

THE ROLE OF FIXX IN ELECTRON BIFURCATION

by

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DEDICATION

This work is dedicated to my Grandmother, Elizabeth St. Pierre, without whom I would have succumbed to circumstance rather than fight and persevere.

“I must not fear. Fear is the mind-killer. Fear is the little-death that brings total obliteration. I will face my fear. I will permit it to pass over me and through me. (. . .) Where the fear has gone there will be nothing. Only I will remain.” –Frank Herbert

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ABSTRACT

Two known methods of physiological energy conservation are substrate level phosphorylation and electron transfer phosphorylation. Recently, electron bifurcation has been established as a third and key mechanism of energy conservation in biological processes. This coupling of endergonic and exergonic reactions allows for utilization of reducing potential to perform energetically expensive physiological reactions. A significant and energetically expensive physiological reaction is nitrogen fixation, which provides a substantial portion of the bioavailable nitrogen that life requires. Electron bifurcation is utilized by the FixABCX system that is up regulated during diazotrophic growth and is suggested to bifurcate electrons from NADH to quinone of the electron transport chain through high potential electron transfer proteins and to nitrogenase through low potential electron transfer proteins. The determination of how cellular mechanisms overcome the energy barriers of high potential electron transfers through electron bifurcation is crucial for our fundamental understanding of energy transfer and energy conservation. The work presented in this thesis aims to progress the present knowledge in this third mechanism of energy conservation and shows support for a protein in the FixABCX complex, FixX, as the low potential electron acceptor in the complex. Numerous organisms were investigated as potential model systems for FixABCX with varying degrees of success. The genome of the organism, *Roseiflexus castenholzii*, contains both the nitrogenase and *fixABCX* genes and has successfully been used to obtain FixX. This protein shows homology to ferredoxin, a physiological reductant of the nitrogenase Fe protein in some organisms. EPR spectroscopy and sequence analysis suggests FixX contains 2 [4Fe-4S] clusters, while a potentiometric titration shows the clusters to have highly negative mid-point potentials. The preliminary evidence supports FixX of the FixABCX system to be a low potential electron transfer protein.

INTRODUCTION

Historical Perspective on Electron Bifurcation

Traditionally, the two fundamental modes of energy conservation in biological systems are substrate level phosphorylation and electron transfer phosphorylation; however, recently, a third mode of biological energy conservation has been determined – electron bifurcation. Now recognized as an essential mechanism of biological energy conservation¹, electron bifurcation maximizes energy efficiency by exploiting reducing agent electrons and coupling exergonic and endergonic reactions without concomitant hydrolysis of a high-energy bond. The coupling of these reactions overcomes the thermodynamic barrier presented by the endergonic reaction, maximizing the conversion of electrochemical potential to chemical bond energy (Figure 1). By coupling a cellular reaction with a significant negative free energy change, to one with a positive free energy change, the extent of energy wasted as heat is limited. The first described system to utilize electron bifurcation was published in 1975 by Peter Mitchell discussing the Q-cycle of complex III². This cycle uses the oxidation of quinol to produce reducing equivalents, which are utilized in two distinct redox chains. This quinone-based system operates at much higher potentials (-100 mV to +300 mV) than do flavin-based systems (-300 mV to -500 mV) on which this thesis focuses.

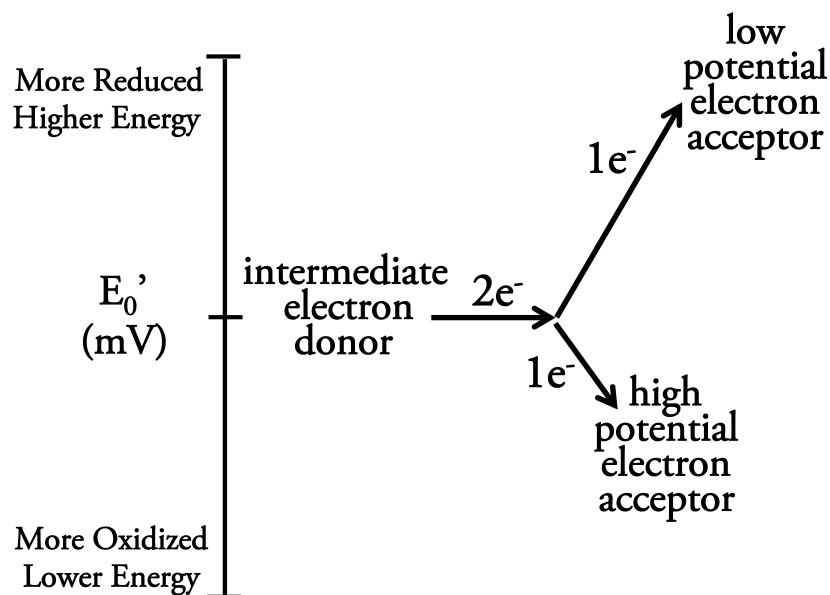


Figure 1: General mechanism showing electron bifurcation from a single electron donor to acceptors at different redox potentials.

From what is currently understood, typical bifurcating systems utilize flavins and Fe-S clusters as the electron transfer molecules with bifurcation centered around versatile flavoproteins. Electrons from a reducing agent such as nicotinamide adenine dinucleotide (NADH) are donated to a bifurcation center, and utilized singly to reduce acceptor molecules with highly separated potentials. This is in stark contrast to how known electron transfer mechanisms operate. Typically, electrons flow in an exergonic manner or are coupled with exergonic reactions, such as adenosine triphosphate (ATP) hydrolysis, to overcome endergonic potentials and are often associated with membrane bound proteins. The electron transfer from a more positive donor to a more negative acceptor, as occurs in electron bifurcation, can occur so long as the overall reaction is thermodynamically favorable³. There are several published examples of enzymatic

systems that take advantage of electron bifurcation to couple endergonic and exergonic reactions. A ferredoxin based system in *Methanothermobacter marburgensis* couples the exergonic reduction of CoM-S-S-CoB with H_2 to the endergonic reduction of ferredoxin with H_2 ⁴. The crystal structure of a flavin based bifurcating system from *Thermatoga maritima*, NfnAB was obtained⁵ and revealed specific differences in residues which either accommodated or excluded the phosphate moiety of NADPH; this system couples the endergonic reduction of ferredoxin with NADPH to the exergonic trans hydrogenation of NADPH to NAD^+ . Structural data from this system provides some insights for how bifurcating systems control electron flow with substrate specificity.

How cellular mechanisms of electron bifurcation overcome energy barriers of high potential electron transfers through electron bifurcation is crucial for our fundamental understanding of energy transfer and conservation. Developers of biomimetic and synthetic catalysts utilize the mechanistic knowledge from these studies for advancing efficiency and effectiveness while decreasing the required input energy and emissions.

Mechanistic Qualifiers of Electron Bifurcation

Thermodynamically speaking, reductions of substrates are dictated by the potentials of the electron donor and acceptor where electron transfer from a lower potential to higher potential are favorable¹. Unusually, in electron bifurcation the mid-point potential of the low potential e^- donor is intermediary between the high potential e^- acceptor and the Fd. Electron bifurcation is highly unique in that the resultant energies of

the bifurcated electrons are not at equal energy levels post electron transfer. These electron transfer reactions can proceed in either direction with the forward being electron bifurcating and the reverse electron confurcating (Figure 2).

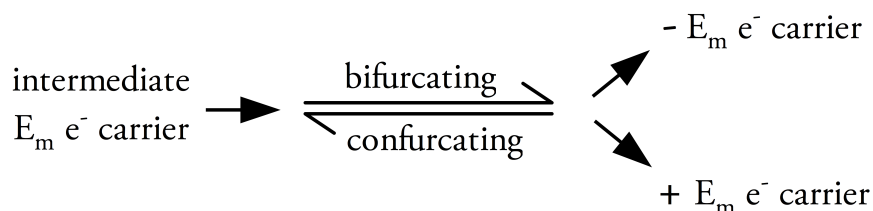


Figure 2: General reaction scheme of electron bifurcation

All known systems include a bifurcating complex containing at least one flavin (FAD or FMN), are ferredoxin-dependent, and proceed according to the above reaction⁵. Typically NADPH or NADH are the electron donors, however H₂ and formate⁶ serve as electron donors, as may other molecules. Follows are detailed examples of bifurcating systems after outlining the basic properties of Fe-S clusters and flavins; the two major electron transfer centers currently known to be involved in electron bifurcation. The mechanism by which electron bifurcation occurs is largely as yet unknown and understudied.

Cofactors: Mid-point Potentials and Spectroscopic Properties

Fe-S Clusters

Iron sulfur clusters are obligate single electron transfer components and exist as sets of inorganic iron and sulfur covalently coordinated to the protein often through several cysteine thiolate ligands. These clusters can be coordinated through the side-chain nitrogen atom of histidine residues, though several other amino acid residues have been

shown to coordinate clusters through mutational studies⁷. Ferredoxins (Fds) are proteins, which contain iron-sulfur clusters generally liganded with conserved cysteine motifs implicating the type of iron-sulfur cluster contained within. The architecture of each type of iron sulfur cluster affects the potentials at which the cluster is oxidized or reduced additionally, the coordinating ligands of each cluster help to tune the redox properties of the protein⁸⁻¹⁴. Fds are the classic example of low molecular weight electron transfer proteins in which two [4Fe-4S] clusters are coordinated¹⁵ that each can accept a single electron. Fds observed to function with high mid-point reduction potentials are referred to as high potential iron-sulfur proteins (HiPIPs). A prominent feature of Fe-S proteins is the observed wide range of operating mid-point reduction potentials from structurally very similar Fe-S clusters such as the oxidized 2 [4Fe-4S] Fd at -427 mV in *Peptococcus aerogenes* and the oxidized [4Fe-4S] HiPIP at +356 mV in *Chromatium*¹⁵. Fe-S cluster architecture can be investigated initially through UV-visible spectroscopy as each cluster has a characteristic maximum absorbance wavelength influenced by the ligand to metal coordination¹⁶. Thorough investigations utilize electron paramagnetic resonance spectroscopy (EPR), which relies on the absorption of electromagnetic radiation by unpaired electrons found in atoms such as transition metals. The absorbed radiation transitions the free electron to a different energy state and transition in energy can then be measured through spectroscopy. As in Figure 3 the reduced and oxidized states of each cluster can be differentiated, as can the cluster types¹⁷. Occasionally the clusters will produce highly similar spectrum at which point the temperature at which the spectrum is obtained can be altered, as these clusters often will then display spectra with different

signal intensities allowing for differentiation. This temperature dependence relates to the different relaxation times of each cluster after radiation absorption. The reported g values for oxidized or reduced clusters relates the property of the electron in a particular environment, specifically the relationship between the microwave frequency (ν), Planck's constant (h), Bohr magneton constant (β), and the magnetic field (B) given by equation 1.

$$g = \frac{h\nu}{\beta B} \quad (1)$$

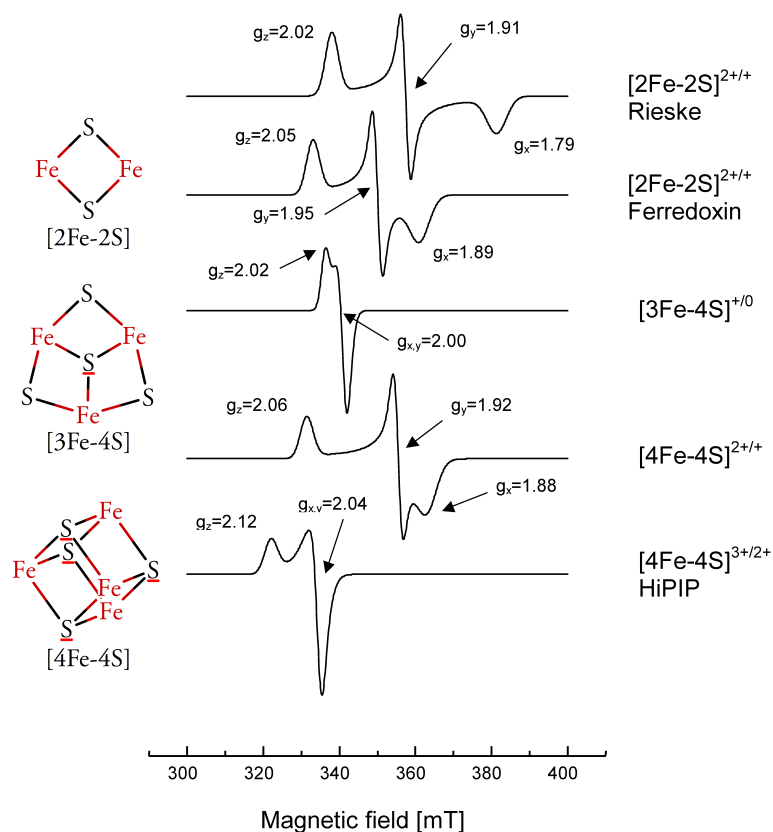


Figure 3: EPR spectra of various Fe-S clusters showing cluster connectivity¹⁸

Flavins: FAD, FMN

Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are coenzymes utilized for electron transfer events. These cofactors arise from the biological conversion of vitamin B₂, riboflavin, a phosphorylated and adenylylated isoalloxazine ring¹⁹. For redox chemistry these are highly advantageous over nicotinamide coenzymes as they are capable of performing one and two electron transfer events as opposed to the obligate two electron transfers as performed by nicotinamide coenzymes. FMN and FAD can serve as mediators between redox coenzymes by accepting two electrons from NADH and singly transfer electrons to obligate single electron acceptors such as Fe-S cluster containing proteins. The hydroquinone/semiquinone couple mid-point potential is lower than semiquinone/quinone couple²⁰, meaning reduction occurs quinone (oxidized, yellow), semiquinone (1 e⁻, blue), followed by hydroquinone (2 e⁻, colorless). The associated electronic configuration of biological flavins are as shown in Figure 4.

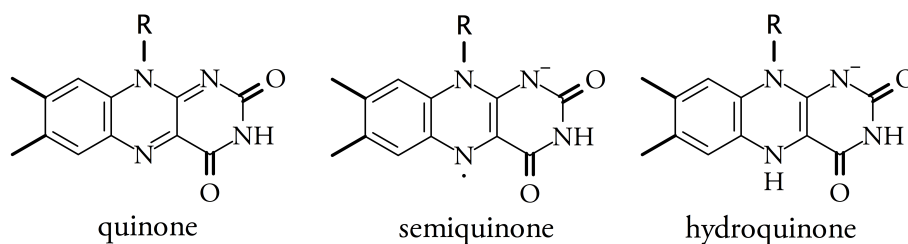


Figure 4: Redox states of flavins in flavoproteins

Flavins can act as both electron acceptors and donors corresponding to the oxidized and reduced states, respectively. Due to the property of the isoalloxazine as a p-quinoid molecule being electron deficient in its oxidized state and can be enhanced by hydrogen bonding, protonation, or alkylation in a protein²¹. Chemical properties of the

two electron reduced 1,5-dihydroflavin state are indicative of a good electron donor. This reduced state has been shown to be bent with the degree of deviation from planarity affecting the electron donor ability¹⁹. A property exploited by biology in flavoproteins where the deviation can be altered by amino acid residue interactions thusly tuning the redox properties of the flavin cofactor as well as altering the affinity of the protein for certain substrates. The study done by Massey and Ghisla in the early 1970's showed substitutions at various sites of a synthetic isoalloxazine ring (Figure 5) to explore potential modifications and the effect on the spectra produced by the substitutions as a way to investigate observed biological flavin spectroscopic properties. None of the studied modifications resulted in reproductions of the biological spectra. Substitutions at N(3), C(7), and C(8 α) produced only minor changes to the absorption properties. Substitutions at N(1) or N(5) resulted in a loss of 450 and 370nm bands and a new band is found at 550nm with N(5) substitution. No long-wavelength absorption is found when N(1), C(2), C(4), and C(8) are substituted while long wavelength absorbance is observed when C(6) and C(9) are substituted with hydroxyl groups. These however are accompanied by a loss of the typical double-banded system of the unsubstituted flavin, suggesting a drastic alteration of electronic structure. The inability to reproduce spectroscopic characteristics of biological flavins by the aforementioned substitutions in addition to a multitude of others leads to the assumption the changes are likely due to charge-transfer donor-acceptor interactions with small molecules rather than covalent modifications.

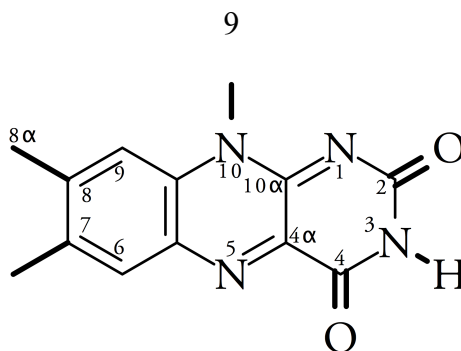


Figure 5: Substitution sites on isoalloxazine rings²¹

Mechanistic Investigations of Electron Bifurcation

Biochemical Analysis with Bcd/EtfAB

An example of a recently investigated bifurcating system is the cytoplasmic butyryl-CoA dehydrogenase/electron transfer flavoprotein (Bcd/EtfAB) in *Clostridium kluyveri*, an anaerobic gram-positive bacterium that produces H₂. It was described as a method of energy conservation in fermentative bacteria²² with further studies supporting a bifurcation mechanism²³. The oxidative portion of the fermentation reaction includes reduction of ferredoxin to (Fd_{ox}) with NADH, an endergonic reaction, to the reduction of crotonyl-CoA with NADH as the exergonic portion of the reaction. It was concluded the hydrogen produced to partial pressures of 10⁵ Pa or higher must be generated from cellular NADH based on intracellular concentrations ten fold higher of NAD⁺ than NADP⁺. *C. kluyveri* contains high concentrations of a 2 [4Fe-4S] cluster containing 6 kDa Fd with mid-point potentials of -320 and -410 mV (Table 1). Additionally, NAD⁺ catalytic efficiency with the aldehyde dehydrogenase is much greater than NADP⁺. Therefore, it stands to reason that the hydrogen produced though this system originates from the oxidation of NADH to NAD⁺ even though the reaction is endergonic at $\Delta G^{\circ} = 20$ kJ/mol and thermodynamically unfavorable under physiological conditions. It was

determined cell extracts performed an acetyl-CoA dependent reduction of Fd via NADH oxidation and production of H₂. This activity was associated by the soluble fraction of cell extracts and did not proceed when acetyl-CoA was substituted with formyl-CoA, monofluoracetyl-CoA, propionyl-CoA, or butyryl-CoA.²³ In confirmation of NADH as the hydrogen source, cell lysates were tested in the absence of NADH and presence of NADPH. No hydrogen production was detected under these conditions.

System	Organism	Operating mid-point reduction potential (mV)		
		Fe-S	FAD	FMN
ferredoxin	<i>Peptococcus aerogenes</i>	2 [4Fe-4S] _{ox} -427	N/A	N/A
HiPIP	<i>Chromatium</i>	[4Fe-4S] _{ox} +356	N/A	N/A
Bcd/EtfAB	<i>Clostridium kluyveri</i>	2 [4Fe-4S] -320 ^{+0/+1} -410 ^{+1/+2} ≤ -500	N/A	N/A
flavodoxin	<i>Acidaminococcus fermentans</i>	N/A	N/A	-60 -430 ≤ -500
Etf _{Me}	<i>Megasphaera elsdenii</i>	N/A	Q/SQ +81 SQ/HQ -136	N/A

Table I. Operating potentials of Fe-S, FAD, and FMN in bifurcating systems. N/A indicates not available.

Subsequent experiments determined that flavodoxin (Fld) could be substituted for Fd in the reaction as the acceptor in the Bcd/EtfAB complex²⁰. The substitution is advantageous in that the single electron reduction of the yellow quinone to the blue

semiquinone form can be observed nearly quantitatively before further reduction to the colorless hydroquinone while the reduced state of Fd is less easily quantified. The Bcd/EtfAB complex was anoxically purified in the presence of FAD and shown to rapidly lose activity when FAD was not added and within several days when stored at 4°. The addition of FMN did not support enzymatic activity when substituted for Fd as observed with FAD. The purified complex was shown to catalyze the reduction of Fd with NADH and crotonyl-CoA. The Bcd/EtfAB complex was shown to catalyze the reduction of crotonyl-CoA (-10 mV) and Fd (-410 mV) through bifurcating electrons from NADH (-320 mV) via the proposed mechanism in Figure 6. It is noted that the reduction from the semiquinone state to the hydroquinone state proceeded 1.4 times faster than the quinone to semiquinone. Presumably this occurs due to the relatively low energy barrier presented by adding an electron to a system with a single unpaired electron versus addition to a closed shell.

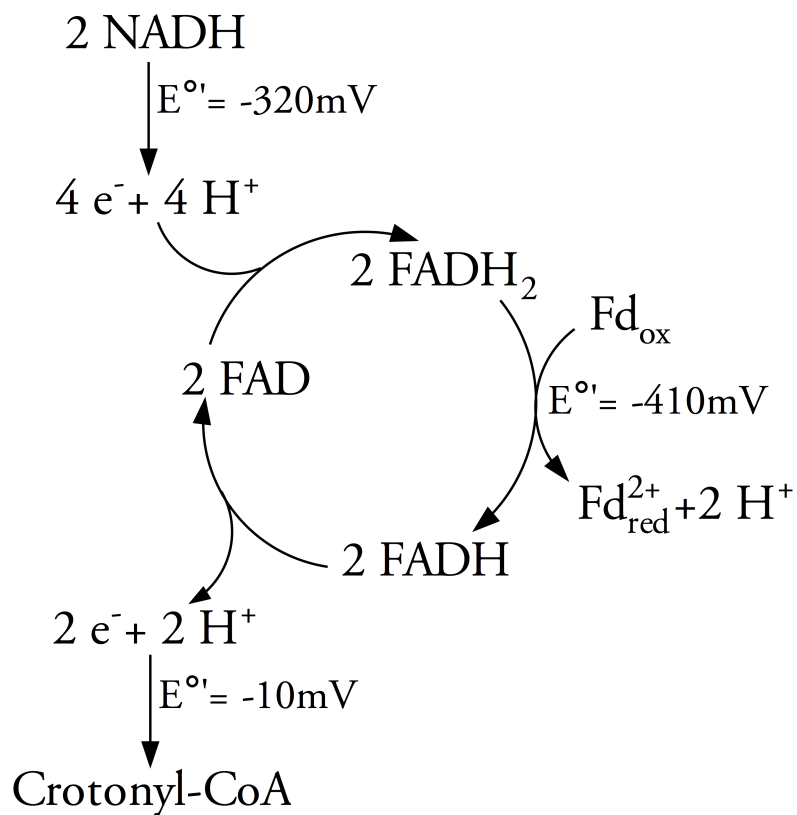


Figure 6: Bcd/EtfAB electron bifurcation mechanism proposed by Li 2008.

Figure 7 depicts a more detailed schematic of electron bifurcation in the Bcd/EtfAB system utilizing data from Fld replaced experiments. Potentials of the Etf FAD as outlined in Table 1 show indications of a significant decrease when the Bcd/EtfAB complex forms.

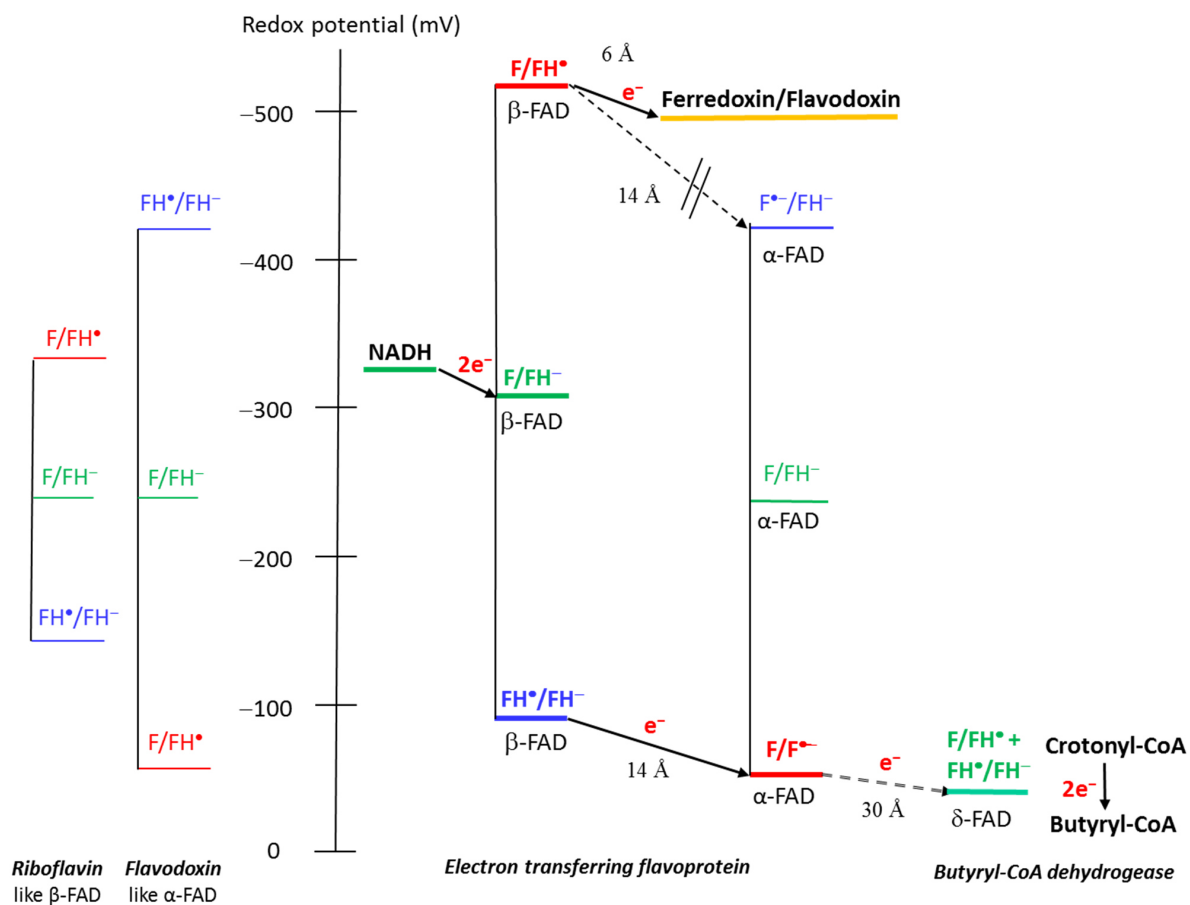


Figure 7: Bcd/EtfAB electron bifurcation scheme proposed by Chowdhury, 2016.

Determination of FixABCX as a Model Bifurcating System

Electron Bifurcation in Nitrogen Fixation

The biochemically characterized bifurcating Etf $\beta\alpha$ systems such as Bcd/Etf balances cellular reductant and ATP pools through coupling the low potential Fd and high potential crotonyl-CoA to NADH oxidation. This system contains two flavins one each in the Etf α and Etf β subunits²⁴ whereas non-bifurcating Etf-QO of the electron transport chain contain a single flavin in Etf α and an AMP in Etf β ^{25,26}. Nitrogen fixation, the reduction of bioavailable dinitrogen to bioavailable reactive nitrogen species, is an

ever-increasing problem as fertilizer demand rises with the population. Cellular growth during nitrogen fixation requires an elegant balance between ATP and reductant pools. As such biological nitrogen fixation is a desirable target for the identification and study of electron bifurcating complexes. In addition to Bcd/Etf another system identified but poorly understood is the *fixABCX* suite, which is up regulated during nitrogen fixation^{27,28} in several diazotrophs. FixABCX is key in the diazotrophic growth in several organisms^{29–34} through electron transfer to nitrogenase^{31,34–36} these select diazotrophs can utilize dinitrogen through biological nitrogen fixation^{37,38}. The expression condition is suggestive of a role in balancing the redox state of the cell through electron bifurcation and indirect association with nitrogenase.

Role of *fixABCX* in Nitrogen Fixation

Fixation of dinitrogen is restricted to prokaryotes and observed within a large number of species within this group including *Azotobacter*, a free-living diazotroph, and members of *Archaeobacteria* and *Eubacteria*³⁹. In addition to *A. vinelandii* as a model organism FixAB in *R. castenholzii* is proposed to be bifurcating through sequence analysis recently done by BETCy collaborators. The *fixABCX* genes in *R. rubrum* are located directly downstream²⁸ of *nifW* while *R. castenholzii* are upstream and on the complimentary strand. The expression of *fixABCX* is increased in both *A. vinelandii* and *R. rubrum* during diazotrophic growth as detected by Northern blot and global transcriptional analysis^{27,28}. FixAB shows sequence homology to the bifurcating Etfβ α suggestive of a two flavin bifurcating system whereas FixCX shows homology to Etf-QO^{29,40,41}. The low potential electron (-420 mV) is putatively transferred to Fe protein

through ferredoxin or FixX (Figure 8) while it's predicted the higher potential electron (+10 mV) is transferred to FixC, a membrane associated protein postulated to associate with quinone of the electron transport chain. Additionally, FixABCX as an important regulator for cellular ATP and reductant pools can be investigated through altering growth conditions in deletion strains. The deletion of most of the *fixABCX* genes (*fix⁻* strain) shows a complete abolishment (*nif⁻*) or defect in the ability to grow diazotrophically in several diazotrophs³⁰⁻³⁴. The *fix⁻* strain utilized in this thesis is a *fixABCX* deletion in *A. vinelandii* whereby the involvement of FixABCX could be investigated under varying growth conditions.

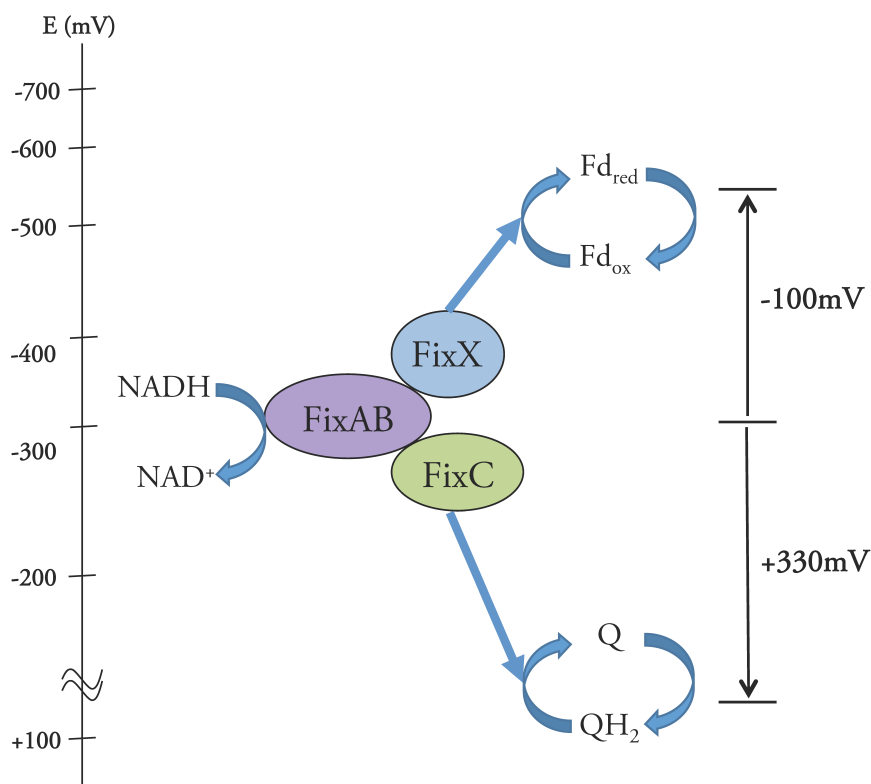


Figure 8: Proposed interactions between the Fix, Nif, and electron transport chain systems.

The Proposed Role of FixX in Electron Bifurcation

In diazotrophs FixX is a protein with some sequence homology to ferredoxins and proposed to contain at least one [4Fe-4S] cluster coordinated by cysteine residues⁴² as suggestive of the sequence similarity to ferredoxin. It has been demonstrated in *Azorhizobium caulinodans* that a mutation in *fixX* abolishes the ability of the organism to grow in the absence of fixed nitrogen (*nif*⁻ phenotype)²⁹. There are many ETFs with similar amino acid sequence motifs and varying ETF function demonstrating that sequence homology is beneficial yet insufficient for determining ETF function⁴³. It is of interest to investigate the biochemical redox potential of FixX to begin supporting the hypothesized roles of FixA, B, C, and X. Coupling the oxidation of NADH (-320 mV) to the reduction of ferredoxin (-420 mV) and quinone (+10 mV) requires electron acceptors with both a highly negative mid-point potential and a much more positive potential within the complex. As FixAB is likely the site of bifurcation, assigning a specific mid-point potential to FixX will support the postulation of FixX acting as the electron donor to Fd/Fld associated with nitrogenase as opposed to quinone. Some demonstration of FixX's importance during nitrogen fixation has been implicated by transcriptional data as evidenced by Hamilton *et al*²⁷ as well as mutational studies^{30,31}. Relative transcript abundance increased 50 fold during alternative nitrogenase (iron only and vanadium) expression and 15 fold during MoFe expressing conditions. In conjunction, the complete loss of diazotrophic growth in a *Bradyrhizobium japonicum* strain³¹ is suggestive of an important role in the reduction of nitrogenase for dinitrogen reduction. It is likely that FixX would show a highly negative mid-point potential in order to act as a reductant for

flavodoxin and/or ferredoxin as those associated with nitrogen fixation are also highly negative. This thesis supports the FixX protein as a redox active Fe-S protein operating at highly negative potentials.

EXPERIMENTAL PROCEDURES

Diazotrophic Growth of *Azotobacter vinelandii* Expressing Alternative Nitrogenases

The *fix⁻* *A. vinelandii* strain was developed and obtained from Amaya Garcia-Costas in the Peters lab. The wild type and mutant strains were propagated on molybdenum free Burke's media plates incubated at 30°C for two days prior to liquid culture inoculation. A 200 mL liquid pre-culture was inoculated with a single loop of cells and allowed to grow until a mid-log OD₆₀₀ shaking at 180 RPM and 30°C. Four separate liquid medias were used for the wild type and *fix⁻* strains; Burke's media (B) to express molybdenum nitrogenase, Burke's media supplemented with nitrogen (BN) for non-diazotrophic growth, Burke's media minus molybdenum (B –Mo) to express the iron only nitrogenase, and Burke's media minus molybdenum supplemented with vanadium (BV) to express vanadium nitrogenase. Media content can be found in the appendix. Mid-log OD₆₀₀ for BV and B –Mo was determined to be 0.2 and 0.4 for BN and B. At the appropriate OD₆₀₀ 25 mL inoculum was added to 250 mL of the same media for the growth curve. Time points were taken at 2 hour intervals for BN and B medias while 4 hour samples were taken for BV and B –Mo. Additionally, the strains were grown in low sucrose (9 mM) Specific growth rate, *k*, was determined by equation 3 where the slope is determined by the logarithmic growth portion of the graphed data with OD₆₀₀ on a base 10 log scale. The generation time, *g*, was determined by equation 4.

$$k = \frac{\text{slope}}{\ln 2} = \text{time}^{-1} \quad (3)$$

$$g = \frac{\ln 2}{k} \quad (4)$$

Development of an Expression and Purification Protocol for *R. castenholzii* FixX

The streptomycin resistant pCDFDuet-1 vector was modified to include a 6x-histidine N-terminal tagged maltose binding protein (MBP) cloned into multiple cloning site I using the restriction sites BamHI and EcoRI for the N and C terminus, respectively. Insertion into this site preserves the vector 6 x histidine tag resulting in a 12 x histidine MBP construct. Subsequently, FixX from *R. castenholzii* was cloned into the modified pCDFDuet-1 vector using the restriction sites EcoRI and HindIII for the N and C terminus, respectively. A alternate version of FixX was also cloned into the MBP modified pCDFDuet-I, which included a C-terminal Strep-Tag sequence. The vector map, protein, and primer sequences are included in the Appendix.

R. castenholzii FixX was expressed in *E. coli* BL21(DE3) cells grown in 2 L flasks with 1 L of volume of LB media with 50 µg/mL streptomycin in a 37°C shaker at 250 RPM until an OD₆₀₀ of 0.4 - 0.5 was reached. These were inoculated with 10% volume of O/N culture grown from a single colony of cells from a fresh transformation. The flasks were then transferred to the bench-top for room temperature induction and media consolidation to 2 L per 2 L flask with the addition of Fe(III) citrate, L-cysteine, sodium fumarate, and isopropyl β-D-1-thiogalactopyranoside (IPTG) as solids to final concentrations of 4 mM, 2 mM, 10 mM, and 1.5 mM, respectively. The flasks are sealed with rubber stoppers with long cannulas bubbling argon into the bottom of the flask and

exhaust tubes flowing into a water trap for an induction period of 4-6 hours. Cells were transferred anaerobically in a Coy chamber (Coy Laboratories) under a nitrogen atmosphere to sealed centrifugation bottles and harvested by centrifugation at 5,000 x G and 4°C for 10 minutes.

Protein was harvested anaerobically according to the following protocol with solutions prepared for either using Strep-Tactin SuperFlow Plus or Amylose resins. Cell pellets were resuspended in 5 mL/gram pellet degassed anaerobic lysis buffer (Strep-Tactin: 50 mM Tris pH 8, 150 mM NaCl, 1 mM NaDT, 5% glycerol, 1 µL/mL supernatant from a super saturated PMSF solution, 1 mg/10 mL DNase, 5 mg/10 mL lysozyme; Amylose: 20 mM Tris pH 8, 150 mM NaCl, 5% glycerol, 1 mM NaDT, 1 mM citrate, 1 uL/mL supernatant from a super saturated PMSF solution, 1 mg/10 mL DNase, 5 mg/10 mL lysozyme) in a Coy chamber under a nitrogen atmosphere for lysis in a cell bomb (Parr Bomb Instrument Co., Moline, IL). The pressure was slowly increased to 1500 PSI and allowed to equilibrate for 30 minutes before slowly collecting the lysate. This process was repeated before harvesting the cleared lysate anaerobically at 18,000 x G and 4°C for 20 minutes. FixX was purified from the cleared lysate using a single step affinity column purification using either Amylose (New England BioLabs) or Strep-Tactin Superflow Plus (Qiagen) resins according to the FixX construct expressed. All purification steps were performed anaerobically using degassed buffers kept under positive pressure with nitrogen or argon. Columns were equilibrated with wash buffer (Strep-Tactin: 50 mM Tris pH 8, 150 mM NaCl, 1 mM NaDT; Amylose: 20 mM Tris pH 8, 150 mM NaCl, 1 mM NaDT, 1 mM citrate) before loading the cleared lysate and

checked for reducing conditions with a 5 mM methyl viologen solution. The column was again washed until the baseline absorbance returned. The was eluted off the column with elution buffer (Strep-Tactin: 50 mM Tris pH 8, 150 mM NaCl, 1 mM NaDT, 2.5 mM D-desthiobiotin; Amylose: 20 mM Tris pH 8, 150 mM NaCl, 1 mM NaDT, 1 mM citrate, 10 mM maltose) as a brown band, was collected anaerobically under an argon atmosphere, and snap frozen with liquid nitrogen for further analysis. Purification columns were washed and regenerated according to their respective standard protocols.

Protein Gel Electrophoresis and Western Blot

Side by side standard SDS-PAGE and western blot protocols were performed to confirm the presence of the FixX protein and determine relative purity. Samples of cell pellets consisted of 1 mL of culture with the addition of 100 μ L 2 x SDS Lamelli sample buffer while liquid samples consisted of equal amounts sample buffer and sample. Samples were prepared and loaded on a 4-20% gradient Protean Mini Pre-cast gel (BioRad). For equivalent loading of cleared lysate and remaining solid cell debris, resuspend cell debris in buffer to the volume of the recovered cleared lysate. The gels should be run simultaneously in duplicate for direct comparison of results with settings of 25 V for 1.5 hours. Continuation of the western blot protocol should immediately follow completion of running the gel(s).

Procedure for Drying Gels

Polyacrylamide gels were dried according to the following procedure⁴⁴.

Equilibrate in a 25% ethanol and 3% glycerol solution for 1 hour. Cellophane sheets were wet with dH₂O. The gel was sandwiched between the cellophane on the gel drying frame with a few milliliters of the equilibration solution. Avoid air bubbles in the system, clip the frame together, and set to dry overnight allowing for air flow above and below the rack. Rehydration can be done by incubating the gel in ethanol followed by dilution with an equal volume of dH₂O over 18 hours.

Determination of Protein Iron Content

The iron content of FixX was determined using the following protocol^{45,46}. Samples were prepared to 1 mL final volume in triplicate. Standards were prepared by diluting a working standard stock solution of 1000 ppm Fe AA standard (Ricca Chemical Co.) to 10 ppm or 10 µg/mL and preparing 0, 40, 80, 120, 160, and 200 µg solutions to 1 mL. Each sample was incubated at 65°C for 2 hours after the addition of 500 µL reagent A (equal volumes 4.5% (w/v) KMnO₄ and 1.2 M HCl). A second incubation at room temperature for 30 minutes was performed after the addition of 100 µL reagent B (4.85 g NH₄ acetate and 4.4 g ascorbic acid dissolved in 5 mL dH₂O to which 40 mg neocuproine dissolved in 50 µL methanol and 40 mg ferrozine dissolved in 1 mL dH₂O are added to a final volume of 12.5 mL). Finally, the absorbance was read at 562 nm. Iron content of the protein samples were calculated based on the generated standard curve.

MBP Cleavage

The cleavage reaction buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM citrate, 5

mM β -mercaptoethanol) was degassed before the addition of the tobacco etch virus (TEV) protease. After TEV addition to a 1:200 TEV:FixX ratio the mixture was degassed again before addition of *R. castenholzii* MBP-FixX. The cleavage reaction was allowed to proceed at 4°C over night.

Fe-S Cluster Reconstitution

The reconstitution of Fe-S clusters into *R. castenholzii* FixX proteins were preformed according to the following protocol⁴⁵. Reconstitution buffer (50 mM Tris pH 8.3) and β -mercaptoethanol are degassed and brought into a Coy anaerobic chamber (Coy laboratories) under a nitrogen atmosphere along with solid $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ and $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$. To a final concentration of 4% β -mercaptoethanol is added to protein solution (1-100 μM final concentration). Reconstitution buffer is added to the solids. All vials are sealed and transferred to 4°C. With a syringe $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ is added slowly with stirring to a final concentration of 800 μM . The $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ is then added slowly with stirring to a final concentration of 800 μM . The vial septa is then covered with parafilm followed by foil and allowed to incubate at 4°C without stirring overnight. The reaction mixture is then brought into a Coy anaerobic chamber under a nitrogen atmosphere to be concentrated to 2.5 mL and passed over a PD-10 desalting column equilibrated with Tris buffer (50 mM pH 8.3). The protein is then eluted and concentrated before flash freezing with liquid nitrogen.

Spectroscopy

UV-visible

Utilize 100 μM final protein concentration to ensure a visibly brown solution and strong absorbance readings. Under a nitrogen atmosphere transfer the protein solution into a sealed cuvette. Utilize a Cary60 spectrophotometer to scan from 300-800 nm with peaks centered around 420 nm and 560 nm corresponding to [4Fe-4S] and [2Fe-2S] clusters, respectively.

Electron Paramagnetic Resonance of FixX

EPR parameters utilized were typical X-band (9.39 GHz, attenuation 23 dB, microwave power 1 mW) at temperatures controlled by a liquid helium cryostat controller. Spectra were recorded with a Bruker ELEXSYS E500 continuous wave X-band spectrometer. Sample concentrations varied from 200 μM to 600 μM . Potentiometric titrations were preformed under strict anaerobic conditions in an Mbraun box under a nitrogen atmosphere. Samples were prepared by initial desalting with a PD-10 to remove NaDT. Samples were reduced by titrating NaDT into the stirred protein solution containing redox mediator cocktail (10% glycerol, 100 mM Tris pH 8, 5 μM indigo carmine, 5 μM methyl viologen, 5 μM benzyl viologen, 5 μM phenosafranin). Analysis of the data utilized the MatLab software (The MathWorks, Inc., Natick, Massachusetts) running the EasySpin script package (Stefan Stoll, Department of Chemistry, University of Washington).

RESULTS AND DISCUSSION

FixABCX in Nitrogen Fixation

Several target organisms have been investigated as potential models for studying the FixABCX system. Some success has been made with *in vivo* mutational studies showing the loss of ability to grow diazotrophically when all or portions of the *fix* system are deleted from the genome³⁰⁻³⁴. Comparative growth curves of *A. vinelandii* wild type and *fix* deletion strains were done to determine if the *fix* system deletion would inhibit diazotrophic growth. Figure 9 depicts the growth of the strains simultaneously non-diazotrophically (WT, *fix*⁻ g(hr) = 3.23, 2.76, single experiment) and diazotrophically expressing vanadium nitrogenase (WT, *fix*⁻ g(hr) = 6.38, 6.72, single experiment), molybdenum nitrogenase (WT, *fix*⁻ g(hr) = 2.66, 2.98, triplicate experiment), and iron only nitrogenase (WT, *fix*⁻ g(hr) = 5.22, 5.89, triplicate experiment). It was determined there were no detrimental effects to the generation time when grown non-diazotrophically as observed in pre-growth curve cultures and trends from previous experiments. The *fix* deletion strain shows a minor increase in generation time under diazotrophic growth conditions suggesting the *A. vinelandii* genome encodes for a system that compensates for *fix* unlike *B. japonicum*, *A. caulinodans* and others³⁰⁻³⁴. Thus far attention has been given to the FixAB proteins as they've been successfully purified for use in preliminary assays. The novel FixX has been mostly overlooked as it has proven to be a challenge to express as occupied or be reconstituted with Fe-S clusters.

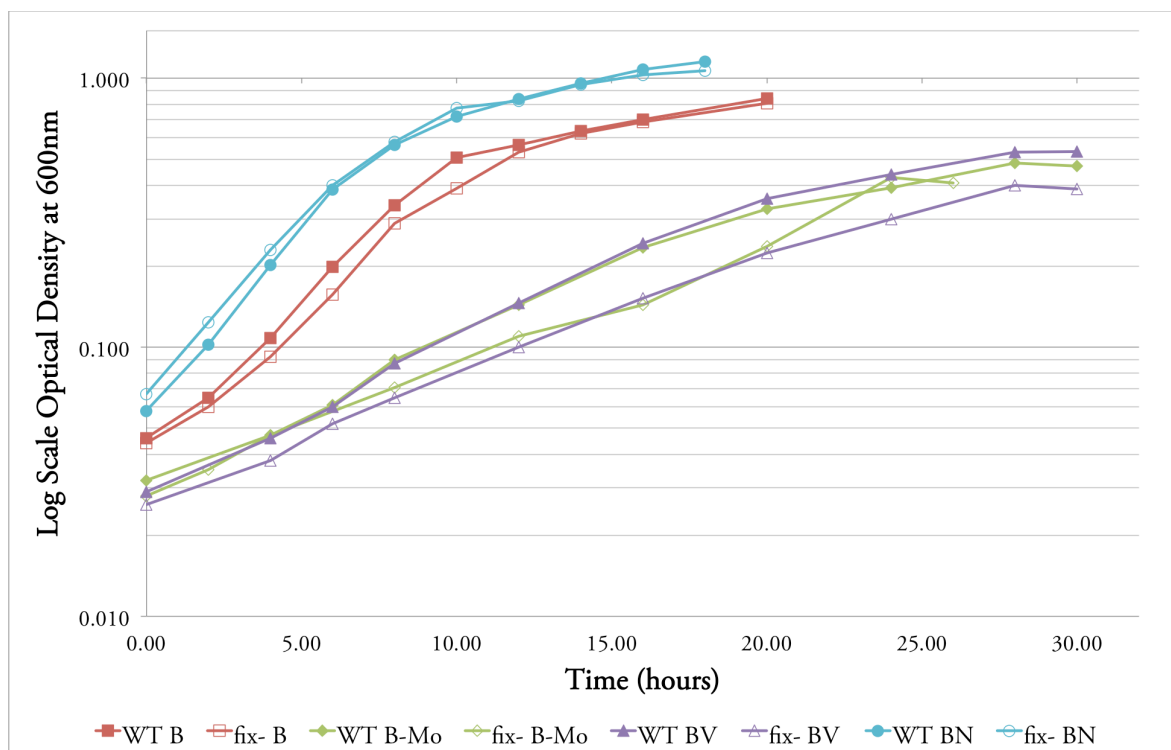


Figure 9: Growth curves of *A. vinelandii* wild type and *fix*⁻ strains grown non-diazotrophically and diazotrophically expressing VeFe, MoFe, and FeFe nitrogenases.

The evidence gathered proved to be inconclusive in *A. vinelandii* as other systems such as *rnf* as in *Rhodobacter capsulatus*⁴⁷ could compensate for the lack of *fix* resulting in a lack of phenotype sufficient for investigation while other systems such as *R. rubrum*⁴⁸ with severe phenotypes make in-depth investigations more challenging. *In vitro* studies of individual proteins are in progress throughout the BETCy center. FixX is a small, novel, Fe-S occupied protein in the *fixABCX* operon that appears to be sensitive to oxygen exposure. It was observed while working with initial preparations of *R. castenholzii* FixX that a loss of protein concentration could be detected by NanoDrop spectrophotometry with a concomitant decrease in visible brown coloration and increase in visible precipitate in the protein solution. Those that have been attempted and

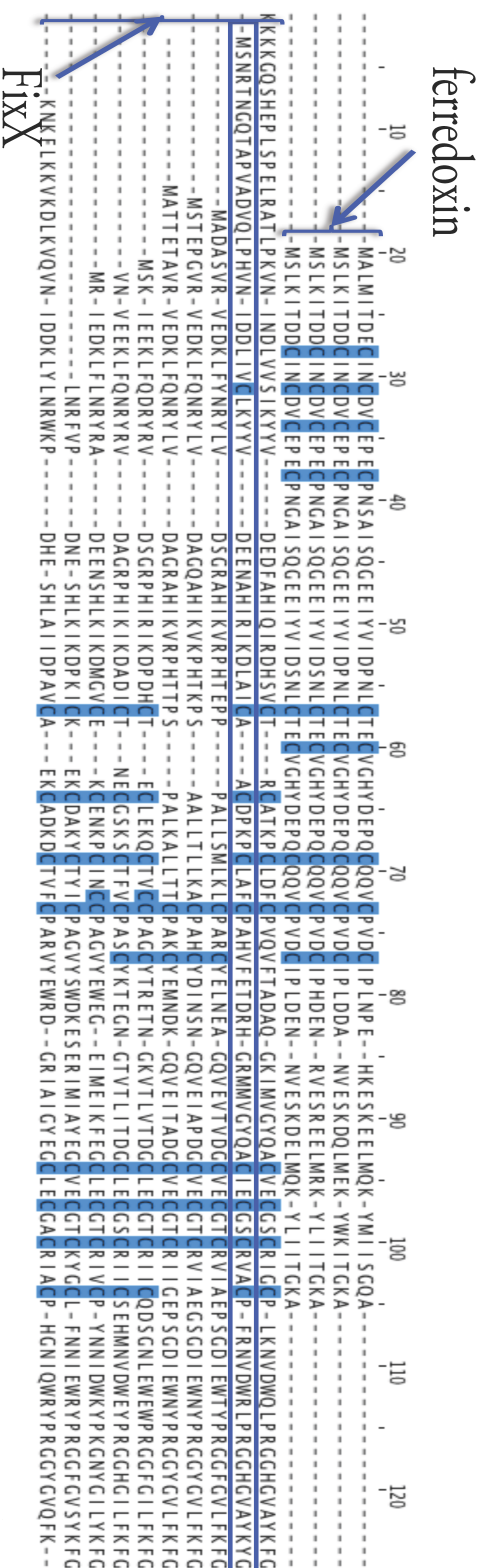
produced insoluble, unoccupied, or non-reconstitutable protein include *R. rubrum* and *A. vinelandii*. We then investigated heterologous expression of FixX in *R. castenholzii*, a filamentous anoxygenic thermophilic phototroph with gliding motility^{49,50}. This versatile bug can grow either photoheterotrophically anaerobically, chemotrophically aerobically, and potentially photoautotrophically by fixing CO₂ through the 3-hydroxypropionate pathway⁵¹ as observed in other purple non-sulfur bacteria such as *Chloroflexus aurantiacus*. *R. castenholzii* was selected as a target for the study of this bifurcating system due to its ability to grow in the conditions under investigation as well as the potential for producing proteins with a higher stability than *A. vinelandii* or *R. rubrum* due impart to its thermophilic nature. Through the BETCy collaboration we have attempted heterologous expression of *A. vinelandii* FixX, which produced insoluble unoccupied protein. Solubility was obtained through the fusion of maltose binding protein to the N-terminus of FixX. Reconstitution experiments were performed according to established protocols^{52–54} on apo-FixX by BETCy collaborators unsuccessfully. Attempts to obtain soluble and Fe-S occupied from organisms, which are likely to produce highly stable proteins lead to the decision to use *R. castenholzii* as a model organism for FixX. We have since successfully cloned, over-expressed, and purified soluble protein confirmed by SDS-PAGE and Western Blot. The protein is associated with iron as would be predicted with an Fe-S cluster protein. Two constructs are currently available for study both are fused to maltose binding protein (MBP) with a MBP N-terminal His-tag and a Nuclear Inclusion a (NIa) protease site from tobacco etch virus (TEV) for cleavage of MBP. One construct contains a FixX C-terminal streptavidin tag

(WSHPQFEK), which was utilized in several studies outlined below. These constructs are expressed using the *E. coli* BL21-DE3 expression system grown as previously described. The MBP-FixX and MBP-FixX-Strep proteins run true to size on SDS-PAGE gels at 56.5 kDa and 57.5 kDa, respectively.

Elucidation of Fe-S in FixX

A sequence alignment between several ferredoxin and FixX (Figure 10) protein sequences reveals a strong homology in the [4Fe-4S] binding region supporting the presumption that FixX also contains this cluster type. Figure 3 highlights the sequence of *R. castenholzii* FixX (blue box) in comparison to ferredoxins and FixX sequences from other organisms. The cysteine residues are also highlighted in blue. The analysis shows the ferredoxins and FixX proteins to both have a conserved cysteine motif consistent with Fe-S cluster redox active proteins. The FixX sequences are highly conserved in the C-terminal region, which does not appear in ferredoxin. Additionally, in ferredoxin there is a set of four highly conserved cysteines not present in FixX while FixX shows a similar set in the C-terminal region of the protein. In *Rhodobacter capsulatus* a [4Fe-4S] cluster is coordinated by CX₂CX₂CX₃C motif⁴⁷ as well as a [2Fe-2S] coordinated by a CX₄CX₂C motif⁵⁵ in another protein. Given the nine cysteine residues in a CX₁₉CX₂CX₄CX₃CX₁₉CX₂CX₂CX₃C it is reasonable to assume one or more clusters would be able to be coordinated. These differences suggest each protein serves a unique physiological function. Determination of the Fe-S architecture in *R. castenholzii* FixX will provide insights of its role during electron bifurcation. The architecture of each type

of iron sulfur cluster affects the potentials at which the cluster is oxidized or reduced additionally, the coordinating ligands of each cluster help to tune the redox properties of the protein. The *fixX* cysteine architecture of *R. castenholzii* is suggestive of at least one Fe-S cluster via sequence analysis.



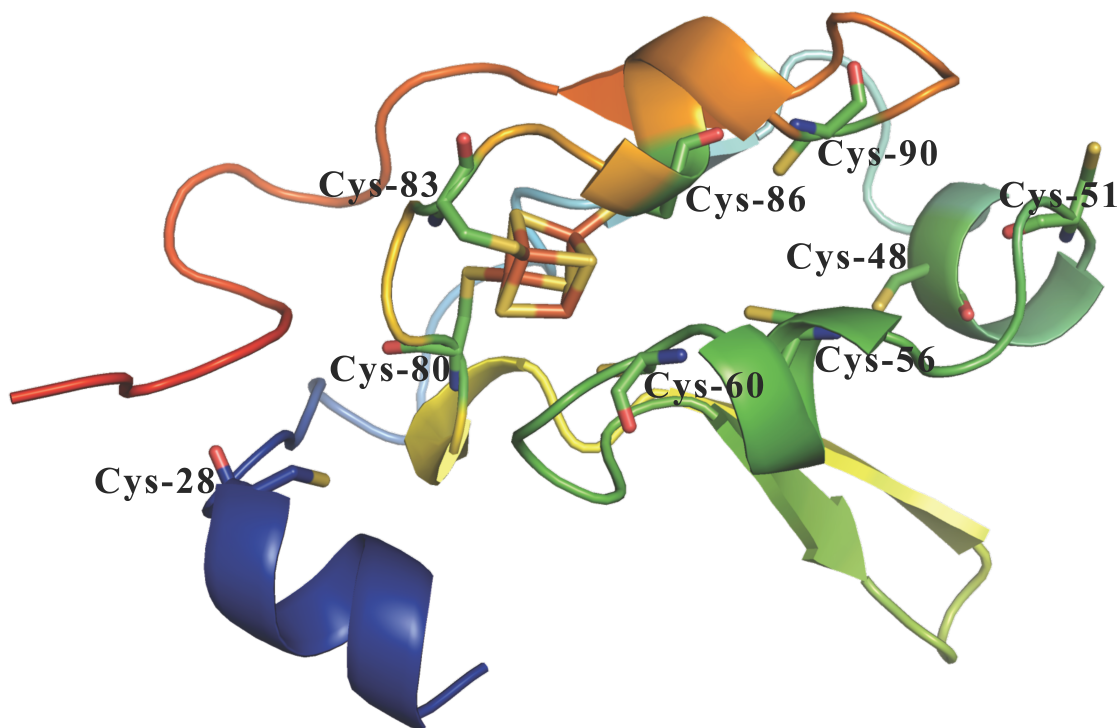


Figure 11: Homology model of *R. castenholzii* FixX based on ferredoxin homology depicting the potential for Fe-S coordination. Figure generated using PyMol.

SWISS-Model results state 30% sequence identity with ETF ubiquinone oxidoreductase from which Figure 11 was generated and analyzed in PyMol using the SWISS-Model homology .pdb file. The homology structure shows a single [4Fe-4S] cluster (orange and yellow stick model) coordinated by cysteine residues. This model also reveals a cleft lined with several cysteine residues where potentially a second [4Fe-4S] cluster could be coordinated.

Colorimetric iron assays^{45,46} were performed as described and optimized for the FixX protein. As purified samples show stoichiometric amounts of iron to protein of 4:1, respectively while reconstituted protein pre-MBP cleavage show ratios of 6:1. Potassium permanganate serves as the colorimetric indicator as an initial assay for iron to protein

stoichiometry^{45,46}. The protein preps have varied from 2-5 Fe : 1 FixX with each protein preparation. The variability may be due by the apparent oxygen sensitivity of FixX or indicative of highly labile clusters.

Reconstitution of FixX was done using an established protocol⁴⁵ as previously described and performed under strict anaerobic conditions. Purified MBP-FixX-Strep was used for reconstitution and determined to contain 2Fe : 1FixX as purified (Figure 12). Color changes were observed with the addition of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ to a dark reddish and dark brown with the addition of Na_2S . Post-reconstitution Fe content was determined in parallel with the as purified sample to be 6 Fe : 1 FixX (Figure 12).

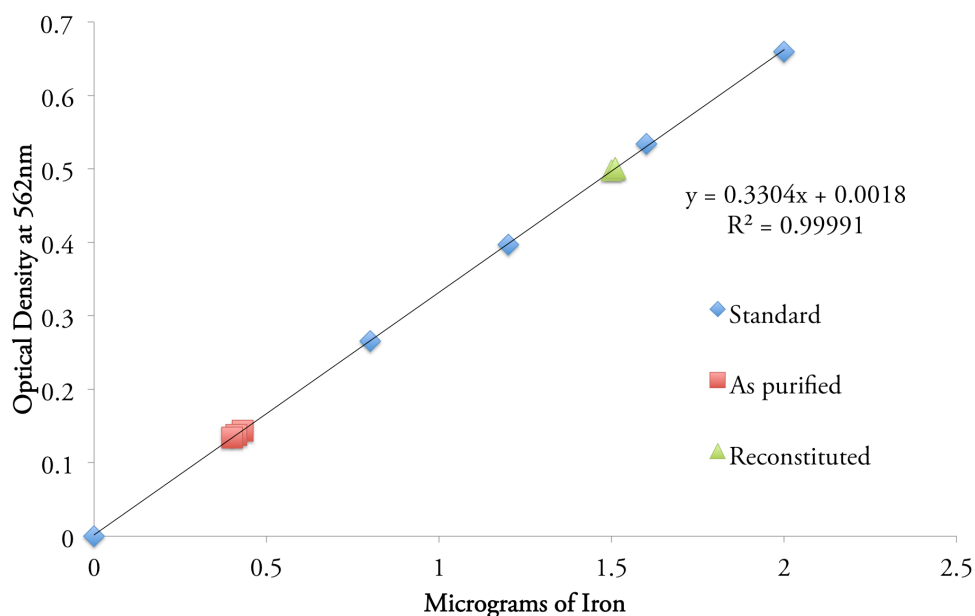


Figure 12: Colorimetric iron assay of *R. castenholzii* MBP-FixX-Strep protein as purified and reconstituted.

Additionally, Fe-S cluster architecture can be investigated through UV-visible spectroscopy as each cluster has a characteristic maximum absorbance wavelength influenced by the ligand to metal coordination¹⁶. Peaks at 420 nm correspond to [4Fe-4S]⁵⁴ and 550 nm to [2Fe-2S]⁵⁶ clusters. This experiment was performed on a protein preparation purified anaerobically using Strep-Tactin Superflow Plus column resin. Figure 13 shows an increase in absorbance around 420 nm as predicted for [4Fe-4S] cluster content for both the as purified (A) and reconstituted (B) protein. Additionally, the spectrum shows a broad absorbance at 550 nm. This could be indicative of a functional [2Fe-2S] cluster, an immature [4Fe-4S] cluster, or a partially degraded [4Fe-4S] cluster.

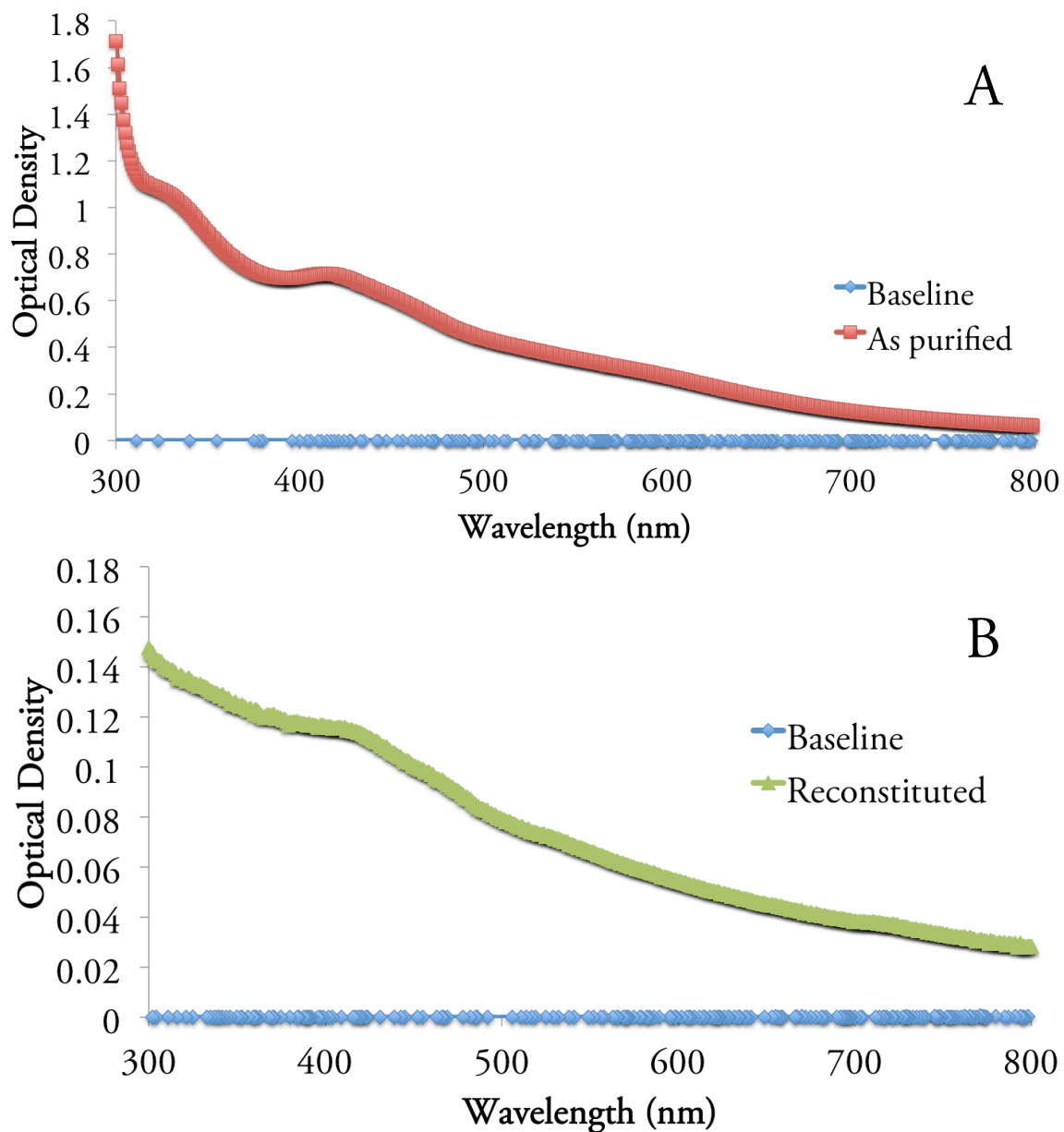


Figure 13: UV-visible spectra of *R. castenholzii* MBP-FixX-Strep fusion protein as purified measured at 100 μ M (A) and reconstituted measured at 50 μ M (B). Wavelengths were scanned using a Cary 60 spectrophotometer from 300 nm to 800 nm. Absorbance at 420 nm indicates [4Fe-4S] cluster content while 550 nm indicates [2Fe-2S] cluster content.

Initial Crystallization Trials of FixX

FixX has yet to be crystallized with attempts ongoing and progress to be reported herein. Optimization of identified crystallization conditions will follow. Additionally, identified key components of the *fix* and *nif* systems will be co-crystallized to complement electron transfer data. Initial crystallization screens were performed using *A. vinelandii* apo-MBP-FixX (22 mg/mL) provided by R. Ledbetter, USU with the Hampton crystallization screen set up aerobically. This screen produced numerous slight brown needle clusters (Figure 14) indicative of Fe-S cluster protein crystals. Initial hits were reproduced anaerobically in a Coy chamber utilizing degassed crystallization solutions with the hanging drop vapor diffusion method in 4 μ L drops with a 1:1 protein:reservoir solution ratio at room temperature. The crystals were reproducible and formed within a week however they subsequently precipitated or dissolved. As this proved unsuccessful attempts turned to an alternative crystallization approach, capillary batch diffusion⁵⁷. The initial screen utilized for this method was the Hampton cryo set up in an Mbraun box in a nitrogen atmosphere with *R. castenholzii* MBP-FixX (19 mg/mL) and MBP-FixX-Strep (28 mg/mL). The formation and subsequent precipitation of crystals was observed for both *A. vinelandii* apo-MBP-FixX as well as *R. castenholzii* holo-MBP-FixX and holo-MBP-FixX-Strep for all crystallization attempts and methods. It is possible the MBP fusion protein interferes with the stability of the protein crystals.



Figure 14: Initial crystals of *A. vinelandii* apo-MBP-FixX at 19°C with 100 mM HEPES pH 7.5, 15% isopropanol, 20 mM MgCl₂ (left) and 100 mM HEPES pH 7.5, 17% isopropanol, 20mM MgCl₂ (right) representative of observed crystal forms for both *A. vinelandii* and *R. castenholzii*.

MBP cleavage was performed to perform crystallization trials without the potential interference of the solubilizing protein. The protein remained soluble post-TEV protease cleavage removing the MBP fusion protein (Figure 15). Optimization experiments were performed and resulted in approximately 90% cleavage efficiency and appears by visual inspection to retain Fe-S occupancy.

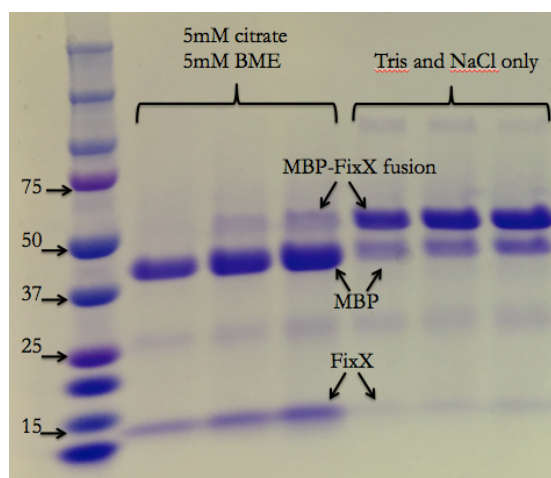


Figure 15: SDS-PAGE gel showing two buffer conditions and successful MBP cleavage.

The Biochemical Oxidation Reduction Potential of FixX

Electron paramagnetic resonance (EPR) spectroscopy ideally provides unique spectra for each type of iron sulfur cluster so long as these clusters have unpaired electrons and so are EPR active. Reduction of ferredoxin and Fe protein requires substantially more negative potentials (-420 mV), while more positive (+10 mV) reduction potentials are likely to reduce quinones in the electron transport chain. These spectra will provide a picture of the iron sulfur cluster type in FixX, oxidation state, and the potentials at which the protein can be poised. Elucidation of the potentials at which FixX can be poised through redox titrations and EPR analysis will help direct physiological function investigations. This technique will be used to answer two separate questions; what is the Fe-S cluster architecture of FixX and what range of potentials does FixX operate. Initial EPR experiments were performed as previously described. The data suggests a system of 2 [4Fe-4S] clusters one with axial symmetry growing in at lower potentials with the associated g values of 2.04, 1.94, 1.94 with a second rhombic signal associated with g values of 2.07, 1.94, 1.90. The strong isotropic signal observed in the titration is likely a radial signal due to mediators with a g value of 2.00. These conclusions are supported by the temperature dependence as both signals appear to have a maximum signal intensity at 15 K classic of [4Fe-4S] clusters. Two distinct [4Fe-4S] clusters are additionally supported though the resolution of the peaks between the increased 30 mM NaDT at pH 10 versus 5 mM NaDT at pH 8 as well as preliminary EasySpin simulations (See appendix). These simulations continue to be refined and show a reasonable fit for three distinct systems with g values directly corresponding to the

isotropic mediator signal and the low potential [4Fe-4S] with the second not yet fully refined.

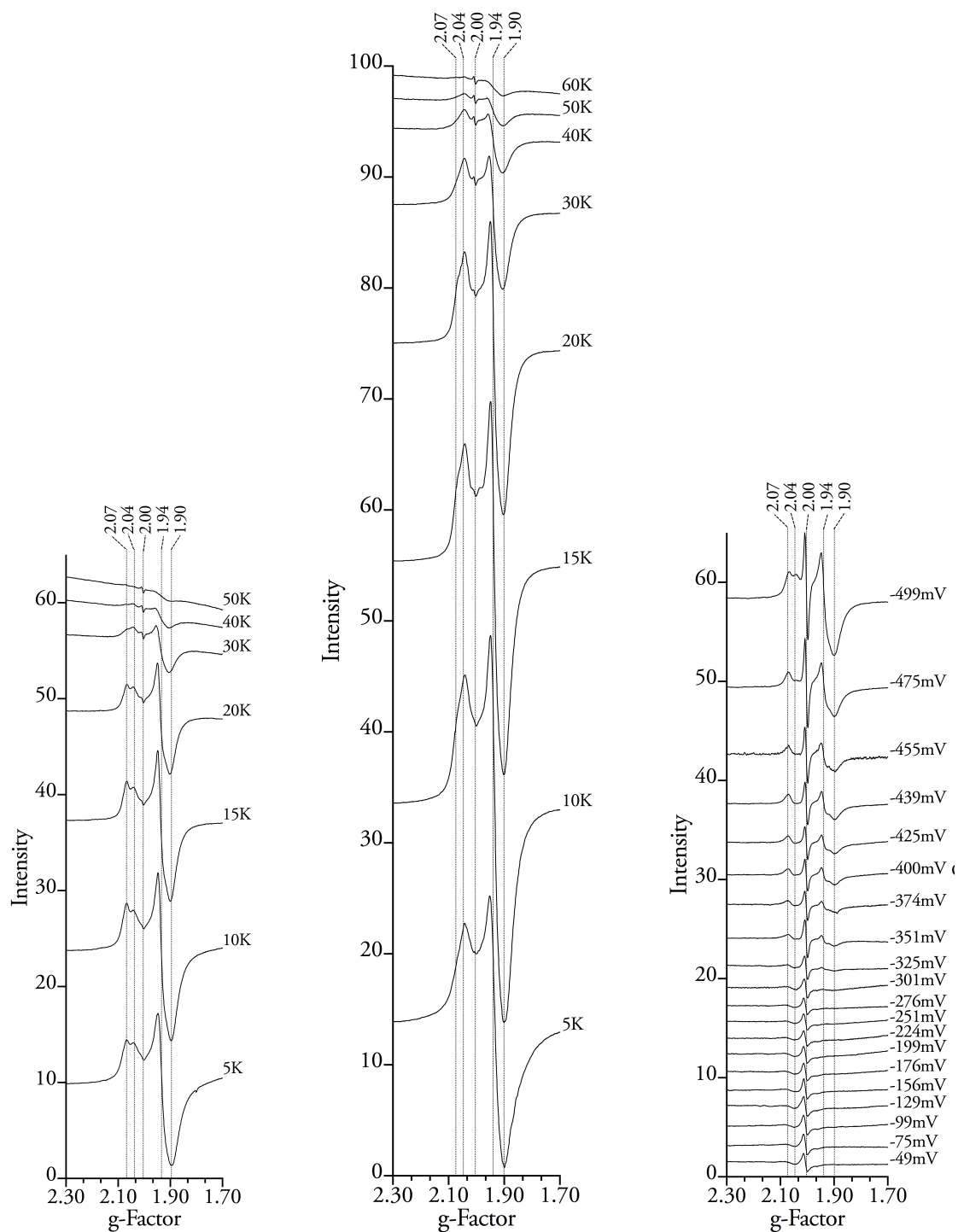


Figure 16: Temperature relaxation profiles of *R. castenholzii* MBP-FixX-Strep 200 μ M at pH 8 with 5 mM NaDT (left), 600 μ M at pH 10 with 30 mM NaDT (middle), and a potentiometric titration at pH 8 (right).

Progress Toward Fe-S Coordinating Ligands in FixX

The sulfur atom within cysteine is the main ligand for Fe-S clusters across all domains of life. With this knowledge, the observed CX₁₉CX₂CX₄CX₃CX₁₉CX₂CX₂CX₃C motif in the sequence, and the iron content of purified protein it is reasonable to assume cysteines are coordinating an Fe-S cluster. To tease apart the involvement of each of the nine cysteine residues, systematic mutagenesis of each to an alanine is in progress (Figure 16). Primers for each individual cysteine to alanine substitution have been designed (see appendix). The mutation to alanine will preserve the geometry of the peptide chain while removing the sulfur atom, which is needed for iron coordination. Mutations resulting in protein expressed with a decreased iron content are likely involved in the coordination of the cluster. It will be more difficult to determine the involvement of any mutations that result in precipitated protein as the protein also precipitates when exposed to oxygen. The residue could also be involved in a disulfide bond and is stabilizing the protein in its thermophilic environment. Any mutations resulting in precipitated protein can be considered critical for protein folding. In the event of ambiguity between iron ligation and disulfide bonding structural data from crystallography work can be used to define the role of the residue.

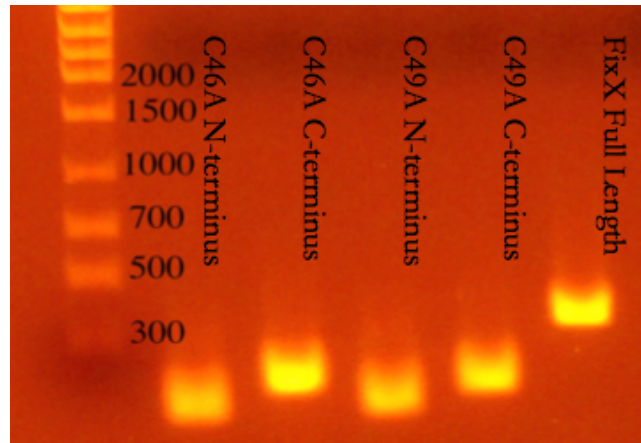


Figure 17: PCR generated DNA fragments for *R. castenholzii* FixX cysteine mutagenesis.

CONCLUDING REMARKS

Possible growth defects of a *fix⁻* *A. vinelandii* strain were investigated with regard to non-diazotrophic growth and diazotrophic growth expressing each of the alternative nitrogenases (VFe, MoFe, and FeFe). No significant phenotype was observed under the experimental conditions and concluded that the *fix* system is unlikely to be the only utilized by *A. vinelandii*. To further investigate FixABCX a method for the overexpression and reconstitution of *R. castenholzii* FixX was developed which allowed for preparation of the protein for biochemical assays. Amino acid sequence homology with ferredoxin suggested a potential for the coordination of 2 [4Fe-4S] clusters. This was further investigated through EPR temperature relaxation profiles and a potentiometric titration. The temperature relaxation profiles allowed for the differentiation between two distinct clusters both of which having a maximum signal intensity around 15K consistent with [4Fe-4S] clusters, while the potentiometric titration shows both of these potential clusters to have highly negative mid-point potentials. With this evidence, it is likely that FixX in *R. castenholzii* is the low potential electron acceptor in the FixABCX system.

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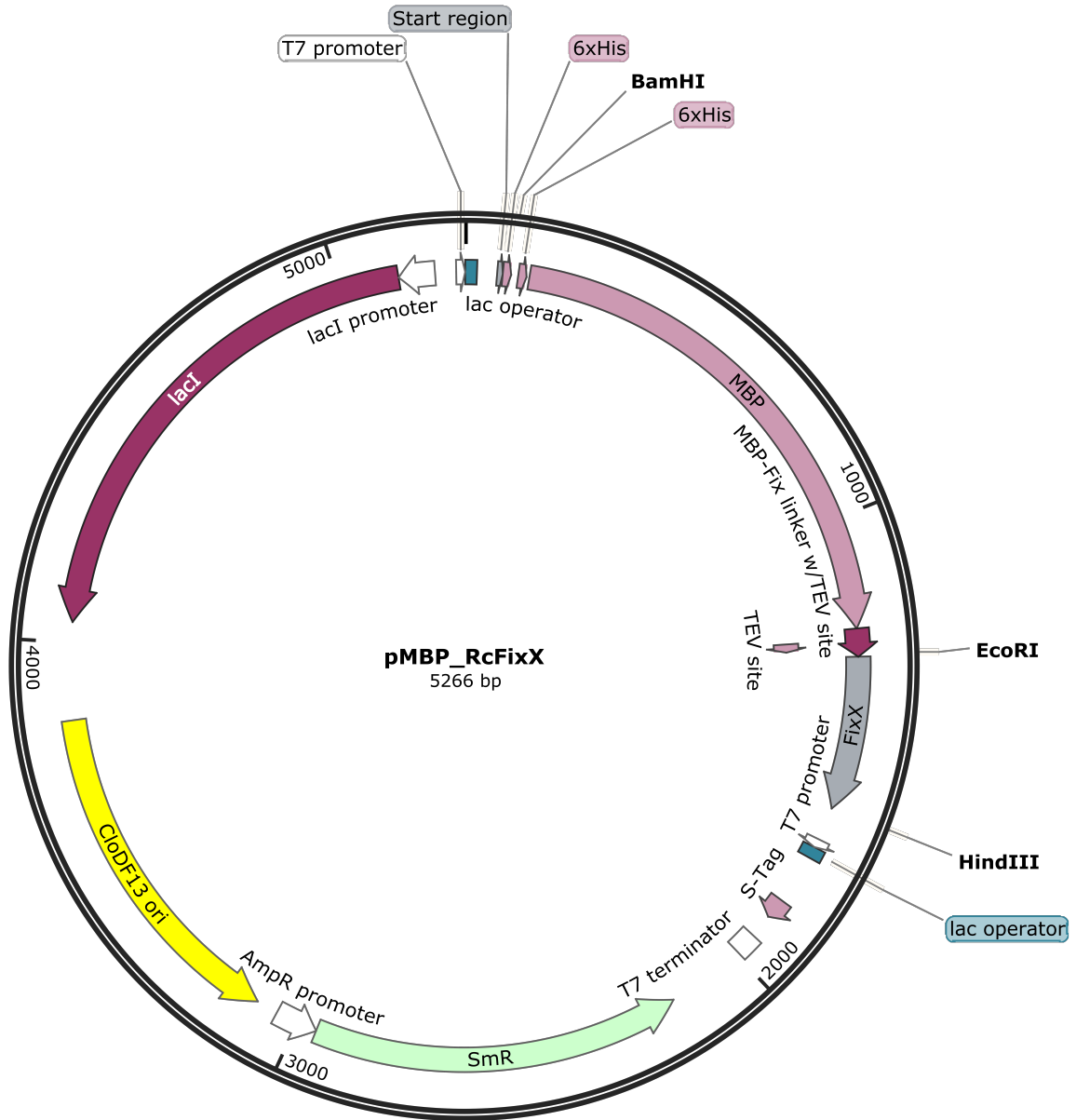
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APPENDIX A

SUPPLEMENTAL MATERIALS

Roseiflexus castenholzii FixX Vector Map

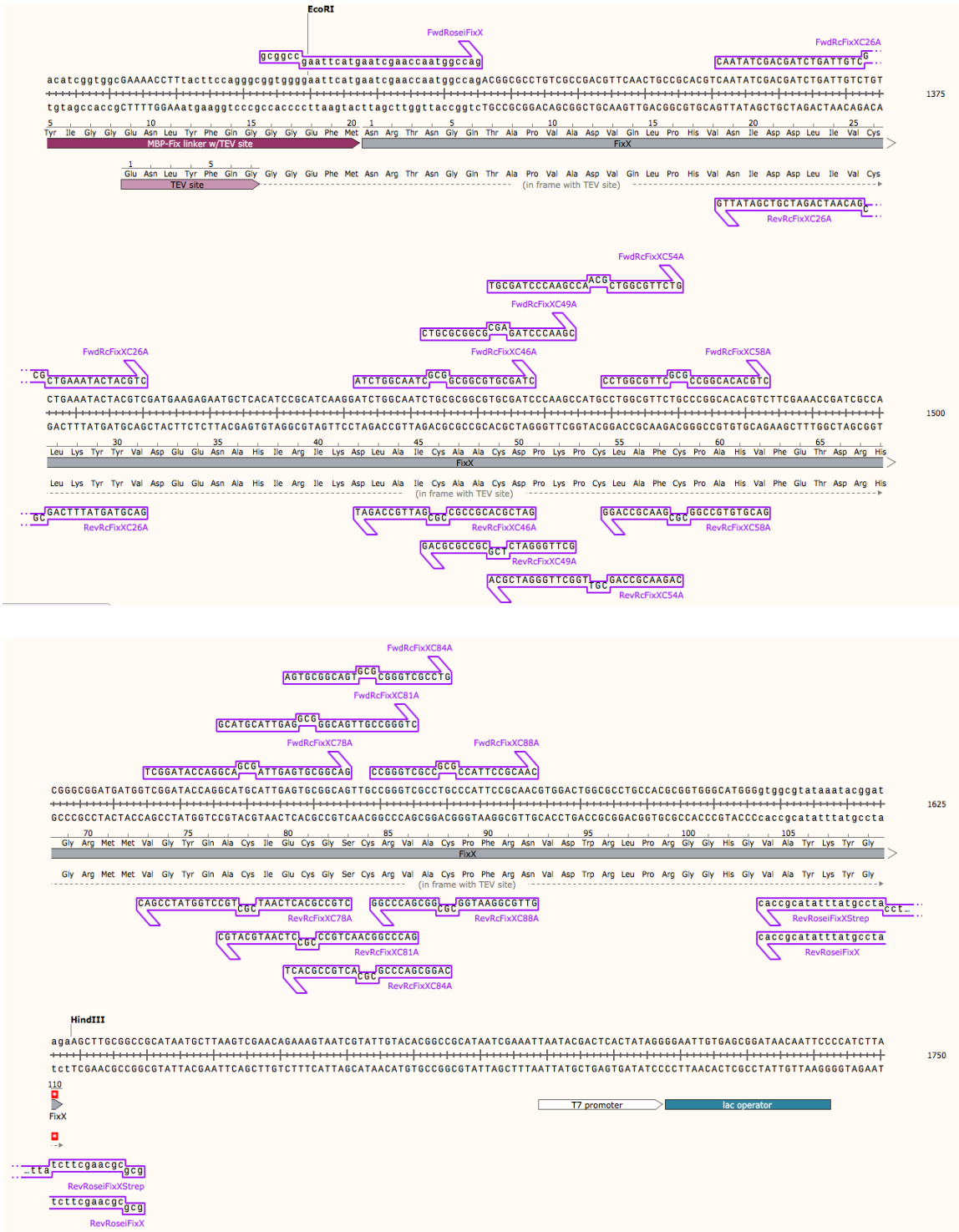
Created with SnapGene®



*Generated with SnapGene software (from GSL Biotech; available at [snapgene.com](https://www.snapgene.com))

Roseiflexus castenholzii Primers

Name	T _m (°C)	Sequence
FwdRcFixXC26A	56	caatatcgacgatctgattgtcgcgctgaaatactacgtc
FwdRcFixXC46A	58	atctggcaatcgcgccggcgtgcgac
FwdRcFixXC49A	61	ctgcgcggcgcgagatcccaagc
FwdRcFixXC54A	59	tgcgatcccaagccaacgctggcgttctg
FwdRcFixXC58A	57	cctggcggttcgcgccggcacacgtc
FwdRcFixXC78A	58	tcggataaccaggcagcgattgagtgccggcag
FwdRcFixXC81A	59	gcatgcattgaggcgggcagttgccgggtc
FwdRcFixXC84A	58	agtgcggcagtcgcggggtcgctg
FwdRcFixXC88A	57	ccgggtcgccgcgccattccgcaac
FwdRoseiFixX	58	gcggccgaattcatgaatcgaaccaatggccag
RevRcFixXC26A	55	gacgtagtagtattcagcgcgacaatcagatcgatgatattg
RevRcFixXC46A	58	gatcgacgcgcgcgcgattgccagat
RevRcFixXC49A	61	gcttgggatctcgcgccgcgcag
RevRcFixXC54A	57	cagaacgccagcgttggcttgggatcgca
RevRcFixXC58A	57	gacgtgtgccggcgcggaacgccagg
RevRcFixXC78A	58	ctgccgcactcaatcgctgcctggtatccgac
RevRcFixXC81A	59	gacccggcaactgcccgcctcaatgcatgc
RevRcFixXC84A	58	caggcgacccgcgcactgccgcact
RevRcFixXC88A	57	gttgcggaatggcgccggcgacccgg
RevRoseiFixX	61	gcgcgcaagcttctatccgtatttatacgccac
RevRoseiFixXStrep	54	gcgcgcaagcttctatttttgaactgcgggtggctccatccgtatttatacgccac
6xHisMBPTEVFwd		gcggccggatcccatgcatcaccatcaccatcacggt
6xHisMBPTEVRev		gcgcgccttaagcccaccgccttgaagta



MBP and FixX Sequences6xHis tag Sequence

CATCACCATCACCATCAC

Translation

H H H H H H

Strep tag Sequence

TGGAGCCACCCGCAGTTCGAAAAA
ACCAGCCTGGGCGTCAAGTTTTT
AAAAACTTGACGCCCAGGCTGGT

Translation

W S H P N F E K

TEV Protease Site Sequence

GAAAACCTTTACTTCCAGGGC

Translation

E N L Y F Q G

6xHis-MBP-TEV Sequence

ATGCATCACCATCACCATCACGGTACCAAACTGAAGAAGGTAAACTGGTAA
TCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAA
ATTCGAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTG
GAAGAGAAATTCCCACAGGTTGCGGCAACTGGCGATGGCCCTGACATTATCT
TCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGCCTGTTGGCTGAA
ATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTTACCTGGGATG
CCGTACGTTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTA

TCGCTGATTTATAACAAAGATCTGCTGCCGAACCCGCCAAAAACCTGGGAAG
 AGATCCCGGCGCTGGATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGA
 TGTTCAACCTGCAAGAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGG
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 CAGTCCGAACAAAGAGCTGGCAAAGAGTTCTCTCGAAAACCTATCTGCTGACT
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 TGAAGTCTTACGAGGAAGAGTTGGCGAAAGATCCACGTATTGCCGCCACCAT
 GGAAAACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCCGCT
 TTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGA
 CTGTGATGAAGCCCTGAAAGACGCGCAGACTGGTACCGGATCTTACATCGG
 TGGCGAAAACCTTTACTTCCAGGGCGGTGGG

Roseiflexus castenholzii FixX Sequence

ATGAGTAATCGAACCAATGGCCAGACGGCGCCTGTCGCCGACGTTCAACT
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 ATGAAGAGAATGCTCACATCCGCATCAAGGATCTGGCAATCTGCGCGGCG
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 CGATCGCCACGGGCGGATGATGGTTCGGATACCAGGCATGCATTGAGTGCG
 GCAGTTGCCGGGTTCGCCTGCCCATTCGCAACGTGGACTGGCGCCTGCCA
 CGCGGTGGGCATGGGGTGGCGTATAAATACGGATAG

Translation

MSNRTNGQTAPVADVQLPHVNIDDLIVCLKYYVDEENAHIRIKDLAICAACDPKP
 CLAFCPAHVFETDRHGRMMVGYQACIEGSCRVACPFRNVDWRLPRGGHGVA
 YKYG

640895718 YP_001433666 4Fe-4S ferredoxin iron-sulfur binding domain protein
 [*Roseiflexus castenholzii* DSM 13941: NC_009767]

Western Blot

1L of 10X Glycine-tricine: 30.3g of Tris (base) + 169.2 g of glycine

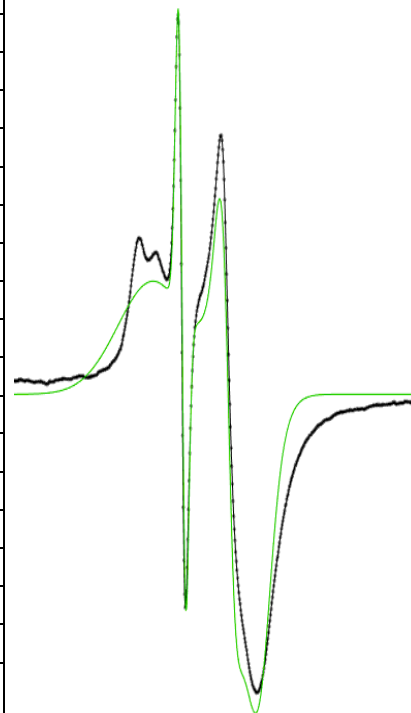
1L of Transfer Buffer: 100 mL of 10X Tris-Glycine + 200 mL methanol + 200 mg of SDS, bring volume up to 1L and stir until fully dissolved

1L of 10X TBS (Tris Buffered Saline): Tris (base) 60.55g, NaCl, 87.66g, pH 7.4 (with HCl), water to 1L

1L of TBST: 100 mL of 10X TBS + 500uL of tween 20 (this is very viscous, cut tip of pipet tip)

EasySpin Simulation Code

System	best	current	center	vary
A.g	2.00188	-	2.0019	0.0001
A.gStrain	0.0133634	-	0.0129	0.001
A.weight	0.577734	-	0.87	0.3
B.g(1)	2.07073	-	2.07	0.001
B.g(2)	1.939	-	1.94	0.001
B.g(3)	1.89901	-	1.9	0.001
B.gStrain(1)	0.123613	-	0.025	0.1
B.gStrain(2)	0.0195381	-	0.025	0.1
B.gStrain(3)	0.0337349	-	0.025	0.1
B.weight	19.466	-	20	15
C.g(1)	2.03912	-	2.04	0.001
C.g(2)	2.0009	-	2	0.001
C.g(3)	1.89952	-	1.9	0.001
C.gStrain(1)	0.119912	-	0.025	0.1
C.gStrain(2)	0.123791	-	0.025	0.1
C.gStrain(3)	0.0473984	-	0.025	0.1
C.weight	15.5264	-	20	15
Nelder/Mead simplex data as is scale & shift (Isq0) center of range RMSD 0.0350627				



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Exp.mwFreq = 9.3845;
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FitOpt.Method = 'simplex';
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EPR/FixXSample20_1mW15K.DSC');
Sys1.g = [2.00]; % Mediator Radical
Vary1.g = [0.0];
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Vary1.gStrain = [0.001];
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Vary1.weight = .3;
Sys2.g = [2.07, 1.94, 1.90]; % Broad [4Fe-4S]

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Vary2.weight = 15;  
Sys3.g = [2.04, 2.00, 1.90]; % Narrow [4Fe-4S]  
Vary3.g = [0.0, 0.0, 0.0];  
Sys3.gStrain = [0.025, 0.025, 0.025];  
Vary3.gStrain = [0.1, 0.1, 0.1];  
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Vary3.weight = 15;  
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