively. Following a 10 hour induction, cells were harvested.

All steps during the purification of Fld were conducted anaerobically under an argon atmosphere. 100 grams of cell paste were resuspended in 50 mM Tris, pH 8 with 2 mM dithiotheritol (DTT) at a biomass to buffer ratio of 1:5 (w/v). The resuspended cells were lysed in a french pressure cell (SLM Aminco FA-078, Rochester, NY) at 200 MPa. The cell lysate was centrifuged (Sorvall Lynx 4000, ThermoScientific, Waltham, MA) at
48,000 x g for 30 minutes at 4°C. Following centrifugation, 0.5% (w/v) streptomycin sulfate was added to the supernatant to precipitate nucleic acids. The precipitation was removed via a second centrifugation as described above. The supernatant was loaded onto a 150 ml Q-sepharose column, which was first washed with two column volumes of Buffer B (50 mM Tris, 1 M NaCl, 1 mM DTT, pH 8) and then equilibrated with two column volumes of Buffer A (50 mM Tris, 1 mM DTT, pH 8). A 15-75% salt gradient was run over 5 column volumes. Fractions containing Fld (blue color) were combined and diluted with Buffer A to a final NaCl concentration of less than 100 mM. FMN was added to a final concentration of 2 mM to increase the percentage of holo-Fld in the presence of 2 mM DT, which slowly changed the color of the protein solution from blue to yellow. The reconstitution was carried out for at least 2 hours at room temperature. The protein was then loaded onto a Q-sepharose column (~30 mL) pre-reduced and equilibrated with Buffer B and Buffer A containing 1 mM DT. After loading, the column was washed with 1 column volume of Buffer A and eluted with 100% Buffer B as a concentrated fraction for loading onto Sephacryl-200 column (~600 ml) equilibrated with an 100 mM HEPES (pH 7.8) with 150 mM NaCl, 0.5 mM DTT, and 1 mM DT. The Fld was followed as a yellow-green band. The protein was concentrated using an Amicon (EMD Millipore, Billerica, MA) concentrator with a 10,000 kDa cutoff membrane and stored in liquid nitrogen. The purity of the Fld was greater than 95% based on SDS-PAGE method described above. The FMN content was determined to be ~70% of the total protein. This was determined by measuring the absorbance of FldQ at 452 nm and using an extinction coefficient of 11.3 mM⁻¹ cm⁻¹. The Fld concentration used in this work refers to the concentration of the holo-Fld with FMN bound.
Steady-state substrate reduction assays for determination of $k_{cat}$ for Fe protein and MoFe protein

Substrate reduction assays were carried out in a 9.4-mL sealed serum vials with a liquid volume of 1 mL in an assay buffer consisting of an MgATP regeneration system (5 mM MgCl$_2$, 22 mM phosphocreatine, 4 mM ATP, 0.2 mg/mL creatine phosphokinase, 1 mg/mL BSA) in 100 mM MOPS buffer (pH 7.3) with 10 mM DT or 600 μM Fld$^{HQ}$-10 to 12 mM DT mixture. After solutions were made anaerobic, the headspace in the reaction vials was adjusted to proper partial pressures for the various gaseous substrates, such as acetylene and N$_2$. The MoFe protein was then added to the designated final concentration. Each reaction vial was pre-incubated in a 30°C water bath for 1 minute before initiation of the reaction by the addition of Fe protein. Reactions were incubated for 30 s at 30°C with a shaking rate of 130 rpm before being quenched by the addition of 500 μL of 400 mM EDTA at pH 8.0 or 500 μL of 1 M formic acid. The products (H$_2$, C$_2$H$_4$, and NH$_3$) from different substrate reduction assays were quantified according to published methods.$^{42}$ Refer to substrate reduction tables and figures for detailed information on assay conditions and concentrations.

Determination of ATP/e$^{-}$ values for different substrate reduction reactions

The total number of electrons transferred for product formation (H$_2$, C$_2$H$_4$, and NH$_3$) from different substrate reduction reactions was determined. The reaction mixture contained 8.5 mM ATP, 9.7 mM MgCl$_2$, and 1 mg BSA in 100 mM MOPS buffer (pH 7.3) with either 10 mM DT or 712 μM Fld$^{HQ}$-10 mM DT. General substrate reduction assays procedures are described above. To quantify the amount of hydrolyzed ATP as formation of ADP, a trace amount of [α-$^{32}$P]ATP with final concentration of about 0.006
μM was added to the reaction mixture. The reactions were then initiated by addition of a mixture of MoFe and Fe protein at a designated molar ratio. An aliquot of 25 μL was taken out of the reaction and quenched with 50 μL of 1 M formic acid. 1 μL of the quenched mixture was spotted onto a silicon-gel thin-layer chromatography (TLC) plate and developed in 0.6 M potassium phosphate buffer, pH 3.4 for 70 min. The plate was dried and exposed overnight to a phosphor screen. The [α-32P]ATP and the [α-32P]ADP were detected with a Storm PhosphorImager and quantified using the ImageQuant software (Molecular Dynamics). The amount of hydrolyzed ATP or produced ADP were quantified based on the density ratio of [α-32P]ATP and [α-32P]ADP spots from each experiment after subtracting no protein controls.

**Stopped-flow (SF) spectrophotometry and reduction of Fe protein by DT and FldHQ**

SF spectrophotometry was conducted using an AutoSF-120 stopped-flow instrument equipped with a data acquisition system (KinTek Corp., Snow Shoe, PA). The change in absorbance was monitored at 426 nm over time. This wavelength detects a decrease in absorbance as the Fe protein [4Fe-4S] cluster becomes reduced. 426 nm rather than 430 nm was chosen since it is the isosbestic point of Fld (Figure B6). Reactions were conducted at 4°C, unless otherwise stated, with a mixing ratio of 1:1. All samples were prepared in 100 mM MOPS buffer (pH 7.3). Fe protein was oxidized in an anaerobic glove box using ~3-fold molar excess of phenazine methosulfate (PMS). The protein-PMS mixture was allowed to incubate for 15 minutes prior to separation with Sephadex G-25 equilibrated with 100 mM MOPS, pH 7.3 with 150 mM NaCl. The oxidized Fe protein was contained in one drive syringe with or without nucleotide. The other drive syringe contained the electron donor, DT or FldHQ-DT mixture, with or
without nucleotide. All reagents were used at the following final concentrations and kept under an argon atmosphere: 40 μM oxidized Fe protein, 10 mM DT, 300 μM Fld, 10 mM ATP or ADP, and 10 mM MgCl₂ (only used in the presence of nucleotides). For the reduction of Fe²⁺ by DT, the data were fit to a single exponential equation to obtain the pseudo-first order rate constants. Due to the presence of both FldHQ and DT in the experiments, the pseudo-first order rate constants for reduction of Fe²⁺ by FldHQ were obtained by fitting the data to a double exponential equation.

**Reduction of Fe²⁺(ADP)₂ by different reductants in the presence of MoFe protein**

The Fe-MoFe dissociation rate constant was determined using SF as described above except that the temperature was 25°C. Fe protein was oxidized (see details above) and MoFe protein was stripped of DT in an anaerobic glove box. DT was removed from MoFe protein using a DOWEX-Sephadex G-25 column equilibrated with 100 mM MOPS, pH 7.3 containing 150 mM NaCl. One drive syringe contained 80 μM Fe²⁺, 80 μM MoFe protein, and 9 mM MgADP. The other syringe contained the reductant mixture: 1) 20 mM DT, 2) 100 μM methyl viologen (MV)-20 mM DT mixture, or 3) 400 μM FldHQ-20 mM DT mixture. All reductants contained 9 mM MgADP. DT data were fit to a single exponential decay equation, and MV-DT and FldHQ-DT data were fit to a double exponential decay equation.

**Primary electron transfer**

Primary electron transfer from the Fe protein to the MoFe protein in the presence of DT and Fld was measured at 25°C using SF spectrophotometry as described above. All mixtures were prepared in 100 mM MOPS, pH 7.3 and were kept under an argon atmosphere. One syringe contained 80 μM Fe protein, 20 μM MoFe protein, 1 mM DT
with or without 200 µM FldeHQ. The other syringe was loaded with 1 mM DT and 20 mM MgATP. As turnover occurred in the [Fe\textsuperscript{red}(MgATP)\textsubscript{2}-MoFe] complex, the oxidation of the [4Fe-4S] cluster of the Fe protein was monitored by an increase in absorbance at 426 nm. Data were fit to a single exponential curve.

**Quench-flow studies for ATP hydrolysis**

Pre-steady state ATP hydrolysis experiments were performed using a rapid chemical quench-flow (KinTek, Snow Shoe, PA) in a Coy chamber (Grass Lake, MI) under a dinitrogen atmosphere. Mixtures were prepared in 50 mM MOPS, pH 7.4. Syringe A contained 80 µM Fe and 20 µM MoFe proteins with either 10 mM DT or 500 µM FldeHQ-10 mM DT mixture. Syringe B contained 6 mM ATP, 8 mM MgCl\textsubscript{2} with [α-\textsuperscript{32}P]ATP. 18 µl from syringe A was mixed with 18 µl from syringe B and then rapidly quenched with 45 µL of 0.75 M formic acid contained in syringe C. Aliquots (1 µl) of the quenched mixture were plated on TLC plates and the ratio of [α-\textsuperscript{32}P]ATP vs [α-\textsuperscript{32}P]ADP formed were analyzed as described above for the steady state ATP hydrolysis experiments.

**Real-time measurement of inorganic phosphate (Pi) release**

Pi release was determined in a stopped-flow (SF) fluorometer (Auto SF-120, KinTek Corp.) using a coumarin (N-[2-(1-maleimidyl)ethyl]-7-(diethylamino) coumarin-3-carboxamide) labeled phosphate binding protein (MDCC-PBP) assay.\textsuperscript{43} Pi was quantified from binding MDCC-PBP that was monitored by an increase in fluorescence (λ-excitation = 430 nm, λ-emission >450 nm), using a standard curve generated with KH\textsubscript{2}PO\textsubscript{4} as described.\textsuperscript{43} The experiments were carried out at 25°C in 25 mM HEPES, pH 7.4 containing 1 mM DT. The SF syringes and flow lines were treated with a Pi mop (25
mM HEPES buffer at pH 7.4 with 320 µM 7-methylguanine (7-meG), and 0.12 U/mL purine nucleoside phosphorylase (PNPase)) before each experiment for 45 minutes to remove contaminating Pi and then rinsed with DT reduced buffer. The same concentration of the Pi mop system was also added to the reaction mixtures. 1 µM MoFe and 16 µM Fe were rapidly mixed with a solution of 25 µM MDCC-PBP, 7.5 mM MgCl₂ and 6 mM ATP and the change in fluorescence was monitored over time. 100 µM FldHQ was added to the MoFe and Fe protein mixture to monitor the effect of FldHQ on Pi release.

**In silico docking study of protein-protein interactions**

*In silico* protein-protein docking simulations were performed using the computational docking program ClusPro 2.0. ClusPro 2.0 uses PIPER, a Fast Fourier Transform-based protein docking program with pairwise potentials to derive the structure model. Flavodoxin II (PDB ID 1YOB) was used as the ligand and Fe protein (PDB ID 1FP6) as the receptor. The final docking model was chosen based on agreement of the electrostatic potentials of the bound complex, which were generated with PyMOL.

**Time resolved proteolysis of flavodoxin and Fe proteins**

Limited proteolysis experiments were performed on Fe protein, Fld and the Fe protein-Fld complex. Protein ratios of 1:1, 1:2, and 2:1 (Fe protein:Fld) were digested with Trypsin Gold (Promega, Madison, WI). The reactions were performed in sealed vials in a 50 mM ammonium bicarbonate, pH 8 buffer with 1 mM sodium dithionite in a total volume of 120 µL and a protease-to-protein ratio of 1:1000 (w/w) at room temperature. 15 µL samples were taken at 0, 5, 10, 20, 40, 60 and 240 minutes. Reactions were quenched with 1µl of 10% formic acid. 10 µL of each sample was used for analysis.
of the trypsin digestion pattern by SDS-PAGE using a 4-20% linear gradient gel (Mini-Protean TGX, Bio-Rad, Hercules, CA).

**Mapping of proteolytic cleavage sites**

The molecular weight of released peptides was determined using an Autoflex III MALDI-TOF/TOF mass spectrometry (Bruker Daltonics, Billerica, MA). 1 μL of each sample was co-crystallized with a saturated solution of α-cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile (Thermo-Fisher Scientific, Waltham, MA) containing 0.1% formic acid. Peptide mass spectra were acquired in positive, reflectron mode with an acceleration voltage of 20 kV. Spectra were an accumulation of 1000 laser shots. Spectra were averaged from three spots for each time point. Tryptic peptides were mapped to the protein sequence using the Protein Analysis Worksheet (PAWS) software package (ProteoMetrics, LLC). Results were mapped onto the ClusPro 2.0 Docking model.

**Chemical cross-linking**

Chemical cross-linking experiments were performed with Fe protein (20 μM) and Fld (Fe protein:Fld ratios were 2:1, 1:1, 1:2) using 10mM glutaraldehyde in 50mM HEPES pH 7.5, 150 mM NaCl, and 1mM sodium dithionite. Reaction conditions were optimized to limit formation of higher order aggregates. Samples were incubated with glutaraldehyde for 10 minutes, at room temperature. The reaction was quenched with 100 mM Tris buffer, pH 8.0. To preserve the native state and conformation of proteins and proteins in complex with their cofactors, all steps were performed under strict anaerobic conditions. Complex formation and crosslinking were initiated at the same time.

Cross-linked samples and controls were analyzed by SDS-PAGE (4-20% linear gradient Mini-Protean TGX, Bio-Rad, Hercules, CA). Protein bands corresponding to
monomers and dimers were digested with trypsin as previously described prior to mass spectrometry analysis. LC/MS/MS measurements were performed using a maXis Impact UHR-QTOF (Bruker Daltonics, Billerica, CA) interfaced with an Agilent 1100 HPLC nanoflow system (Agilent Technologies, Santa Clara, CA). Peptide mixtures were separated on a Dionex column (20 mm x 100 μm, 5 μm, 100Å, Acclaim PepMap100, C18, Dionex), kept at 40°C, and eluted with flow of 800nl/min in solvents A (0.1% formic acid) and B (acetonitrile/0.1% formic acid) gradient: 3–30% B in 16 min, followed by 3 min 30-95% B and 5 min 95% B column wash step. The column was equilibrated with 3% B for 2 minutes prior to the next injection. Electrospray conditions in both MS and auto MS/MS modes were: drying gas flow 4.0 L/min at 100°C, capillary 1600 V. Data was collected over the m/z range of 300–1700 at 2 spectra/s acquisition rate for MS and MS/MS, respectively. A linear voltage gradient depending on mass to charge ratio was applied for peptides fragmented in auto MS/MS experiments with a decreasing order of preference +2> +3> +4 > +1 charged parent ions. Raw data were converted to mgf format using MS Convert and uploaded to SearchGUI, (version 1.26.6.) for sequence identification and visualized and validated in PeptideShaker (version 0.40.1). Validated peptides with the identification confidence higher than 95% were selected for further analysis.

RESULTS AND DISCUSSION

Establishing $k_{cat}$ with DT or FldHQ

In the two-component catalytic system of nitrogenase, both the Fe protein and the MoFe protein are catalysts. The rate limiting step for the overall reaction is held to be the dissociation step in the Fe protein cycle (Figure B2). This means that the rate constant of
the rate limiting step should be the same as the \( k_{\text{cat}} \) for both Fe protein and MoFe protein cycles in terms of electrons donated/accepted per active site per unit time. Earlier studies established a turnover number (\( k_{\text{cat}} \)) for electron flow to substrate reduction of between 5-10 s\(^{-1}\) regardless of substrate.\(^{31,46}\) This value is approximately the same as the first order rate constant for dissociation of the Fe\(^{\text{ox}}\)(MgADP)\(_2\) from the MoFe protein of \(~6\) s\(^{-1}\), leading to the conclusion that the overall reaction rate limiting step is dissociation (Figure B2).\(^{1,16–18,31}\) However, a few previous studies have shown that the rate of dissociation of the Fe-MoFe protein complex using DT is slower than the \( k_{\text{cat}} \) under saturating (‘high flux’) conditions.\(^{40,46}\)

Here, we determined the \( V_{\text{max}} \) for both Fe protein and MoFe protein cycles using either DT or Fld as reductant by varying the ratio of Fe protein to MoFe protein (called the “electron flux”). The \( V_{\text{max}} \) values for the Fe protein cycle were determined under low flux conditions with a molar ratio of 1:1([Fe]:[MoFe]) for acetylene reduction and 1:2 for proton reduction using either DT or Fld\(^{\text{HQ}}\)-DT as a reductant. These ‘low-flux’ conditions saturated the Fe protein with MoFe protein and resulted in a Fe protein cycle \( k_{\text{cat,Fe}} \) of \(~6\) s\(^{-1}\) with DT as reductant. When Fld\(^{\text{HQ}}\)-DT was used as the reductant, a \( k_{\text{cat,Fe}} \) of \(10-11\) s\(^{-1}\) was observed (Table B1 and C1, Figures C1-C4). These results reveal that using Fld as reductant speeds up the overall reaction, suggesting that the rate limiting dissociation step has been accelerated by about 2-fold when Fld is the reductant compared to the dissociation rate constant when DT is the reductant.\(^{40}\) This enhancement effect on the \( V_{\text{max}} \) and \( k_{\text{cat}} \) of both Fe and MoFe protein is dependent on the electron flux, with lower flux showing the largest effect (Figures C2-C5). When the electron flux is increased to
Table B1. Steady-state \( k_{\text{cat}} \) values for Fe protein and MoFe protein with different reductants.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reductant</th>
<th>( k_{\text{cat}} ) (s(^{-1})) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe Protein</td>
<td>DT</td>
<td>5-6</td>
</tr>
<tr>
<td></td>
<td>Fld(^{HQ})</td>
<td>10-11</td>
</tr>
<tr>
<td>MoFe protein</td>
<td>DT</td>
<td>10-11</td>
</tr>
<tr>
<td></td>
<td>Fld(^{HQ})</td>
<td>10-11</td>
</tr>
</tbody>
</table>

\(^a\)The steady-state \( k_{\text{cat}} \) values for Fe protein and MoFe protein were estimated based on the \( V_{\text{max}} \) of Fe protein (MW \( \approx \) 64,000 Da) under low electron flux conditions and \( V_{\text{max}} \) of MoFe protein (MW \( \approx \) 240,000 Da) under high flux conditions. For detailed reaction conditions refer to the figure legends for Figures C1-C4 and Table C1.

To saturate the MoFe protein with Fe protein (‘high flux’, [Fe]:[MoFe] = 16-20:1) to measure the MoFe protein cycle, the \( k_{\text{cat}} \) is found to be \( \sim 10-11 \, \text{s}^{-1} \) with both DT and Fld as reductant. The increase in \( k_{\text{cat,Fe}} \) observed for Fld\(^{HQ}\) compared to DT as reductant could be explained by an increase in the rate of dissociation of the Fe protein from MoFe protein when Fld\(^{HQ}\) is the reductant under low flux condition. To test this, we determined an apparent dissociation rate constant for \( \text{Fe}^{\text{ox}}(\text{ADP})_2 - \text{MoFe protein} \) with either DT or Fld as reductant.

**Dissociation of the \( \text{Fe}^{\text{ox}}(\text{ADP})_2 \) from the MoFe protein**

To determine the apparent dissociation constant using SF spectrophotometry, the \( \text{Fe}^{\text{ox}}(\text{ADP})_2 - \text{MoFe protein} \) complex was pre-formed in one syringe of the SF spectrophotometer and rapidly mixed against a reductant mixture. Here, such experiments were conducted with either DT or Fld\(^{HQ}\) in the second syringe. The reduction of the \( \text{Fe}^{\text{ox}} \) was monitored at 426 nm, an isosbestic point for reversible conversion between Fld\(^{SQ}\) and Fld\(^{HQ}\) (Figure C6). As shown in Figure B3, the estimated first order rate constant for
Figure B3. Reduction of Fe\textsuperscript{ox}(ADP)\textsubscript{2} protein by DT (red), MV (green), or Fld\textsuperscript{HQ} (blue) in the presence of MoFe protein. The reduction of Fe\textsuperscript{ox}(ADP)\textsubscript{2} by different reductants was monitored as the decrease of the absorbance at 426 nm as a function of time. The data are displayed as gray dots and were fit to different equations as described in the Materials and Methods section to obtain the pseudo-first order $k_{obs}$. Syringe 1 contained 20 mM DT with 100 μM MV, or 400 μM Fld\textsuperscript{HQ} with 20 mM DT. Syringe 2 contained 80 μM Fe\textsuperscript{ox} and 80 μM MoFe. MgADP (9 mM) was present in both syringes.

Dissociation ($k_{obs}$) with DT as reductant was ~4 s\textsuperscript{-1}. When Fld\textsuperscript{HQ} was the reductant, the dissociation and reduction of Fe\textsuperscript{ox}(ADP)\textsubscript{2} was much more rapid, with the majority of the reduction (~65%) occurring in less than 1 ms. A $k_{obs}$ of >760 s\textsuperscript{-1} was estimated from a fit of the points captured after the dead time. These findings reveal that with Fld\textsuperscript{HQ} as reductant, the reduction of Fe\textsuperscript{ox} is much faster than the observed $k_{cat}$, implying that dissociation of Fe\textsuperscript{ox}(ADP)\textsubscript{2} from MoFe protein is not the rate limiting step when Fld\textsuperscript{HQ} is used as the reductant. These results are consistent with the previously reported data from similar experiments.\textsuperscript{25} To further test the effect of reductant on the apparent rate of dissociation, another non-physiological reductant, methyl viologen was tested.\textsuperscript{47} As can
be seen in Figure B3, this electron donor gave a $k_{\text{obs}}$ of 100 s$^{-1}$, again much faster than the $k_{\text{cat}}$.

The faster reduction of Fe$^{\text{ox}}$(ADP)$_2$ in the presence of MoFe protein by Fld$^\text{HQ}$ could be explained by two possible mechanisms: (i) the very rapid dissociation of the Fe$^{\text{ox}}$(ADP)$_2$ from the MoFe protein when Fld$^\text{HQ}$ is the reductant, pointing to a different rate limiting step,$^{40}$ or (ii) Fld$^\text{HQ}$ reduction of the Fe protein while still complexed to the MoFe protein,$^{48}$ a mechanism proposed earlier by Haaker et al.$^{25}$ To test the second model, the ability of Fld$^\text{HQ}$ to reduce the Fe$^{\text{ox}}$(ADP)$_2$ while still bound to the MoFe protein was examined.

**Effect of Fld$^\text{HQ}$ on the primary electron transfer of nitrogenase**

The pre-steady state ET from the Fe protein to the MoFe protein offers a straightforward way to monitor the possibility of ET from Fld$^\text{HQ}$ to Fe$^{\text{ox}}$ while still bound to the MoFe protein. When the protein mixture of Fe$^{\text{red}}$ and MoFe protein is mixed with MgATP in a SF spectrophotometer, an apparent first order ET event is monitored by the increase in absorbance (oxidation of the Fe protein) that can be fit to a single exponential to yield a rate constant for ET ($k_{\text{ET}}$). With DT as the reductant, a $k_{\text{ET}}$ of 173 s$^{-1}$ was observed (Figure B4), consistent with literature values that range from 140-200 s$^{-1}$.14–16,22,25 Important for this study, the absorbance value plateaus starting at 10 ms and stays roughly flat up to 30 ms. This plateau in absorbance reflects no reduction by DT of the Fe$^{\text{ox}}$ protein while still in the complex. At much later times (100 ms), the absorbance does change, reflecting a complex set of events as the Fe protein dissociates from the MoFe protein, is reduced, and rebinds to the MoFe protein. When the ET study is conducted with Fld$^\text{HQ}$ as reductant, nearly identical primary ET kinetics are observed (Figure B4).
Figure B4. Primary ET from \( \text{Fe}^{\text{red}}(\text{ATP})_2 \) protein to MoFe protein in presence of DT or FldHQ with DT. Syringe 1 contained 80 μM \( \text{Fe}^{\text{red}} \) protein, 20 μM MoFe protein, 1 mM DT and 10 mM MgATP. Syringe 2 had 10 mM DT or 200 μM FldHQ with 1 mM DT in the presence of 10 mM MgATP.

Importantly, no reduction of the \( \text{Fe}^{\text{ox}} \) protein is observed during the 10 to 30 ms time frame. Given the earlier observation of reduction of \( \text{Fe}^{\text{ox}}(\text{ADP})_2 \) protein-MoFe protein with FldHQ of greater than 760 s\(^{-1}\), an on-complex reduction of the Fe protein in the ET study should have resulted in a significant (if not complete) reduction of the Fe protein before 10 ms. The lack of any observed reduction of the \( \text{Fe}^{\text{ox}} \) protein in the ET experiment reveals that the FldHQ protein reduction of the \( \text{Fe}^{\text{ox}}(\text{ADP})_2 \) protein on complex with the MoFe protein must be very slow, as has been reported earlier.\(^{48}\) Thus, these studies, coupled with the dissociation studies presented in the previous section, rule out FldHQ reduction of the Fe protein while still bound to the MoFe protein,\(^{25}\) and instead
favor a rapid dissociation of Fe\textsuperscript{ox}(ADP)\textsubscript{2} protein from the MoFe protein when \textit{Fld}\textsuperscript{HQ} is the reductant.

\textbf{Reduction of Fe\textsuperscript{ox} protein by DT and Fld\textsuperscript{HQ}}

The studies, to this point, favor rapid dissociation of the Fe\textsuperscript{ox}(ADP)\textsubscript{2} protein from the MoFe protein when \textit{Fld}\textsuperscript{HQ} is the reductant. The observed lower rate of dissociation when DT is the reductant could indicate slow reduction of Fe\textsuperscript{ox} protein, rather than slow dissociation of the Fe protein from the MoFe protein.\textsuperscript{17,23,25} Several previous studies have illustrated low activity for nitrogenase at low concentration of SO\textsubscript{2}- as the reductant.\textsuperscript{17–19,22,49} To determine the rates of reduction of Fe\textsuperscript{ox} protein by different reductants, the pre-steady state studies were conducted using Fe\textsuperscript{ox} in the absence and presence of nucleotides (ADP or ATP). Fe\textsuperscript{ox} protein in one syringe was rapidly mixed against either DT or Fld\textsuperscript{HQ} in the other syringe. Additionally, these studies were conducted at 4\textdegree{}C to slow the reactions down enough to observe. Consistent with previous studies, nucleotides significantly slow down the rates of reduction of Fe\textsuperscript{ox} protein by DT (by ~100-fold).\textsuperscript{22,23} In contrast, with Fld\textsuperscript{HQ} as reductant, the rates of reduction remained fast and roughly unchanged with or without nucleotide present (Figure B5).\textsuperscript{25,27}

Taken together, the studies presented so far suggest that when Fld\textsuperscript{HQ} is the reductant, the Fe\textsuperscript{ox}(ADP)\textsubscript{2} dissociation from the MoFe protein is not rate limiting and does not correspond to the overall \(k_{\text{cat}}\) for substrate reduction. Rather, the dissociation step when Fld\textsuperscript{HQ}, is used as the reductant is much more rapid than the \(k_{\text{cat}}\).
Figure B5. Kinetics of the reduction of Fe$^{3+}$ protein by DT and Fld$^{HQ}$ with DT in the presence and absence of nucleotides. ET is monitored by observing the change in absorbance at 426 nm as a function of time. Panel A: Reduction of Fe protein by DT and Fld$^{HQ}$ with DT with no nucleotide. Panel B: Reduction of Fe protein by DT and Fld$^{HQ}$ with DT in the presence of MgADP. Panel C: Reduction of Fe protein by DT and Fld$^{HQ}$ with DT in the presence of MgATP. Syringe 1 contained 80 μM Fe$^{3+}$. Syringe 2 contained 20 mM DT or 600 μM Fld$^{HQ}$ with 20 mM DT. MgADP and MgATP, when included, were present in both syringes at a final concentration of 10 mM. The $k_{obs}$ values were averaged from two independent experiments except for Fld with MgATP.

Protein-protein interaction between Fe protein and Fld

The results presented so far indicate that Fld$^{HQ}$ does not reduce the Fe protein while still complexed to the MoFe protein, suggesting that reduction only occurs once the Fe protein is free from the MoFe protein. We next employed molecular modeling to predict where the Fld would bind to the Fe protein to achieve ET. The docking model produced by ClusPro 2.0 predicts the binding of 1 Fld monomer to a dimer of Fe protein (Figure B6, Panel A). The distance between the [4Fe-4S] cluster at the active site of the
Fe protein and the FMN of the Fld measured in PyMOL was within electron transfer distance (<10Å) (Figure B6, Panel B).

Analysis of electrostatic potentials performed in PyMOL demonstrated qualitative agreement with the ClusPro 2.0 docking model. The highly negative patch at the FMN cofactor of Fld sits on top of the highly positive patch at the location of the [4Fe-4S] cluster of the Fe protein (Figure C7). Salt bridge interactions were identified in the proposed complex binding region on either side of the FMN cofactor of Fld and the [4Fe-4S] cluster of the Fe protein (Figure B7, Panel A). The identified residues are Arg100 on one of the Fe protein subunits and Glu104 of Fld and between Arg140 of the Fe protein and Asp154 of Fld. These salt bridge interactions further support the analysis that electrostatic interactions between the Fe protein and Fld enable complex formation.

**Figure B6.** Interaction of Fe protein and Fld for ET. Panel A: The docking model from ClusPro 2.0 predicts the binding of one Fld monomer to a dimer of Fe protein. The complex structure shows the nitrogenase Fe protein with subunits in yellow and green (PDB ID: 1FP6) and Fld (NifF) is in blue (PDB ID: 1YOB). Panel B: Close-up of the docked proteins. The distance between the [4Fe-4S] cluster at the active site of the iron protein and the FMN cofactor at the active site of the flavodoxin is predicted to be 6.4Å.
Interestingly, the Fe protein Arg100 residue is the site of ADP ribosylation, which controls association of the Fe protein with the MoFe protein in response to N status of the cell.\textsuperscript{50,51}

Time-resolved limited proteolysis experiments were subsequently performed to test the docking model. These experiments involve incubation of proteins with proteases for varying lengths of time and then mapping the observed cleavages on the protein sequences. This approach facilitates identification of kinetically favored sites of protease cleavage. By comparing the peptide fragments before and after formation of a complex, regions that change conformation or are protected in the complex can be identified. Reactions were performed at Fe protein/Fld ratios of 1:1, 1:2, and 2:1, and with the two proteins alone. Sites of proteolysis were identified using MALDI-TOF and LC/MSMS and mapped onto the docking model. Cleavage sites on Fe protein were located on solvent exposed surfaces under all conditions (Figure B7). Fewer sites were found on Fld, however, upon formation of the complex, Fld cleavage sites directly adjacent to the proposed binding surfaces (Figure B7, Panel B) were protected. Specifically, Fld tryptic fragment Lys15-Lys22 was no longer present when bound to Fe protein (Figure B7, Panel B). Furthermore, regions near the [4Fe-4S] cluster of the Fe protein and the FMN cofactor of Fld were not observed, indicating that these regions were also protected from proteolysis (Figure B7, Panel B). The proteolysis data are consistent with the docking model with respect to the surfaces involved in mediating protein-protein interactions.
**Figure B7.** Mapping of the Fe protein-Fld interaction site. Panel A: Salt bridge interactions were identified in the proposed binding region on either side of the FMN cofactor of Fld (blue) and the [4Fe-4S] cluster of the Fe protein (FeP)(green and yellow). The identified residues were Arg-100 on one of the Fe protein subunits (green) and Glu-104 of flavodoxin and between Arg-140 on the other Fe protein subunit (yellow) and Asp-154 of Fld. Panel B: Time resolved proteolysis experiments show changes in Fld upon binding to Fe protein. Fe protein and Fld were digested with trypsin before (Panel B, left) and after formation of a complex (Panel B, right). Mapping of the kinetically favored sites of cleavage revealed different patterns. One of the favored sites on Fld, which mapped near the binding surface with Fe protein (black arrow), was protected from cleavage in the complex (red arrow). Panel C: Addition of cross-linking reagent to Fe protein-Fld complex (1:1 w/w protein ratio) resulted predominantly in formation of monomers (intraprotein linkages) and dimers (interprotein linkages between Fe protein-Fe protein and Fe protein-Fld) (Panel C, left). In addition to cross-linked complex, the following controls were run: Fe protein, Fld, and Fe protein-Fld non cross-linked, and Fe protein and Fld cross-linked individually. To identify interacting domains, corresponding monomers and dimmers were digested with trypsin and identified peptides (red) were mapped onto the ClusPro 2.0 Docking model (Panel C, middle and right show Fld and Fld in complex with Fe protein after exposure to glutaraldehyde, respectively). Fragments colored in orange denote peptides underrepresented in cross-linked sample with respect to unlabeled protein. Red arrow, points to this part of Fld structure which was absent after crosslinking.
While implied by our data and previous models, the stoichiometry of the solution phase complex has not been directly addressed. To investigate this, chemical cross-linking experiments were performed. Fe protein alone, Fld alone, and after complex formation were covalently cross-linked using 20 mM glutaraldehyde (GA). The proteins were exposed to GA for 10 min before the reaction was quenched. Analysis of the samples by SDS-PAGE showed that the predominant species present was consistent with an Fe protein-Fld binary complex (Figure B7, Panel C, left). Mass spectrometry analysis confirmed the presence of both proteins in the gel band of interest. However, some peptide fragments were no longer detectable or were present in much lower quantity when Fe protein-Fld were engaged in complex and exposed to GA (Figure B7). For example, based on Fld (Figure B7, Panel C, middle and left), there are three distinguished regions: (1) colored in red, this part of the protein chain was easily digested by trypsin and was identified by LC/MSMS with high confidence. One of the fragments, Ile125-Lys146, was always detectable regardless of GA treatment, suggesting lack of involvement in any kind of interactions (within Fld itself and/or between Fld and Fe protein); (2) colored in orange, these parts of protein sequence were also identified, however, they were found in significantly lower concentrations in cross-linked samples what implies more structured regions (within Fld itself) that were in close proximity and were linked with GA and/or protein sections potentially engaged in interactions with Fe protein; colored in blue, this part of Fld chain was not detected in any sample. Taken together our observations from chemical labeling, Fld peptide fragment Phe147-Lys160 is directly involved in contact with Fe protein, however more Fld sequence parts might be involved in complex formation through subtle conformation adjustments.
The results from the *in silico* modeling, proteolysis, and cross-linking experiments all support the predicted 1:1 stoichiometry for the Fe protein-Fld interaction with an interface bringing the two cofactors ([4Fe-4S] cluster and FMN) close together to favor the ET (Figures B6, B7, and C7). The interaction site on the Fe protein surface with Fld is the same as that with MoFe protein. To test this prediction, we carried out the proton reduction assay under both low and high electron flux conditions. The results display a significant inhibitory effect of Fld on the proton reduction activity (Figure C8) in the presence of 2.4 mM Fld\textsuperscript{HQ}, compared to those in the presence of 600 μM Fld\textsuperscript{HQ} (Figure C1). The observed inhibition from Fld further supports the transient interaction between the Fe protein and Fld during nitrogenase catalysis.\textsuperscript{27}

**Efficiency of ATP hydrolysis per electron transferred for substrate reduction**

It has been reported that Fld\textsuperscript{HQ} can reduce the Fe\textsuperscript{ox} protein by 1 electron to the [4Fe-4S]\textsuperscript{1+} state\textsuperscript{25,27} or by two electrons to the all ferrous [4Fe-4S]\textsuperscript{0} state.\textsuperscript{33,34} From the all ferrous state, the Fe protein could deliver two electrons to the MoFe protein per association, resulting in 2 ATP hydrolyzed/2 e\textsuperscript{-} transferred.\textsuperscript{33,34,40} This possibility is contrary to the consensus that each association event results in 2 ATP hydrolyzed per 1 e\textsuperscript{-} transferred. Using DT as the reductant, the ATP/e\textsuperscript{-} ratio was found to be ~2:1 with three different substrates and under high or low electron flux conditions (Table B2, Figure C9). This result is consistent with the previously reported data. Using Fld\textsuperscript{HQ} as the reductant, the ratio of ATP/e\textsuperscript{-} ratio was also found to be ~2:1 under high and low electron flux with three substrates. The results clearly indicate that ~2 ATP were hydrolyzed per electron transferred (ATP/e\textsuperscript{-}= 2, Table B2), which was independent of substrate, reductant (DT or Fld\textsuperscript{HQ}), and electron flux condition. These findings are consistent with a
Table B2. Number of ATP hydrolyzed per electron transferred for reduction of different substrates by nitrogenase under different electron flux conditions.

<table>
<thead>
<tr>
<th>Reaction Conditions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reductant</th>
<th>ATP/e Ratio&lt;sup&gt;d&lt;/sup&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proton (Ar)</td>
<td>N₂ (1 atm)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Acetylene&lt;sup&gt;f&lt;/sup&gt; (0.11 atm)</td>
</tr>
<tr>
<td>Low Flux&lt;sup&gt;b&lt;/sup&gt;</td>
<td>DT</td>
<td>1.87 ± 0.04</td>
<td>1.85</td>
<td>2.17 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Fld&lt;sup&gt;HQ&lt;/sup&gt;</td>
<td>1.91 ± 0.05</td>
<td>1.91</td>
<td>2.26 ± 0.12</td>
</tr>
<tr>
<td>High Flux&lt;sup&gt;c&lt;/sup&gt;</td>
<td>DT</td>
<td>1.63 ± 0.12</td>
<td>1.82</td>
<td>1.89 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Fld&lt;sup&gt;HQ&lt;/sup&gt;</td>
<td>1.96 ± 0.17</td>
<td>1.78</td>
<td>2.74 ± 0.21</td>
</tr>
</tbody>
</table>

<sup>a</sup>All assays for product quantification were done in a buffer containing 8.5 mM MgATP without a regeneration system with 12 mM DT or 712 μM Fld<sup>HQ</sup> plus 10 mM DT. For ADP quantification assays, trace concentration of α-<sup>32</sup>P-ATP was added to the reaction mixtures. <sup>b</sup>Low flux assays were done using 1.95 μM of Fe protein with 2.08 μM of MoFe protein for proton and acetylene reduction, and 4.16 μM of MoFe protein for N₂ reduction. <sup>c</sup>High flux assays were done with 0.41 μM MoFe protein and 8 μM Fe protein. <sup>d</sup>The ATP/e<sup>-</sup> ratios for proton and acetylene reduction assays were averaged from the data at three different reaction times (Figure S1) with the standard deviation shown. N₂ reduction values were calculated from assays with a reaction time of 60 sec. <sup>e</sup>Total electrons for H₂ and NH₃ production were counted. <sup>f</sup>Only electrons for ethylene production were counted as proton reduction was below the detection limit.

Based on the findings presented to this point, it is concluded that the Fe protein-MoFe protein dissociation step is not rate-limiting. What then is the rate limiting step in the overall reaction cycle? In the Fe protein cycle using DT as the reductant (Figure B2), the ATP hydrolysis \( (k_{ATP} = 50-70 \text{ s}^{-1}) \) and the Pi release \( (k_{Pi} = 16-22 \text{ s}^{-1}) \) steps both are slower than the ET \( (k_{ET} = 140-200 \text{ s}^{-1}) \).

Comparing rate constants for ATP hydrolysis from pre-steady state studies with DT or Fld<sup>HQ</sup> as reductant and a 1:4 ([MoFe]:[Fe]) electron flux revealed similar values.
(\(k_{\text{ATP,DT}}\) and \(k_{\text{ATP,Fld}}\)) of 40 s\(^{-1}\) and 44 s\(^{-1}\) (25\(^{\circ}\)C), respectively (Figure B8). These rate constants are about double the \(k_{\text{cat}}\) (10-11 s\(^{-1}\)) when considering that 2 ATP molecules are hydrolyzed per electron, indicating that ATP hydrolysis is not the rate-limiting step for the overall reaction. The steady state (linear) rates of ATP hydrolysis are found to be \(k_{\text{ATP,DT}} = 10\text{ s}^{-1}\) and \(k_{\text{ATP,Fld}} = 20\text{ s}^{-1}\) (25\(^{\circ}\)C), consistent with the \(k_{\text{cat}}\) values reported here.

The slow steady-state ATP hydrolysis rate using DT matches with the slow reduction rate of \(\text{Fe}^{\text{ox}}(\text{ADP})_2\) and \(\text{Fe}^{\text{ox}}(\text{ATP})_2\) \((k_{\text{obs}} = 5-6\text{ s}^{-1})\) by DT. Whereas, the faster \(k_{\text{ATP,Fld}}\) is consistent with the faster reduction of the \(\text{Fe}^{\text{ox}}(\text{ADP})_2\) and \(\text{Fe}^{\text{ox}}(\text{ATP})_2\) states by Fld\(^{\text{HQ}}\).

**Figure B8.** Time course of pre-steady state and steady state ATP hydrolysis during nitrogenase catalysis under Ar with DT (open squares) or Fld\(^{\text{HQ}}\) with DT (closed squares) as reductant at 25\(^{\circ}\)C. The hydrolysis of ATP was monitored with [\(\alpha\)-\(^{32}\)P]ATP as a tracing reagent. The ratio of ADP/MoFe protein was plotted as a function of time. The data were fitted to two phases: a burst exponential followed by a linear steady state for both DT and Fld\(^{\text{HQ}}\) with DT. Pre-steady state burst phase gave first order rate constants of \(k_{\text{ATP,DT}} = 40\text{ s}^{-1}\) and \(k_{\text{ATP,Fld}} = 44\text{ s}^{-1}\), while the steady state linear phase gave rate constants of \(k_{\text{ATP,DT}} = 10\text{ s}^{-1}\) and \(k_{\text{ATP,Fld}} = 20\text{ s}^{-1}\).
The ATP hydrolysis rate constant obtained under high flux conditions ([MoFe]:[Fe] = 1:16) showed no difference in the pre-steady state rate constants for ATP hydrolysis with DT or Fld as reductant (data not shown). However, the steady state rate constant for ATP hydrolysis using DT is significantly increased to 25 s\(^{-1}\), which is about the same as the value when using Fld\(^{HQ}\) (27 s\(^{-1}\)) under same conditions. It is interesting that the steady-state ATP hydrolysis rate constant (25-27 s\(^{-1}\)) is about the same as those previously reported for the pre-steady state Pi release step (16-22 s\(^{-1}\), Figure B2).\(^{16,18}\) Given that two Pi are released per electron transferred, the Pi release rate constant should be double the \(k_{cat}\) value (about 20 s\(^{-1}\)) if this step is the overall rate limiting step.

**Pi release events**

Pi release was measured using an established fluorometric method\(^{43}\) with either DT or Fld\(^{HQ}\) as reductant under high electron flux conditions ([Fe]:[MoFe] = 16:1). The data showed an initial lag phase followed by a linear phase with both reductants, with the linear phases having rate constants for Pi release of \(k_{PL,DT} = 27\) s\(^{-1}\) and \(k_{PL,Fld} = 25\) s\(^{-1}\) (Figure B9). These rate constants are about double the overall reaction rate constant \(k_{cat}\) of 10-11 s\(^{-1}\) per electron. Two Pi are released for each electron transferred, showing that the overall rate limiting step is likely events associated with Pi release. We have no data on the ADP release event, so the position of this event in the cycle remains unknown.

**Establishing the rate-limiting step in the Fe protein cycle**

Considering all of the rate constants for the steps in the Fe protein cycle with Fld\(^{HQ}\) as reductant: electron transfer \((k_{ET} = 173\) s\(^{-1}\)), ATP hydrolysis \((k_{ATP} \sim 40-44\) s\(^{-1}\)),
Figure B9. Real-time measurement of Pi release during nitrogenase catalysis using DT (gray) or Fld\textsuperscript{HQ} with DT (black) as reductant at 25°C. The Pi release was monitored by a fluorescence increase caused by the binding of Pi to MDCC-PBP. The ratio of Pi/MoFe protein is plotted as a function of time. After the initial lag phase (~30 ms), the data collected from 40 to 200 ms were fitted to a linear equation (solid line), giving rate constants of $k_{\text{Pi,DT}} = 27 \text{ s}^{-1}$ and $k_{\text{Pi,Fld}} = 25 \text{ s}^{-1}$.

Pi release (25-27 s\(^{-1}\)) and re-reduction of Fe\textsuperscript{ox}(ADP)\(_2\) ($k_{\text{obs}} > 1200 \text{ s}^{-1}$), it is concluded that the overall rate limiting step for the reaction are events associated with the Pi release step, not complex dissociation. Our findings are consistent with other ATP hydrolyzing systems, including helicases\textsuperscript{53,54} and myofibrillar ATPases,\textsuperscript{55-58} where Pi release is the rate limiting step.

Summary

The Fe protein cycle can now be updated with the rate constants determined here using Fld\textsuperscript{HQ} as the reductant, as shown in Figure B10. The cycle begins with the rapid equilibrium docking of Fe\textsuperscript{red}(ATP)\(_2\) to MoFe protein, which is followed by the
conformationally gated ET events. Next occurs ATP hydrolysis, which is followed by events associated with Pi release. It is unknown if the Pi release event is conformationally gated, but it is clear that events associated with this step are rate limiting for the overall Fe protein cycle. The dissociation of the Fe\textsuperscript{ox}(ADP)\textsubscript{2} is fast, with rapid reduction by the Fld\textsuperscript{HQ}. Finally, ATP replaces ADP in the free Fe protein. The order of these last two events is not established, but the rapid reduction by Fld\textsuperscript{HQ} suggests that reduction should occur before nucleotide exchange.

**Figure B10.** Updated Fe protein cycle with Fld\textsuperscript{HQ} as reductant.
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the nitrogenase metal centers as a result of iron protein–molybdenum-iron protein

kinetic studies of dithionite utilization, component protein interaction, and the formation
of an oxidized iron protein intermediate during \textit{Azotobacter vinelandii} nitrogenase


APPENDIX C

SUPPORTING INFORMATION

EVIDENCE THAT THE Pi RELEASE EVENT IS THE RATE LIMITING STEP IN THE NITROGENASE CATALYTIC CYCLE
Table C1. Electron distribution for $\text{H}_2$ and $\text{NH}_3$ production under different electron flux conditions with DT or Fld as reductants.

<table>
<thead>
<tr>
<th>Reaction Conditions$^a$</th>
<th>1 atm $\text{N}_2$</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$e^-$ for $\text{H}_2$ (nmol)</td>
<td>$e^-$ for $\text{NH}_3$ (nmol)</td>
<td>% of $e^-$ for $\text{NH}_3$</td>
<td></td>
</tr>
<tr>
<td>Low Flux$^b$</td>
<td>DT</td>
<td>241.68 ± 4.19</td>
<td>231.94 ± 5.05</td>
<td>48.97 ± 1.26</td>
</tr>
<tr>
<td></td>
<td>Fld$^{HQ}$</td>
<td>329.72 ± 2.59</td>
<td>292.70 ± 2.55</td>
<td>47.03 ± 0.49</td>
</tr>
<tr>
<td>High Flux$^c$</td>
<td>DT</td>
<td>172.17 ± 8.71</td>
<td>171.83 ± 1.29</td>
<td>49.95 ± 1.33</td>
</tr>
<tr>
<td></td>
<td>Fld$^{HQ}$</td>
<td>157.79 ± 4.36</td>
<td>130.47 ± 5.76</td>
<td>45.26 ± 2.30</td>
</tr>
</tbody>
</table>

$^a$All reactions were done in a buffer containing a MgATP regeneration system. For assays using dithionite as the reductant, 10 mM DT was used. And for assays using flavodoxin as the reductant, 600 µM of flavodoxin and 10 mM DT were used. All reactions were done at 30°C for 30 sec. $^b$Protein concentrations were 1.95 µM of Fe protein and 2.08 µM of MoFe protein ([Fe]:[MoFe] ≈ 1:1). $^c$Protein concentrations were 8 µM of Fe protein and 0.41 µM of MoFe protein ([Fe]:[MoFe] ≈ 20:1).
**Figure C1.** Acetylene reduction by wild-type Mo-nitrogenase using DT or Fld as reductants under different electron flux conditions. Low flux: 1.95 µM of Fe protein and 2.08 µM of MoFe protein ([Fe]:[MoFe] ~ 1:1) in the reaction mixture. Units for specific activity of acetylene reduction are nmol ethylene/min/mg Fe protein. High flux: 8 µM of Fe protein and 0.41 µM of MoFe protein ([Fe]:[MoFe] ~ 20:1). Units for specific activity of acetylene reduction are nmol ethylene/min/mg MoFe protein. All assays were done in the presence of 0.1 atm of acetylene and 0.9 atm of argon. Reaction mixtures contained 12 mM DT or 600 µM FldHQ with 12 mM DT as reductants.
Figure C2. Electron flux dependence on proton reduction activity by wild-type Mo-nitrogenase using DT or Fld as reductants. The assays were done in the presence of 1.95 µM of Fe protein with varied MoFe protein concentrations under argon. See Figure B3-1 for additional information on reaction conditions.
Figure C3. Electron flux dependence on \( \text{N}_2 \) reduction by wild-type Mo-nitrogenase using DT or Fld as reductants. Specific activities are plotted in terms of \( \text{NH}_3 \) (left panel) and \( \text{H}_2 \) (right panel) production. Assays were done at 1 atm \( \text{N}_2 \). See Figure B3-1 for additional information on reaction conditions.
**Figure C4.** Electron flux dependence on the total activity of proton and N\(_2\) reduction by wild-type Mo-nitrogenase using DT or Fld as reductants under N\(_2\). The total activity of Fe protein with different MoFe protein concentration was calculated by adding the electrons diverted to NH\(_3\) production to that for H\(_2\) production in **Figure B3-3**, and is plotted here as H\(_2\) evolution activity.
Figure C5. Electron flux dependence on MoFe protein activity for N\textsubscript{2} reduction using DT or Fld as reductants. The assays were done in the presence of 0.41 µM of MoFe protein with varied Fe protein concentrations under 1 atm N\textsubscript{2}. Other conditions are the same as that used in Figure B3-1.
Figure C6. Determination of the isosbestic point in UV-vis spectrum for the reversible conversion of $\text{Fld}^\text{HQ}$ to $\text{Fld}^\text{SQ}$. The spectra were recorded at a scan frequency of 1 min$^{-1}$ from the oxidation of $\text{Fld}^\text{HQ}$ by O$_2$ with the blue trace showing the first no O$_2$ spectrum (time 0 min) and the red trace showing the last spectrum after incremental oxidation by O$_2$ for 16 min.
**Figure C7.** Left: Front and side view of electrostatic potentials for the bound complex between the Fe protein and Fld generated in PyMOL. Right: Side and top view of electrostatic potentials for the individual proteins with complex docking sites shown by the arrows.
Figure C8. Inhibition of nitrogenase activity by high concentration of flavodoxin under different electron flux conditions. The electron flux condition and protein concentrations for low flux and high flux experiments are the same as in the Figure B3-1 legend. Reductants are 12 mM DT or 2.4 mM Fld plus 12 mM DT. The experiments were carried out for proton reduction under argon, and the total amount of H₂ after a 60 second incubation at 30⁰C were plotted.
Figure C9. Time course study of ATP hydrolysis and electron utilization for proton reduction under low electron flux (panel A, [Fe]:[MoFe] $\approx$ 1:1) and high electron flux (panel B, [Fe]:[MoFe] $\approx$ 20:1) conditions using DT or Fld as reductants. The amounts of produced ADP and electrons for H$_2$ evolution under argon are plotted versus reaction time. The reaction conditions are shown in Table 3-2.
SUMMARY

Dedicated and enthusiastic scientist and teacher. Experience in the areas of biochemistry, microbiology, general biology, and general chemistry. Innovative and dynamic instructor with solid teaching experience at the collegiate level, including classroom instruction and training undergraduate and graduate researchers. Applies a number of different strategies to enhance student experience and performance. Strong science communication skills, serving as a science reporter and editor. Self-starter who finds reward in focusing on task, yet remains resilient and adaptable to changing needs of the job. A team player with well-developed communication and management skills. International work experience in Australia and China. Distinguished recipient of several awards and grants.

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Utah State University, Logan, Utah
Department of Chemistry and Biochemistry  2013-2018
Guest Lecturer, 2016-2018
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Graduate Teaching Assistant, 2013-2014
• Instructed Chemical Principles Laboratory II
Mohave Community College, Bullhead City, Arizona 2011-2013

Resident Science Faculty
- Designed course materials and taught Introductory Biology and Microbiology Lectures and Laboratories
- Produced curriculum for summer forensic science course aimed at high school students
- Received a $10,000 National Park Teachers’ Grant in collaboration with the National Park Service to enhance student field microbiology experience

Duke Talent Identification Program, Sarasota, Florida 2012

Summer Program Instructor
- Developed curriculum and taught an Introduction to Laboratory Sciences course for gifted junior high school students

Jiaxing University, Jiaxing, China 2010

Department of Foreign Languages
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- Taught English as a Second Language

Question Master, Jiaxing University English Speaking Contest and 5th Annual English Speaking Contest of Zhejiang Province
- Evaluated ESL students on prepared and impromptu speeches

Idaho State University, Pocatello, Idaho 2005-2009

Department of Biological Sciences
Assistant Lecturer, 2007-2009
- Taught Introductory Microbiology Laboratory, General Microbiology Laboratory, Microbial Physiology Lecture and Laboratory, and Special Topics in Microbiology

Microbiology Lab Coordinator, 2007-2009
- Supervised Microbiology Laboratory teaching assistants
- Developed and updated Microbiology Laboratory course curricula on a continuous basis
- Maintained live culture collection
- Ordered and organized Introductory and General Microbiology Laboratory materials

Graduate Teaching Assistant, 2005-2007
- Facilitated the Introductory Microbiology and Microbial Physiology Laboratories

RESEARCH EXPERIENCE

Utah State University, Logan, Utah 2013-2018
Department of Chemistry and Biochemistry
Graduate Research Assistant, Advisor Dr. Lance Seefeldt
- Investigates electron flow and management in living systems
- Produces value-added products from yeast grown on industrial effluent
- Mentors undergraduate and new graduate students
- Writes and contributes scientific data to peer-reviewed manuscripts

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 2014
Summer Course Participant, Instructors Dr. Maitreya Dunham, Dr. Marc Gartenberg, and Dr. Grant Brown
- Performed experiments focused on classical and modern yeast genetics techniques
Idaho State University, Pocatello, Idaho  
Department of Biological Sciences

Graduate Research Assistant, Advisors Dr. Tim Magnuson and Dr. Gene Scalarone, 2005-2007
• Characterized a novel arsenic-metabolizing bacterium
• Developed new methods to detect enzyme activities in microbial biofilm samples
• Mentored undergraduate students working in the laboratory

Undergraduate Research Assistant, Advisor Dr. Tim Magnuson, 2003-2005
• Discovered a novel arsenic-metabolizing bacterium
• Assisted research scientists with media preparation and other basic tasks

University of Melbourne, Melbourne, Australia  
Department of Microbiology and Immunology
Summer Research Assistant, Advisor Dr. Peter Janssen
• Conducted studies on how to increase the culturability of soil microorganisms

Savannah River Ecology Laboratory, Aiken, South Carolina  
Summer Research Assistant, Advisors Dr. Andrew Neal and Dr. Christopher Bagwell
• Researched the impacts of metal contamination on microbial populations at the Savannah River Site

SCIENCE COMMUNICATION EXPERIENCE

Utah Public Radio  
National Public Radio Affiliate
Volunteer Science Reporter
• Shares science occurring throughout the state of Utah with the general public through written articles and radio stories

Frontiers in Energy Research  
Department of Energy
Volunteer Writer and Editor
• Showcased the accomplishments and activities of Energy Frontier Research Centers to the scientific community and general public through written articles

PEER-REVIEWED PUBLICATIONS


**BOOK CHAPTERS**


**NEWS ARTICLES AND RADIO STORIES**


Ledbetter, R.N. 2017. Shower the National Weather Service with data by being a backyard weather observer. *Utah Public Radio.*

Ledbetter, R.N. 2017. Dr. Scott the Paleontologist keeps on inspiring young minds with new book. *Utah Public Radio.*


Ledbetter, R.N. 2017. Protecting teens from HPV: The vaccine and what one parent learned on her journey to know more. *Utah Public Radio.*


Ledbetter, R.N. 2016. Alan Alda takes a leading role, inspires scientists to be effective communicators. *Utah Public Radio.*


Ledbetter, R.N. 2016. Getting fired up for the next wave of space exploration. *Utah Public Radio*.


Ledbetter, R.N. 2016. The story behind the scientist: One who beat the odds. *Frontiers in Energy Research*.


**ORAL PRESENTATIONS**


Ledbetter, R.N. 2012. Bringing instruction to life. Mohave Community College All Staff Day Meeting, Lake Havasu City, AZ.


**POSTER PRESENTATIONS**


James, M., R.N. Ledbetter, A.L. Neal, and T.S. Magnuson. 2006. Biogenic mineral production as evidence for life. Idaho State University Undergraduate Research Symposium, Pocatello, ID.


PROFESSIONAL DEVELOPMENT


Science Communication Workshop, Alan Alda Center for Communicating Science, 2016.


The Teaching Professor Workshop: Infusing Learner-Centered Strategies, Austin, Texas. 2013.


SERVICE


SCIENCE GRANTS, AWARDS, AND HONORS

Thomas F. Emery Outstanding Graduate Student in Biochemistry, Utah State University, 2018.

Student Travel Award, American Society for Microbiology, 2014.

Next Generation Scientists for Biodiesel Scholarship, National Biodiesel Board, 2014.

Park Teachers’ Grant, National Park Foundation, 2012.

Goldschmidt Travel Grant, Geochemical Society and the National Science Foundation, 2007.

Goldschmidt Travel Grant, Geochemical Society and the National Science Foundation, 2006.

Graduate Research Fellowship, NASA Idaho Space Grant Consortium, 2006.

East Asia and Pacific Summer Institutes for U.S. Graduate Students, University of Melbourne, National Science Foundation, 2006.

Graduate Research Fellowship, NASA Idaho Space Grant Consortium and NASA Idaho EPSCoR Fellowship Program, 2005.

Research Experience for Undergraduates, Savannah River Ecology Laboratory, University of Georgia, National Science Foundation, 2004.

RADIO AWARDS

Mark of Excellence Award: Radio Feature, 1st place (Intermountain West) for “‘They’re built on love’: Raising children with Williams syndrome,” Society of Professional Journalists, 2018.

