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UNIVERSITY OF CALIFORNIA SAN DIEGO

Light-Driven Uncoupling of Nitrogenase Catalysis from ATP Hydrolysis

A dissertation submitted in partial satisfaction of the requirements for the
degree Doctor of Philosophy

in

Chemistry

by

Lauren E. Roth

Committee in charge:

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Professor Stanley Opella
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2012

The Dissertation of Lauren E. Roth is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2012

Table of Contents

Signature Page	iii
List of Abbreviations	vi
List of Figures.....	vii
List of Tables.....	x
Acknowledgements.....	xi
Vita	xiii
Abstract of the Dissertation	xiv
Chapter 1.....	1
Introduction	
A Brief History of Early N ₂ Fixation Research	2
The Haber-Bosch Process.....	2
The Impact of Ammonia Production	4
Biological Nitrogen Fixation	5
Goals of Dissertation	7
References.....	14
Chapter 2.....	19
Modification of MoFeP with Photosensitizers	
Introduction	20
Materials and Methods	23
Results and Discussion	31
Conclusions.....	33
References.....	46
Chapter 3.....	49
Light-driven Two-Electron Reduction Reactions Catalyzed by MoFeP-RuBP	
Introduction	50
Materials and Methods	52
Results and Discussion	57

Conclusions.....	61
References.....	78
 Chapter 4.....	 81
Modified Methods for Site-Directed Mutagenesis of MoFeP from <i>A. vinelandii</i>	
Introduction	82
The Nif Gene Cluster.....	83
Genetic Engineering in <i>Azotobacter vinelandii</i>	83
Materials and Methods	86
Results and Discussion	91
Conclusions.....	96
References.....	109
 Chapter 5.....	 112
The Light-Driven, Six-Electron Reduction of HCN to CH ₄ by Photosensitized MoFeP	
Introduction	113
Materials and Methods	115
Results and Discussion	118
Conclusions.....	122
References.....	132
 Chapter 6.....	 134
Dissertation Conclusions	
References.....	138
 Appendix 1	 139
Calculating the Quantum Yield of α -C158-RuBP Photoreduction	
 Appendix 2	 140
Mutation Schemes for Successfully Transformed MoFeP Variants	

List of Abbreviations

ATP	Adenosine Triphosphate
ET	Electron Transfer
FeMoco	Iron Molybdenum Cofactor
FeP	Iron Protein
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
IA-Phen	Iodoacetamido-1,10-phenanthroline
IA-RuBP	$\text{Ru}(2,2'\text{-bipyridine})_2(5\text{-iodoacetamido-1,10-phenanthroline})(\text{PF}_6)_2$
MES	2-(N-morpholino)ethanesulfonic acid
MoFeP	Molybdenum-Iron Protein
MOPS	3-(N-morpholino)propanesulfonic acid
PAGE	Polyacrylamide Gel Electrophoresis
PT	Proton Transfer
SDS	Sodium Dodecyl Sulfate
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol

List of Figures

Figure 1.1.	FeP-MoFeP AMPPCP complex structure	11
Figure 1.2.	Interactions between MoFeP and FeP during electron transfer.....	13
Figure 2.1.	Nucleotide dependent FeP-MoFeP docking geometries	35
Figure 2.2.	Protein environments around the P-cluster and FeMoco.....	36
Figure 2.3.	Proposed modification sites on the MoFeP surface	37
Figure 2.4.	α -C158 MoFeP growth curve.....	38
Figure 2.5.	Anion exchange purification of α -C158 MoFeP.....	39
Figure 2.6.	Ni affinity column purification of α -C158 MoFeP.....	40
Figure 2.7.	Cartoon representations of MoFeP labeling sites.....	41
Figure 2.8.	Modeled structures of α -C196-RuBP and α -C158-RuBP.	42
Figure 2.9.	UV-vis absorbance spectra of RuBP labeled MoFeP.	44
Figure 3.1.	Reaction scheme for eosin-mediated nitrogenase reduction	64
Figure 3.2.	Illumination set-up for photoreduction activity assays.....	65
Figure 3.3.	H ⁺ photoreduction activity.....	66
Figure 3.4.	C ₂ H ₂ photoreduction activity.....	67
Figure 3.5.	X-band EPR spectra of illuminated α -C158-RuBP	68
Figure 3.6.	Specific bleaching of the RuBP absorbance feature.	69
Figure 3.7.	α -C158-RuBP H ⁺ and C ₂ H ₂ photoreduction assays.....	70
Figure 3.8.	C ₂ H ₂ photoreduction at different protein concentrations.	71
Figure 3.9.	H ⁺ photoreduction at different dithionite concentrations.....	72
Figure 3.10.	Proposed reaction scheme for H ⁺ and C ₂ H ₂ photoreduction	73
Figure 3.11.	C ₂ H ₂ photoreduction action spectrum.	74

Figure 3.12.	LED light-driven C ₂ H ₂ reduction	75
Figure 3.13.	CO inhibition of light-driven C ₂ H ₂ reduction	76
Figure 3.14.	H ₂ and NH ₃ production in an N ₂ atmosphere	77
Figure 4.1.	Genes in the major nif cluster from <i>A. vinelandii</i>	97
Figure 4.2.	Cartoon schematic of congression screening	98
Figure 4.3.	Cartoon representations of mutagenic plasmids	98
Figure 4.4.	Kpn1 digest of <i>nifHDK</i> genes	99
Figure 4.5.	Transformation strategy for <i>A. vinelandii</i> mutants.	100
Figure 4.6.	Screening methods used to identify <i>A. vinelandii</i> mutants.....	101
Figure 4.7.	Alternate labeling sites α -C159 and β -C157	103
Figure 4.8.	α -A45-C158-RuBP activity under ATP turnover conditions.	104
Figure 4.9.	SDS-PAGE gel unmodified and RuBP-labeled α -A45-C158.....	105
Figure 4.10.	α -A45-C158-RuBP H ⁺ photoreduction.	105
Figure 4.11.	SDS-PAGE gel unmodified and RuBP-labeled β -C157.....	106
Figure 4.12.	Initial tests of β -C157-RuBP and α -A45-C159-RuBP activity.....	107
Figure 4.13.	Conformational changes in the P-cluster.	108
Figure 5.1.	Modified version of the Thorneley-Lowe scheme.....	124
Figure 5.2.	GC-MS analysis of HCN photoreduction products.....	125
Figure 5.3.	Characterization of products from OPT reaction in ditioninte	126
Figure 5.4.	HCN photoreduction activity.	127
Figure 5.5.	Proposed photocatalytic scheme for HCN photoreduction.	127
Figure 5.6.	Specific bleaching of α -A45-C158-RuBP absorbance.....	128
Figure 5.7.	H ₂ production during HCN photoreduction.	129

Figure 5.8.	HCN reduction at different pH values.	130
Figure 5.9.	H ⁺ reduction at different pH values	131
Figure A2.1.	Crystal structure of α -A45-C158	147
Figure A2.2.	Crystal structure of α -A45-C158-Q196.....	149

List of Tables

Table 1.1.	Alternate substrates reduced by nitrogenase	12
Table 2.1.	ICP-OES analysis of RuBP labeled MoFeP variants.....	43
Table 2.2.	Quantification of RuBP labeling by UV-vis absorbance.....	43
Table 2.3.	Activity under turnover conditions for MoFeP variants	45
Table 4.1.	Mutagenesis scheme used to create MoFeP variants.....	102
Table 4.2.	ICP-OES analysis of α -A45-C158 and α -A45-C158-RuBP.....	104
Table 4.3.	ICP-OES analysis of RuBP labeled β -C157 MoFeP	106
Table A2.1.	Collection and refinement statistics for the structure of α -A45-C158 MoFeP.	146
Table A2.1.	Collection and refinement statistics for the structure of α -A45-C158 -Q196 MoFeP.....	148

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1. Roth, Lauren E.; Tezcan, F. Akif. "ATP-Uncoupled, Six-Electron Photoreduction of Hydrogen Cyanide to Methane by the Molybdenum-Iron Protein", *Journal of the American Chemical Society* (2012), DOI: 10.1021/ja303265m.
2. Roth, Lauren E.; Tezcan, F. Akif. "Light-Driven Uncoupling of Nitrogenase Catalysis from ATP Hydrolysis", *ChemCatChem* (2011), 3(10), 1549-1555.
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4. Roth, Lauren E.; Nguyen, Joey C.; Tezcan, F. Akif. "ATP- and iron-protein-independent activation of nitrogenase catalysis by light", *Journal of the American Chemical Society* (2010), 132(39), 13672-13674.

Fields of Study

Major Field: Biochemistry

Biochemistry: Bioinorganic chemistry, Enzymology

ABSTRACT OF THE DISSERTATION

Light-Driven Uncoupling of Nitrogenase Catalysis from ATP Hydrolysis

by

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University of California, San Diego, 2012

Professor F. Akif Tezcan, Chair

The Haber-Bosch process carries an enormous industrial and agricultural importance but also has a largely negative economic and environmental impact. Understanding the mechanistic details of biological nitrogen fixation catalyzed by the enzyme nitrogenase would be beneficial both for designing cleaner or more efficient catalysts for small molecule activation and for understanding biological multi-electron/proton redox processes. The enzyme's catalytic mechanism, however, is poorly understood because it relies upon ATP hydrolysis and complex protein-protein interactions to coordinate electron transfer and substrate activation. We have proposed that uncoupling MoFeP catalysis from ATP- and FeP-dependent electron transfer, specifically through

light-driven electron injection, would enable the direct study of catalysis at the enzyme active site and the population of discrete reaction intermediates for structural or spectroscopic investigation.

The experimental results discussed herein describe the design and characterization of a photosensitized MoFeP capable of reducing substrates independent of ATP hydrolysis and FeP, which have previously been believed essential for catalytic turnover. To achieve light-driven electron injection and substrate activation, MoFeP variants were labeled with a Ru photosensitizer at three different exposed cysteine residues on the protein surface. A particular MoFeP-Ru construct with the Ru photosensitizer located directly above the P-cluster was found to catalyze the 2-electron reduction of protons and acetylene in a light-dependent manner. A modified version of the same construct was able to catalyze the 6-electron reduction of HCN into CH₄ and likely also NH₃.

Currently, none of the MoFeP-Ru constructs can catalyze the light-driven reduction of N₂ to NH₃, most likely as a result of inefficient electron transfer to the FeMoco in the absence of FeP. In light of recent findings that certain amino-acid substitutions in MoFeP may enable efficient electron transfer into the MoFeP active site (FeMoco) in the absence of FeP, we have initiated efforts focused on modifying the MoFeP through site-directed mutagenesis. Incorporating these substitutions into our MoFeP-Ru constructs should increase the efficiency of electron transfer to FeMoco such that light-driven N₂ reduction is realized and discrete catalytic intermediates for this reaction can be populated in sufficient quantities for structural or spectroscopic examination.

Chapter 1

Introduction

A Brief History of Early N₂ Fixation Research

Dinitrogen (N₂) fixation has captivated scientists for more than two hundred years. One of the first examples in the literature detailing the activation of atmospheric N₂ appears in Henry Cavendish's 1784 paper "Experiments on Air" in which he describes how N₂ can be combined with O₂ and trace amounts of H₂ to produce nitric acid.¹ Nearly forty years later, chemist Johann Wolfgang Döbereiner discovered that platinum could be used to convert N₂ and H₂ into trace amounts of ammonia (NH₃), thereby beginning the scientific quest to understand and improve N₂ reduction to NH₃.^{2,3}

It was at the start of the twentieth century that the field of catalytic NH₃ synthesis had its defining breakthrough. By 1900, it was apparent that current agricultural production would not be able to sustain an ever increasing global population.⁴ Natural fertilizer sources necessary to increase the amount of arable land were either limited, as in the case of salpeter deposits in Chile, or insufficient, as with ammonium sulfate produced from coal distillation.⁵ Atmospheric N₂ was an obvious source of readily available starting material for synthetic fertilizer production since it had been shown that N₂ could be activated to produce NH₃, however, there lacked a cost effective process to run this reaction on an industrial scale.⁶

The Haber-Bosch Process

In 1909, Fritz Haber demonstrated that the conversion of atmospheric N₂ to NH₃ would be possible on a large scale. Using an osmium-based catalyst and

a high pressure autoclave, Haber was able to convert gaseous N_2 and H_2 into NH_3 at a rate of 80 g of NH_3 per hour. It was then the work of Carl Bosch and Alwin Mittasch, which turned Haber's scientific discovery into a commercially successful process. Bosch was responsible for developing the extremely high pressure technology required to adapt NH_3 production on the laboratory scale to the industrial scale while Mittasch's studies on the effects of various additives on metal based catalysts resulted in the generation of a promoted iron based catalyst similar to those used in current NH_3 production.^{7,8}

The combination of these discoveries resulted in an effective method for NH_3 synthesis that is today known as the Haber-Bosch process. In 1913, when the first NH_3 production plant based on the Haber-Bosch process opened, it produced 30 metric tons of NH_3 per day.⁵ As of 2010, the global production of NH_3 was estimated at over 350,000 metric tons per day, with 87% of NH_3 produced in the United States used for fertilizer production.⁹

In its current form the Haber-Bosch process involves reacting N_2 and H_2 at high pressures and temperatures over a Fe based catalyst to produce NH_3 :⁷

The most common Haber-Bosch catalysts are composed of primarily Fe_2O_3 with various structural and electronic promoters such as K, CaO and Al_2O_3 . Several alternative compounds able to catalyze the conversion of N_2 to NH_3 have also been discovered, such as Ru-based complexes¹⁰, molybdenum [HIPTN₃N] compounds¹¹, and bimetallic nitrides, yet none so far can match the high-activity and low cost of traditional iron based Haber-Bosch catalysts.⁵

The Impact of Ammonia Production

Haber and Bosch's development of an industrially viable process that could efficiently produce NH_3 directly from N_2 and H_2 is arguably one of the most important scientific breakthroughs of the last century. It has been suggested that no other discovery has had such a clear impact on both the global population and science as a whole.¹² NH_3 produced through the Haber-Bosch process can be used to synthesize a number of industrially important molecules, in particular those in the synthetic fertilizers that today sustain more than 40% of the global population.¹³ While fixed nitrogen species from the Haber-Bosch process are clearly essential, the industrial reaction to produce these molecules is also responsible for considerable amounts of energy consumption and greenhouse gas emissions.¹⁴

Although the Haber-Bosch process is relatively efficient, with an overall yield of approximately 35%,¹⁵ it requires extremely high temperatures (600-800K) and pressures (~300 atm) to overcome the inertness of the N_2 molecule. While the synthesis of NH_3 from its elements is thermodynamically favorable at room conditions and the equilibrium strongly favors product formation, the reaction's rate limiting step is dissociation of the N-N triple bond. Even with a Fe based catalyst, this step has high activation energy, necessitating high temperatures to achieve a useful rate of N_2 dissociation. However, the reduction of N_2 to NH_3 is an exothermic reaction and increasing the reaction temperature to increase the rate also dramatically reduces the product yield. To overcome this, the reaction is run under high pressure to drive the reaction toward product formation.¹⁶

The preparation of starting materials combined with the high temperature and pressure necessary to drive NH_3 synthesis are estimated to account for more than 1% of total global energy consumption each year^{16,17} and that percentage will continue to increase as the global population grows. It is projected that the world population will reach or exceed 10 billion by the year 2050, bringing the need for industrially produced nitrogen to 160 million tons per year. Generating such quantities of fixed nitrogen using the currently available methods would require burning approximately 270 million tons of coal annually.¹⁸ Therefore, the design of a cleaner and more efficient method for NH_3 production is important both environmentally and industrially. Such a method might include a biocatalyst that mimics that of biological N_2 reduction, a process that occurs at ambient conditions.

Biological Nitrogen Fixation

Biological N_2 fixation is catalyzed by the enzyme nitrogenase, a two component protein capable of reducing atmospheric N_2 to NH_3 at room temperature and pressure. There are three main classes of nitrogenases. These enzymes all contain the same basic two component proteins, but have different heteroatoms in the enzyme active site (Mo, V or Fe).¹⁹ The Mo-containing nitrogenase is the best studied among the three forms and is the enzyme system that has been used in all the experiments described herein.

The component proteins of Mo-nitrogenases are known as the iron protein (FeP) and the molybdenum iron protein (MoFeP) (Figure 1.1). MoFeP is a $\alpha_2\beta_2$

heterotetramer that contains two metal clusters, the P-cluster, a [8Fe:7S] cluster, and the FeMoco, a [7Fe:1Mo:9S:1C] cluster^{20,21} with a coordinated homocitrate molecule. FeP is a dimeric ATPase that transfers electrons to the MoFeP. Based on crystal structures of the complexed nitrogenase proteins, electron transfer is believed to proceed from a [4Fe:4S] cluster in the FeP to the P-cluster and finally to the FeMoco, where it is proposed that substrate binding and activation occur (Figure 1.1).²²⁻²⁶

According to the standard model, FeP transfers one electron at a time to the MoFeP, coupling this transfer to the hydrolysis of 2 ATP molecules and the obligatory reduction of two protons (H⁺) to H₂.^{27,28} Since 8 electrons are required to fully reduce N₂ and 2 H⁺, the overall enzymatic reaction is depicted as:

In addition to N₂, nitrogenase is able to reduce a number of other substrates, including H⁺, acetylene (C₂H₂), hydrazine (N₂H₄) and several multiply bonded species that can fit in its binding pocket.²⁹⁻³¹ (Table 1.1) Reducing any of these substrates requires at least 2 electrons, therefore FeP and MoFeP must undergo multiple association and dissociation cycles to accumulate enough electrons on the FeMoco for catalysis. Based on stop-flow experiments and crystal structures of nitrogenase complexes with nucleotide-dependent docking geometries, it is proposed that the following cycle occurs during nitrogenase turnover (Figure 1.2). First, a reduced FeP with two molecules of MgATP bound will associate with the MoFeP surface. It will then transfer one electron into the MoFeP as it hydrolyzes both MgATP molecules. The oxidized FeP bound to two MgADP

molecules will then dissociate from the MoFeP in what is likely the rate limiting step in nitrogenase catalysis.³² Oxidized FeP will then be reduced in solution by other electron transfer proteins in the cell or by small molecule reductants *in vitro*, and the nitrogenase cycle can begin again.³³ It is proposed that every stage in nitrogenase catalyzed reduction–protein interactions, electron transfer, substrate binding and reduction–is synchronized through the ATPase activity of FeP.

Goals of Dissertation

ATP-dependent multi-electron transfer activity makes nitrogenase rare, though not unique,³⁴⁻³⁷ among redox enzymes but is also the reason why, despite decades of research, very few concrete details are known about the enzyme's mechanism.³⁸ Several key questions about the mechanism of nitrogenase catalysis still remain unresolved:

- 1) How do ATP hydrolysis and the interactions between FeP and MoFeP regulate the delivery of multiple electrons and protons to FeMoco and the substrate?
- 2) Why is FeP the only reductant that can activate N₂ reduction by MoFeP?
- 3) What are the binding modes of substrates to the FeMoco?
- 4) What intermediates are generated during nitrogenase catalyzed substrate reduction, specifically those of N₂ reduction to NH₃?

The structures of nitrogenase complexes have outlined the possible path of electron flow but they have not explained how ATP-hydrolysis and FeP-MoFeP

interactions are involved in controlling the electron flow during catalysis. Biochemical assays have led to working models for nitrogenase catalysis^{32,39-41} such as the Thorneley-Lowe model but these models have not been structurally validated. EPR/ENDOR studies have revealed possible modes of substrate interactions with FeMoco, yet the requirement for ATP turnover to sustain catalysis and the transient nature of substrate binding have made it challenging to populate discrete substrate-bound states for structural or spectroscopic investigation.^{33,42}

With so many key questions still unanswered despite so much experimental work, it is clear that new experimental approaches are needed to study nitrogenase. One promising alternate route involves decoupling nitrogenase catalysis from ATP-dependent electron transfer. This approach requires constructing an entirely new electron transfer system into the MoFeP, one which could be accomplished using transition metal-based photosensitizers attached to the surface of MoFeP. Covalently linking these molecules to the MoFeP through surface exposed residues would enable direct electron transfer to the enzyme active site, thereby removing the complication of ATP hydrolysis and allowing the population of discrete reaction intermediates for structural or spectroscopic investigations that were previously inaccessible.

Photoreduction is also ideally suited to study proton and electron transfer in nitrogenase because it can easily be combined with the more traditional methods used to study nitrogenase, in particular site directed mutagenesis. By generating different variants through mutagenesis, photosensitizers can be

placed almost anywhere on the MoFeP surface, enabling the identification of residues that are critical for controlling electron flow both to and between the P-cluster and the FeMoco. In addition, photoreduction and mutagenesis can be used to characterize MoFeP variants with perturbed electron or proton transfer pathways, perhaps helping to locate the purported "conformational gate"^{43,44} within the protein that enables efficient electron flow from P-cluster to FeMoco for catalysis. Furthermore, it has been shown that MoFeP with specific mutations near the active site can bind but not fully reduce certain substrates.^{45,46} Such intermediates may be generated and trapped through photo-induced electron transfer, which in turn may permit a thorough characterization of their identity and properties through an array of spectroscopic and structural techniques.

By utilizing photo-induced electron transfer into the MoFeP, we hope to identify the normally ATP-regulated electron and proton transfer pathways in nitrogenase and be poised to manipulate them in order to delve deeper into the mechanism of N₂ activation. Not only will the insights gained here enhance our fundamental knowledge of how nature couples electron and proton movement for the activation of inert substrates, but they may also open up new avenues for light-driven activation of inert molecules. As previously mentioned, nitrogenase is a powerful catalyst that can reduce almost any multiply bonded species that can fit in its substrate binding pocket.^{30,47,48} What is more, the vanadium containing nitrogenase has recently been shown to catalyze the conversion of carbon monoxide (CO) into higher order alkanes⁴⁹⁻⁵¹, thereby expanding the scope of nitrogenase catalysis to C-C bond coupling reactions. A full

mechanistic understanding of nitrogenase coupled with light activation may enable the design of nitrogenase variants that can perform pharmaceutically and industrially important reduction or C-C and C-N coupling reactions in a cleaner and more efficient manner than those currently available.

Figure 1.1. FeP-MoFeP AMPPCP complex structure (PDB ID: 2AFK) highlighting each protein's metal clusters, their relative positions to each other, and the proposed electron transfer pathway in the nitrogenase complex. The FeP dimer is depicted in gray, and the MoFeP α and β subunits are shown in purple and green, respectively. Only one $\alpha\beta$ dimer of MoFeP is shown for clarity. The approximate distances between the clusters are based on the distances between the cluster centroids reported in Ref. 17. (adapted from Ref. 36)

Table 1.1. Alternate substrates reduced by nitrogenase enzymes and the proposed stoichiometry of the reaction not including the number of ATP molecules hydrolyzed during electron transfer.

substrate	reaction
protons	$2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2$
acetylene ^{52,53}	$\text{C}_2\text{H}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{C}_2\text{H}_4$
diazene ⁵⁴	$\text{N}_2\text{H}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{NH}_3$
hydrazine ⁵⁵	$\text{N}_2\text{H}_4 + 2\text{H}^+ + 2\text{e}^- \rightarrow 2\text{NH}_3$
azide ^{56,57}	$\text{N}_3^- + 3\text{H}^+ + 2\text{e}^- \rightarrow \text{NH}_3 + \text{N}_2$
	$\text{N}_3^- + 7\text{H}^+ + 6\text{e}^- \rightarrow \text{N}_2\text{H}_4 + \text{NH}_3$
	$\text{N}_3^- + 9\text{H}^+ + 8\text{e}^- \rightarrow 3\text{NH}_3$
diazirene ⁵⁸	$\text{N}_2\text{CH}_2 + 8\text{H}^+ + 8\text{e}^- \rightarrow \text{CH}_4 + 2\text{NH}_3$
nitrite ⁵⁹	$2\text{NO}_2^- + 8\text{H}^+ + 6\text{e}^- \rightarrow 4\text{H}_2\text{O} + \text{N}_2$
nitrous oxide ^{60,61}	$\text{N}_2\text{O} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{O} + \text{N}_2$
carbon dioxide ³⁰	$\text{CO}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{CO} + \text{H}_2\text{O}$
carbonyl sulfide ⁶²	$\text{COS} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{CO} + \text{H}_2\text{S}$
thiocyanate ⁶³	$\text{SCN}^- + 3\text{H}^+ + 2\text{e}^- \rightarrow \text{HCN} + \text{H}_2\text{S}$
hydrogen cyanide ⁶⁴⁻⁶⁶	$\text{HCN} + 4\text{H}^+ + 4\text{e}^- \rightarrow \text{CH}_3\text{NH}_2$
	$\text{HCN} + 6\text{H}^+ + 6\text{e}^- \rightarrow \text{CH}_4 + \text{NH}_3$

Figure 1.2. Interactions between MoFeP and the different nucleotide bound states of FeP during electron transfer for substrate reduction.

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Chapter 2

Modification of MoFeP with Photosensitizers

Introduction

The recalcitrance of nitrogenase to detailed mechanistic studies has led to creative methodologies aimed at studying what intermediates might be formed during catalysis, including the use of less-active nitrogenase cluster homologues¹ or the study of the relaxation of trapped intermediates rather than their generation.² In addition, an extensive set of mutational studies targeting the FeMoco active site has provided glimpses into possible modes of substrate interactions on FeMoco.³⁻⁸ These strategies, however still depend on turnover conditions in solution, which necessitates an ATP-regeneration system in addition to FeP, MoFeP and a constant supply of electrons, and ultimately cannot be fully synchronized to produce high concentrations of isolated intermediates. An experimental "cure" to this problem would be to uncouple MoFeP catalysis from FeP-dependent reduction, thereby removing the necessity for ATP hydrolysis and protein-protein interactions. Only in that case can electron transfer to FeMoco be initiated in a synchronized fashion through stopped-flow mixing, electrochemical techniques or, as is our goal, triggering with light.

Until very recently, MgATP-bound FeP was the only known electron donor that had been shown to effect the reduction of any substrate by MoFeP, despite its modest reduction potential that ranges from -280 mV in its isolated form to -690 mV when it is complexed to MoFeP.⁹ Even much stronger small-molecule reductants such as $\text{Ti}^{\text{IV/III}}[\text{citrate}]$ ($E^0 \leq -800$ mV) and $\text{Cr}^{\text{III/II}}\text{-EDTA}$ ($E^0 \sim -1$ V)^{10,11} are incapable of supporting catalysis in the absence of FeP. This resistance to small molecule reduction strongly suggested that electron transfer and subsequent

substrate reduction are somehow “gated” in the MoFeP, perhaps through conformational changes initiated by protein-protein interactions formed during FeP-MoFeP complex formation.

Evidence for this gating mechanism comes from the observation that, when nitrogenase activity assay solutions are altered, enzymatic activity is sensitive to changes in osmotic pressure but not viscosity.¹² Furthermore, following a screening of their site-directed mutant library, Seefeldt, Dean and colleagues have shown that a MoFeP variant, β -Y98H, could be reduced in solution by electrochemically-generated Eu^{II} -EGTA ($E^\circ = -0.88 \text{ V}$) and Eu^{II} -DTPA ($E^\circ = -1.14 \text{ V}$) complexes and could catalyze the reduction of N_2H_4 to NH_3 at nearly the same rate as the wild type protein under turnover conditions.¹³ β -Y98 lies in the intervening region between the P-cluster and FeMoco, and is implicated in electron transfer between the two clusters.¹⁴ Although the reason for the particular reactivity of this mutant is not immediately apparent, it could feature perturbed reduction potentials of each cluster or mimic a conformational state induced by FeP in which electron transfer from the P-cluster to FeMoco is activated.¹⁴

Despite these results, when examining the crystal structures of FeP-MoFeP complexes obtained at various stages of MgATP hydrolysis there is little to suggest even a subtle conformational change occurring in the MoFeP. The complexed nitrogenase structures revealed large conformational changes in FeP that enable it to assume different docking geometries on MoFeP and modulate the distance (thus, electronic coupling) between the $[\text{4Fe:4S}]$ cluster and the P-

cluster (Figure 2.1). Nevertheless, these structures also showed that there are no discernible conformational changes within MoFeP that would readily indicate an internal electron transfer gate (Figure 2.2).¹⁵ This raises the possibility that efficient electronic coupling from the surface of MoFeP to its core clusters is the only requirement for catalysis, which may be overcome with a surface-immobilized redox photosensitizer.

Designing a MoFeP Photoreduction Construct

We propose that an appropriately chosen photosensitizer could be used to drive catalysis in the MoFeP. Furthermore, covalently linking the photosensitizer to the surface of the MoFeP should mimic the highly-coupled and low-potential form of the FeP [4Fe:4S] cluster formed within the MgATP-bound FeP-MoFeP complex, thereby potentially uncoupling nitrogenase reactivity from ATP hydrolysis.

Photosensitizers can be coupled to the surface of proteins in many ways, most common among them being chemical modification of reactive amino acids. Analysis of the surface of the wild type MoFeP based on its highest resolution crystal structure¹⁶ revealed too many surface-exposed glutamate, aspartate or lysine residues for unambiguous labeling and analysis of potential electron transfer pathways. There are fewer surface exposed histidine residues but the crystal structure still showed at least three likely labeling sites. Cysteine however seemed the best candidate for specific functionalization since only one, α -Cys45, is appreciably solvent exposed. Furthermore, studies designed to

identify reactive amino acids in an apo-FeMoco version of MoFeP showed that α -Cys45 could be modified with the cysteine specific fluorophore I-AEDANS.¹⁷ Modifying MoFeP through cysteine residues was also an attractive option because two mutant versions of the MoFeP, α -C158 and α -C196, were available, each which have an additional cysteine residue located closer to the P-cluster or the FeMoco, respectively (Figure 2.3). Here we describe the design, construction and initial characterization of three MoFeP constructs modified with a cysteine-specific ruthenium photosensitizer, Ru(2,2'-bipyridine)₂(5-iodoacetamido-1,10-phenanthroline)](PF₆)₂ (IA-RuBP).

Materials and Methods

Unless otherwise noted, all chemicals and reagents were purchased from Fisher Scientific, VWR International or Sigma-Aldrich. All work with active nitrogenase proteins was performed either in an anaerobic tent (Coy Laboratories) with an oxygen level less than 4 ppm or on the bench top using standard Schlenk line techniques.

Sequencing A. vinelandii MoFeP mutant strains. *A. vinelandii* strains DJ1194 and DJ1191, which express His-tagged α -C158 and His-tagged α -C196 MoFe protein, respectively, were kindly provided by Dr. Dennis R. Dean. MoFeP mutations were confirmed by sequencing conducted by the Retrogen Company. *nifD* and *nifK* genes isolated according to the following procedures. Genomic DNA was extracted from 1.5 mL *A. vinelandii* cultures grown without a fixed nitrogen source, using standard phenol/chloroform extraction protocols.

The *nifD* and *nifK* genes were isolated with PCR using the Epicentre® Biotechnologies FailSafe™ PCR PreMix Selection Kit and the following primers:

<i>nifD</i> residues 1-241	5'-GAGCTCGAAGAGCTGCTGAT-3'
	5'-TAGCAGTGAACCAGGTTTCAGC-3'
<i>nifD</i> residues 238-492	5'-CATCGGCGACTACAACATC-3'
	5'-TCCTGGTATTCCTGGTGG-3'
<i>nifK</i> residues 1-380	5'-ATGCACTCCTGGGATTATCC-3'
	5'-CACTTCTCCGGATCGGAGA-3'
<i>nifK</i> residues 242-524	3'-CGAGACCTACCTGGGCAAC-3'
	5'-TCATGCTCCAGGGACACC-3'

No additional mutations were observed in either *nifD* or *nifK*.

Azotobacter vinelandii cell growth. Wild type and mutant strains of *A. vinelandii* cells were cultured using either 1 or 2 L cultures of Burk's medium in 2.8 L or 6 L flasks respectively and were shaken at 220 rpm at 30°C in a New Brunswick shaker for 12 to 16 hours. Burk's media contained 60 mM sucrose, 1.67 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.9 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 36 μM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 2 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. Cell cultures also contained 10 mM KH_2PO_4 buffered at pH 7.5 and 2 mM NH_4Cl . The addition of a fixed nitrogen source to Burk's media represses the expression of nitrogenase genes and *A. vinelandii* growth proceeds with a doubling time of approximately 3.2 hours¹⁸. Once the growing cells consume the added NH_4Cl , cell division slows and nitrogenase expression begins, a stage of growth referred to as derepression¹⁹. With the addition of 2 mM NH_4Cl , nitrogenase expression is suppressed until the cells reach an optical density (OD)

at 600 nm of approximately 1 (Figure 2.4). At this point nitrogenase expression can be monitored by the C_2H_2 reduction activity of whole cells.

To determine when cells had entered the derepression stage, culture samples were taken approximately every 15 to 30 minutes starting after cultures had grown for 10 hours. The OD of the culture was measured at both 600 and 660 nm on a Hewlett Packard 8452A diode array spectrophotometer. C_2H_2 reduction activity assays were done with 1 mL samples of cell cultures in 14-mL septum sealed glass vials. 1 mL of 1 atm C_2H_2 was injected into the sealed vial with a gas-tight syringe after which the vial was placed in a 30°C water bath with a shaking speed of 120 rpm. Reactions were allowed to proceed for 15 minutes and were then quenched by the addition of 250 μ L of glacial acetic acid. A 50 μ L sample of the vial headspace was removed with a gas-tight syringe and C_2H_4 production was detected by gas chromatography with a flame ionization detector connected to a SRI Instruments 8610 gas chromatography instrument. C_2H_4 was separated on an Alumina column (5'x1/8"x0.85" SS) (Alltech) using He as the carrier gas at an oven temperature of 150°C. Injection volumes were normalized by C_2H_2 injection peak areas and product concentrations were determined using calibration curves constructed from C_2H_2 standards. Maximum activity from whole cells was typically between 100 to 150 nmol C_2H_4 produced per mL of cell culture. A separate batch of α -C158 MoFeP was also grown in a 200-liter New Brunswick fermentor in the laboratory facilities of Dr. Markus Ribbe at UC Irvine.

Once cultures had entered derepression, cells were harvested by centrifugation, frozen in a dry ice and ethanol slurry and stored at -80°C until protein purification. Wild type and mutant MoFe proteins were isolated and purified under anaerobic conditions according to published protocols by Burgess *et al.* with slight modifications.²⁰ Cell pellets were resuspended in 50 mM tris(hydroxymethyl)aminomethane (Tris), at pH 8.2, 100 mM NaCl, 5 mM sodium dithionite (J. T. Baker) buffer with 40% glycerol to swell the cells and then centrifuged at 12,000 rpm for 30 minutes. This process was repeated twice. Cell pellets were then resuspended in 50 mM Tris at pH 8.2, 100 mM NaCl, and 5 mM sodium dithionite and mechanically lysed by vigorous shaking with marbles. Crude cell extracts were not heat treated prior to purification and any crystallization procedures were omitted. All chromatography was performed on a Bio-Rad BioLogic DuoFlow F10 FPLC system with a standard 280 nm UV filter and a QuadTech UV-vis detector.

Cell lysates were first purified on a weak anion exchange chromatography resin which accomplishes the separation of MoFeP and FeP and eliminates high concentration contaminants present in the cell extract. Clarified cell lysates in a 50 mM Tris and 100 mM NaCl solution buffered at pH 7.75 were loaded onto a GE Healthcare Life Sciences XK-26 chromatography column packed with diethylaminoethyl-Sepharose® (DEAE) resin from Sigma-Aldrich (Figure 2.5). Protein separation was achieved through gradient elution with a starting buffer of 50 mM Tris and 100 mM NaCl and an elution buffer of 50 mM Tris and 500 mM NaCl, both at pH 7.75. The NaCl salt gradient was applied to the

column at a flow rate of 2 mL per min over a total volume of 900 mL. Typical DEAE elution profiles showed 5 peaks that could be identified by running eluent samples on 15% acrylamide SDS-PAGE gels. Based on buffer conductivity at elution time and molecular weight estimates from Coomassie-stained gels these peaks represent the *A. vinelandii* NifEN protein²¹, MoFeP, FeP, and unidentified ferredoxins²²⁻²⁴ and flavodoxins^{25,26}. MoFeP and FeP fractions were further purified with size-exclusion chromatography on a GE Healthcare Life Sciences 100 cm XK-26 column packed with Superdex200 resin (GE Healthcare Life Sciences) with a 50 mM Tris and 200 mM NaCl mobile phase buffered at pH 7.75. His-tagged proteins were further purified on a GE Healthcare HisPrep™ FF 16/10 Ni affinity column using a step gradient with a 25 mM Tris and 500 mM NaCl loading buffer, a 25 mM Tris, 500 mM NaCl and 40 mM imidazole wash buffer and a 25 mM Tris, 500 mM NaCl and 250 mM imidazole elution buffer, all at pH 7.9 (Figure 2.6) Protein purity was determined by SDS-PAGE with 15% acrylamide and 8% acrylamide gels used for FeP and MoFeP samples, respectively (Figure 2.5 and Figure 2.6).

For both wild type and mutant MoFe proteins, C₂H₂ reduction activities under turnover conditions were determined as described in the literature²⁰. Briefly, activity assay reactions were done in 14-mL septum sealed glass vials flushed with Ar. Assay mixtures contained 0.2 µM MoFeP in 1 mL of a 5 mM MgCl₂·6H₂O and 50 mM Tris reaction buffer at pH 8.0 which also contained 5 mM ATP(Sigma-Aldrich), 20 mM phosphocreatine(Sigma-Aldrich) and 0.125 mg creatine phosphokinase (Sigma-Aldrich). 15 µL of 1 M sodium dithionite and 1 mL

of 1 atm C_2H_2 were added to each septum sealed vial with gas-tight syringes and solutions were allowed to equilibrate in a 30°C shaking water bath set to 120 rpm for 15 minutes. Reactions were initiated by the addition of between 0 and 6 μM FeP to the vial and were placed back in the shaking water bath. Reactions were allowed to proceed for 10 minutes and were then quenched with 250 μL of glacial acetic acid. The total C_2H_4 produced was determined by GC analysis as described previously. Typical C_2H_2 reduction activity for wtMoFeP is approximately 2000 to 3000 nmol C_2H_4 produced per mg of MoFeP in the reaction per minute²⁰. The C_2H_2 reduction activities were as follows: 2500 nmol C_2H_4 /mg wt-MoFeP/min, 2400 nmol C_2H_4 /mg α -C158 MoFeP/min, 2300 nmol C_2H_4 /mg α -C196 MoFeP/min.

Synthesis of 5-Iodoacetamido-1,10-phenanthroline (IA-Phen). 0.5 g of 5-amino-1-10-phenanthroline (Polysciences) was dissolved in 90 mL of acetonitrile with slight heating. To this stirred solution, a freshly prepared solution of iodoacetic acid anhydride in 10 mL of acetonitrile was added. The mixture was allowed to react in the dark overnight. The precipitated product was isolated by filtration and washed with cold 5% sodium bicarbonate and then water, and dried in vacuo.

Synthesis of IA-RuBP. $[Ru(2,2'\text{-bipyridine})_2(5\text{-iodoacetamido-1,10-phenanthroline})](PF_6)_2$ (IA-RuBP) was synthesized as described by Castellano et. al.²⁷ All procedures involving the synthesis of or modification with IA-RuBP were done in the dark. Briefly, 50 mg of $Ru(bpy)_2Cl_2$ (Alfa Aesar) and 38.4 mg of 5-IA-Phen were dissolved in 2.5 mL of methanol and refluxed for 3 hours with stirring.

After refluxing, the reaction was cooled to room temperature and filtered to remove any insoluble materials. IA-RuBP was precipitated with a saturated solution of NH_4PF_6 in water, and used without further purification.

Modification of MoFeP with IA-RuBP. Purified MoFeP was diluted to 20 mg/mL ($\sim 86 \mu\text{M}$) with a solution of 50 mM Tris at pH 7.75, 200 mM NaCl, and 5 mM sodium dithionite. While stirring, solid IA-RuBP was added to the MoFeP solution, so that the final concentration of the label was in 10-fold excess over the concentration of surface exposed Cys residues (1 per $\alpha\beta$ subunit of wt MoFeP, and 2 per $\alpha\beta$ subunit of α -C158 and α -C196 MoFeP). Reactions were allowed to proceed at room temperature in the dark for two hours and then stopped by separating MoFeP from any unreacted label using a Bio-Rad Econo-Pac 10-DG desalting column. No further purification of the modified MoFeP was carried out. Labeling of MoFeP was confirmed by UV-vis absorbance (Figure 2.9). The extent of labeling was determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) as described below (Table 2.1).

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). ICP-OES measurements were carried out to determine the integrity of the MoFeP metal clusters after Ru-modification and the stoichiometry of RuBP bound to MoFeP. The metal contents of MoFeP (1 Mo and 15 Fe's per $\alpha\beta$ -dimer) were used to calculate the ratio of RuBP bound per $\alpha\beta$ -dimer. Samples of both modified and unmodified protein were prepared for ICP-OES by diluting appropriate protein stocks to a final volume of 1.2 mL with a solution of 50 mM Tris, pH 7 and 200 mM NaCl. 0.8 mL of 10% reagent grade nitric acid (Fluka) and 2 mL of 8 M guanidine-

HCl were added to each sample to achieve a final concentration of 2% nitric acid and 4 M guanidine-HCl and ensure the denaturation of the protein and release of metal ions. Standard solutions were prepared from 1000 ppm certified ICP-OES metal stock solutions (Ricca) by mixing equal volumes of all metal analytes and diluting to a final concentration of 10 ppm of each metal. A calibration curve with 10 points between 0.01 and 5 ppm was then constructed by diluting appropriate volumes of the 10 ppm stock to 6 mL with 2% nitric acid in deionized water. Data were collected on a Perkin-Elmer Optima 3000 DV ICP-OES spectrometer located at the Analytical Facility of the Scripps Institution of Oceanography. Wavelengths used for the detection of various metal ions were as follows: Fe (234.349, 238.204, 259.939 nm), Mo (202.031, 203.845, 204.597 and 281.616 nm), Ru (240.272 and 349.894 nm). Values reported for each metal are averages of those for all indicated wavelengths.

Calculating labeling efficiencies with UV-vis absorbance. In addition to ICP-OES, the extent of labeling on MoFeP was also calculated based on the increase in absorbance between 400 to 500 nm (Figure 2.9). Since MoFeP also has a strong absorbance in this region the ratio was calculated based on changes in absorbance at 410 nm, where the MoFeP absorbs maximally, and at 450 nm, where the RuBP absorbs maximally using the following pair of equations:

$$Abs_{410\text{ nm}} = \epsilon_{410\text{ MoFeP}}[\text{MoFeP (M)}] + \epsilon_{410\text{ RuBP}}[\text{RuBP (M)}]$$

$$Abs_{450\text{ nm}} = \epsilon_{450\text{ MoFeP}}[\text{MoFeP (M)}] + \epsilon_{450\text{ RuBP}}[\text{RuBP (M)}]$$

The molar extinction coefficients used in the calculations were $\epsilon_{410} = 76000\text{ M}^{-1}\text{ cm}^{-1}$ and $\epsilon_{450} = 46100\text{ M}^{-1}\text{ cm}^{-1}$ for MoFeP and $\epsilon_{410} = 11,300\text{ M}^{-1}\text{ cm}^{-1}$ and $\epsilon_{450} = 16,600$

$\text{M}^{-1} \text{ cm}^{-1}$ for RuBP. Spectra were baseline corrected by subtracting the absorbance at 820 nm.

Results and Discussion

Designing MoFeP-RuBP constructs. After reviewing the MoFeP mutant library assembled by the lab of Dr. Dennis Dean at Virginia Polytechnic Institute, we decided to create initial photosensitizer-linked protein constructs based on three MoFeP variants – wild type (wt), α -L158C, and α -H196C. As previously mentioned, wild-type (wt) MoFeP contains several cysteine residues, however, only one (α -C45) is appreciably solvent exposed to be a candidate for functionalization with IA-RuBP. The additional cysteine residues on each of the other two mutants, which also include α -C45, are in relatively solvent exposed positions, with α -C196 in the immediate vicinity of FeMoco and α -C158 in a cleft right above the P-cluster (Figure 2.3). Based on a crystal structure of wt MoFeP (PDB ID: 1M1N) we could estimate the distances between the attached RuBP labels and the MoFeP metal clusters. At distances of 27 Å and 37 Å, α -C45 is too distant from both the P-cluster and FeMoco, respectively, to factor in the redox activation of nitrogenase, thus this construct should serve as a control in photoreduction reactions. On the other hand, Ru-labels on α -C158, at 14 Å, and α -C196, at 17 Å, should be coupled to either the P-cluster or FeMoco, respectively (Figure 2.7). A RuBP label attached at α -C158 should closely mimic the position of the FeP [4Fe:4S] cluster when FeP is docked to MoFeP in the central conformation. Attaching a RuBP label at α -C196 will allow the

examination of alternative electron transfer routes directly to the FeMoco. (Figure 2.8)

Covalent labeling of wt and mutant MoFeP with RuBP. A two-hour incubation of each MoFeP variant with a 10-fold molar excess of IA-RuBP lead to quantitative labeling of MoFeP as determined by inductively coupled optical emission spectroscopy. wt MoFeP was labeled with 1 molecule of RuBP per $\alpha\beta$ -dimer while both α -C158 and α -C196 had two labels per $\alpha\beta$ -dimer, as predicted. Labeled constructs will be referred to as wt-RuBP, α -C158-RuBP, and α -C196-RuBP. ICP-OES data also showed that IA-RuBP did not react with any additional cysteine residues in MoFeP, particularly those responsible for metal cluster binding. A properly formed MoFeP is expected to have 15 Fe atoms and 1 Mo atom per $\alpha\beta$ -dimer. ICP-OES data revealed that all three labeled MoFeP variants maintained an approximate 15 to 1 ratio of Fe to Mo atoms (Table 2.1).

The extent of labeling on MoFeP can also be determined by UV-vis spectroscopy because the RuBP molecule has a distinct absorbance feature between 400 and 500 nm. (Figure 2.9) Ratios determined from absorbance match well with those from ICP-OES with approximately 1 label per $\alpha\beta$ -dimer for wt-RuBP and 2 labels per $\alpha\beta$ -dimer for both α -C158-RuBP and α -C196-RuBP (Table 2.2). While we were unable to crystallize the RuBP labeled MoFeP variants to confirm labels were attached to the intended cysteine residues, SDS-PAGE gels showed RuBP-dependent fluorescence isolated on the MoFeP α -subunit.

C₂H₂ reduction activity under turnover conditions. ICP-OES results showed that modifying the surface of MoFeP with IA-RuBP did not perturb cluster binding

in the protein. However, measuring the C_2H_2 reduction activity of each labeled MoFeP variant under turnover conditions (in solution with FeP, ATP, dithionite) did reveal changes in catalytic capability. The maximum rates of C_2H_4 production from wt-RuBP and α -C196-RuBP were approximately one half and one quarter of the activity of the corresponding unmodified proteins, respectively. The H^+ reduction capability of wt-RuBP is similarly diminished, with one third the maximum rate of H_2 production compared to wt MoFeP (Table 2.3).

The decreased activity in wt MoFeP is surprising since α -C45 is far removed from both the MoFeP metal clusters and the FeP docking sites. Therefore, the reduced catalytic ability of the labeled construct may be the result of a perturbation occurring during the labeling reaction that does not affect the integrity of the metal clusters but still inactivates the enzyme. The sharp decrease in activity seen with α -C196-RuBP may be the result of the label location since the native amino acid in that position, α -histidine-196, has been implicated in proton transfer to the FeMoco.²⁸ The maximum rate of both H_2 and C_2H_4 production from α -C158-RuBP was comparable to the activity of unlabeled α -C158, suggesting that RuBP in that position does not interfere with FeP and MoFeP interactions (Table 2.3).

Conclusions

We set out to discover whether it would be possible to mimic the highly coupled state of the FeP [4Fe:4S] cluster within the MgATP-activated nitrogenase complex by attaching a transition metal photosensitizer to the surface of MoFeP,

thereby eliminating the need for FeP and ATP hydrolysis to initiate reactivity. With this aim in mind, we labeled three different MoFeP variants – wt, α -C158 and α -C196 – with a cysteine specific photosensitizer, IA-RuBP. Labeling proved efficient and specific with the expected number of RuBP molecules per $\alpha\beta$ -dimer for all 3 constructs. While attaching RuBP to the MoFeP surface had a significant effect on the turnover activity of wt-RuBP and α -C196-RuBP, ICP-OES measurements indicated that labeling did not affect the integrity of MoFeP metal clusters, paving the path to our investigations of photo-activated catalysis by this enzyme.

Figure 2.1. Docking geometries in the (a) nucleotide free (PDB ID: 2AFH), (b) MgAMPPCP (a non-hydrolyzable ATP analogue mimicking the ATP bound state) (PDB ID: 2AFK), and (c) MgADP-bound (PDB ID: 2AFI) FeP-MoFeP complexes. While the FeP shows large conformational differences between the three nucleotide dependent docking states, the MoFeP is unchanged. The coloring scheme is the same as in Figure 1.1 and the second $\alpha\beta$ -dimer of MoFeP is again omitted for clarity.

Figure 2.2. Overlay of the protein environments around the P-cluster and FeMoco from crystal structures of the nucleotide free (green), MgAMPPCP-bound (purple), and ADP-bound (cyan) FeP-MoFeP complexes. There are no large conformational changes in the surrounding residues that may indicate the location of an internal electron transfer gate.

Figure 2.3. MgAMPPCP-bound FeP-MoFeP complex showing the three proposed modification sites of the surface of MoFeP and their position relative to key components in the protein.

Figure 2.4. Growth curve for the *A. vinelandii* strain expressing the α -C158 version of MoFeP. Nitrogenase expression occurs during the derepression stage in which cell growth slows, indicated by a plateauing of OD at 600 and 660 nm and increased C_2H_2 activity from whole cell samples.

Figure 2.5. Initial purification of α -C158 MoFeP on a DEAE column. (a) Elution profile of *A. vinelandii* cell lysate separated on a DEAE ion exchange column. Protein elution is monitored by changes in absorbance at 280 nm (blue trace) and 410 nm (red trace) over the course of a 100 to 500 mM NaCl gradient. (b) Coomassie stained 15% acrylamide SDS-PAGE analysis of fractions from DEAE column trace peaks.

Figure 2.6. Further purification of the α -C158 MoFeP fractions isolated from DEAE purification on a Ni-affinity column. (a) Elution profile of purification using a step gradient with increasing imidazole concentrations to elute proteins. Protein elution was monitored by changes in absorbance at 280 nm (blue trace) and 410 nm (red trace). (c) Coomassie-stained 8% acrylamide SDS-PAGE analysis of fractions from Ni-column trace peaks.

Figure 2.7. Cartoon representations of labeling sites on (a) wt, (b) α -C196, and (c) α -C158 MoFeP and corresponding edge-to-edge distances to MoFeP clusters. (d) Structure of IA-RuBP.

Figure 2.8. Models showing RuBP attached to (a) α -C196 in the vicinity of the FeMoco and (b) α -C158 in the vicinity of the P-cluster. Cluster binding helices of the MoFeP α - and β -subunits are shown in black and gray respectively. Measurements represent the edge to edge distance between the phenanthroline and a sulfur atom in the corresponding metal cluster.

Table 2.1. ICP-OES analysis of the metal content of RuBP labeled MoFeP variants.

Protein	Fe/Mo Ratio	Expected Fe/Mo Ratio	Ru/Mo Ratio	Expected Ru/Mo Ratio	Total Points Measured
wt-RuBP	17.49 ± 4.76	15	1.14 ± 0.19	1	9
α -C196-RuBP	14.83 ± 0.21	15	1.81 ± 0.03	2	6
α -C158-RuBP	13.88 ± 0.36	15	1.87 ± 0.04	2	8

Table 2.2. Quantification of RuBP labeling per $\alpha\beta$ -dimer as determined by UV-vis absorbance of the labeled MoFeP variants.

Protein	Ru/ $\alpha\beta$ Ratio	Expected Ru/ $\alpha\beta$ Ratio	Total Points Measured
wt-RuBP	1.11 ± 0.43	1	3
α -C196-RuBP	1.95 ± 0.15	2	3
α -C158-RuBP	2.00 ± 0.71	2	8

Figure 2.9. UV-vis absorbance spectra of approximately 4 μ M of (a) wt-RuBP, (b) α -C196-RuBP and (c) α -C158-RuBP MoFeP (orange lines). Each figure also includes a trace of 4 μ M wt MoFeP (black) and either 4 μ M (a) or 8 μ M (b and c) RuBP (gray) for comparisons. All spectra were collected in the presence of 5 mM dithionite, which leads to intense absorption below 400 nm. The higher amount of Ru-label in the C158 and C196 mutants compared to labeled wt MoFeP can be visually deduced from the relative intensities of the 420 and 450 nm absorption features of the labeled proteins.

Table 2.3. H^+ and C_2H_2 reduction activity under turnover conditions for unlabeled and labeled MoFeP variants.

Protein	V_{max} Activity (nmol/mg MoFeP/min)	
	H₂	C₂H₄
wt MoFeP	2704	2534
wt-RuBP	950	1100
α -C196	-----	2348
α -C196-RuBP	-----	717
α -C158	2930	2407
α -C158-RuBP	2481	2395

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Chapter 3

Light-Driven Two-Electron Reduction Reactions Catalyzed by MoFeP-RuBP

Introduction

Photo-triggering approaches to enzymatic studies have occupied a central place in the study of biological redox systems, including Photosystem II,¹⁻³ the bacterial photosynthetic reaction center^{4,5} and DNA photolyase,⁶ which feature natural light-activated cofactors, as well as countless electron transfer proteins and enzymes, which have been decorated with artificial photosensitizers like Ru-, Re- and Os-polypyridyl complexes.⁷⁻¹⁴ Hydrogenases, in particular, are a class of enzymes somewhat similar to nitrogenase in that they also contain complex and spectroscopically challenging Fe-S clusters and an as-of-yet incompletely understood mechanism of H⁺ reduction.¹⁵ Several groups have demonstrated that hydrogenases can be readily activated for catalysis through photoactive Ru complexes coupled to electron carriers such as methylviologen^{16,17} or TiO₂ nanoparticles.¹⁸ While these efforts have primarily been motivated by the desire to develop cheap and efficient biocatalysts for H₂ production, light activation methods may help uncover the hydrogenase mechanism.

Before discussing the photoreduction of MoFeP in isolation, it is important to mention a previous light-activated system for driving nitrogenase catalysis. Druzhinin and co-workers reported that a combination of eosin (photosensitizer), NADH (reductive quencher/sacrificial donor) and MgATP-bound FeP could sustain N₂ or C₂H₂ reduction reactions by MoFeP under irradiation.¹⁹ The ATP-dependent nature of the photoreduction system indicates that the semi-reduced form of eosin most likely reduces the FeP first, which can then transfer

an electron to MoFeP along the standard pathway (Figure 3.1).¹⁹ While this approach circumvents the use of dithionite as an electron source, it still depends on FeP as the ultimate mediator of MoFeP reduction under constant ATP turnover.

Photo-inducing electron transfer in the isolated MoFeP, however, will allow us to study the mechanism of substrate reduction at the FeMoco without interference from effects related to protein interactions, for example the rate-limiting dissociation of FeP. By uncoupling nitrogenase catalysis from FeP ATPase activity, we may be able to directly study aspects of the reaction, such as reduction rates, pH dependencies, or catalytic changes from amino acid substitutions, all of which may be masked under turnover conditions. Furthermore, photoreducing MoFeP can potentially allow us to investigate two atypical aspects of nitrogenase catalysis, the possibility of non-sequential electron transfer and the existence of a protein interaction dependent, conformational change in the MoFeP that efficiently couples electron transfer from the P-cluster to FeMoco.²⁰ In this chapter, we describe the characterization of light-driven, ATP-independent catalytic reactions by MoFeP-RuBP constructs, focusing on the two-electron reduction of alternative nitrogenase substrates, H^+ and C_2H_2 .

Materials and Methods

Creating and characterizing MoFeP-RuBP constructs. Nitrogenase MoFeP purification from *A. vinelandii* cells and subsequent reaction with $\text{Ru}(\text{bpy})_2(\text{phenIA})$ was done as described in the previous chapter. The three constructs mentioned herein, wt-RuBP, α -C158-RuBP and α -C196-RuBP were characterized using UV-Vis absorbance and ICP-OES as also previously described. The results from assays to determine turnover activity with H^+ and C_2H_2 substrates are reported in the previous chapter.

Assays for light-driven catalysis by nitrogenase. All photoreduction experiments, controls and CO inhibition assays were carried out in 14-mL rubber septum (VWR) sealed glass vials flushed with Ar. Unless otherwise noted, reaction mixtures contained 2.76 mg of MoFeP, 200 mM dithionite, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and 200 mM NaCl in a final volume of 9 mL and a pH of 7.75. In experiments determining the effect of dithionite concentration on activity, buffer pH was varied to account for increasing acidity with higher dithionite concentrations so that the final reaction pH was near 7.75. Samples testing the effect of alternate sacrificial electron donors on photoreduction activity contained 200 mM of either triethanolamine (TEOA), 2-(*N*-morpholino)ethanesulfonic acid (MES) or 40 mM NADH, due to the lower solubility of this molecule. CN^- inhibition and protein concentration dependence assays were carried out in 2.8-mL septum sealed glass vials flushed with Ar. The effect of protein concentration on substrate photoreduction was determined using 0.1, 0.2, 0.3 and 0.6 mg/mL MoFeP (0.4, 0.9, 1.3 and 2.5 μM) in

a solution of 100 mM Hepes, 200 mM NaCl and 200 mM dithionite with a final reaction volume of 1.68 mL. The final solution pH was 7.75. In CN^- inhibition assays, 50 mM sodium cyanide (NaCN) was first dissolved in the reaction buffer, the pH was adjusted back to 7.7 by the addition of 5 M HCl and subsequently added to assay vials, such that the final CN^- concentration was either 0.25 or 1 mM. Photoreduction assay vials were submerged in a temperature-controlled water bath equipped with a Neslab water circulator and the temperature was maintained at 20°C. For C_2H_2 photoreduction assays, the vials contained either 0.2, 0.1 or 0.05 atm C_2H_2 in Ar, achieved by adding appropriate volumes of a 1 atm C_2H_2 stock with a gas-tight syringe, for standard C_2H_2 photoreduction, CO inhibition and CN^- inhibition experiments, respectively. For H^+ photoreduction experiments, no other gas than Ar was present. After addition of substrates or inhibitors, vials were vented to 1 atm and allowed to equilibrate for 15 minutes. Photoreduction assays were performed using illumination with a 400-W Oriel66023 Hg/Xe lamp (Newport Corporation). Light was passed through a 75-mm plano convex lens (Rolyn Optics) to focus the beam and remove UV light <280 nm, and a 3.4-inch liquid filter (Oriel, model 6214) to remove infrared radiation and prevent sample heating (Figure 3.2). The final power at the sample was measured to be 0.69 watts between 380 and 500 nm. Alternatively, a 455-nm LED unit (Thor Labs) was utilized for specific excitation of the Ru-photosensitizer. The LED output was 0.31 watts.

Reaction progress was monitored continuously by removing (with gastight syringes) either 0.5 mL or 50 μL from the vial headspace at predetermined times

to measure H_2 or C_2H_4 produced, respectively. For H^+ photoreduction experiments, 0.5 mL of Ar (or 0.5 mL of appropriate gas mixture, such as Ar/CO or Ar/CO/ C_2H_2) was added back after each sampling point to compensate for the drop in sample pressure. H_2 and C_2H_4 were detected by a thermal conductivity and a flame ionization detector, respectively, on a SRI Instruments 8610 gas chromatography instrument. H_2 was separated on a 5-Å molecular sieve column (10'x1/8"x0.085" SS) (Alltech), using N_2 as the carrier gas at an oven temperature of 80°C. C_2H_4 was separated on an Alumina column (5'x1/8"x0.85" SS) (Alltech) using He as the carrier gas at an oven temperature of 150°C. Injection volumes were normalized by Ar and C_2H_2 injection peak areas. Product concentrations were determined using calibration curves constructed from H_2 and C_2H_2 standards.

Synthesis of $\text{NH}_2\text{-RuBP}$. Synthesis of a non-reactive RuBP derivative, $[\text{Ru}(2,2'\text{-bipyridine})_2(5\text{-amino-1,10-phenanthroline})](\text{PF}_6)_2$ ($\text{NH}_2\text{-RuBP}$) was done according to the same procedure used for (IA-RuBP) as described by Castellano *et. al.*, except 5-amino-1,10-phenanthroline was used instead of 5-Iodoacetamido-1,10-phenanthroline.²¹

EPR analysis. EPR data were collected at 5 K on a Bruker ElexSys 580 spectrophotometer equipped with a liquid helium continuous flow cryostat located at the CalEPR facility at the University of California, Davis. All EPR spectra were recorded in the perpendicular mode with a microwave frequency of 9.6 GHz, a microwave power of 1 mW, a modulation amplitude of 8.06 G, a conversion time of 4.10 ms/point and a time constant of 8.20 ms. Each spectrum

is the average of 5 scans. All EPR samples contained 20 mg/mL MoFeP in 200 mM dithionite, 100 mM Tris buffered at pH 7.7 and 200 mM NaCl (similar to photoreduction assay conditions). Samples were prepared in standard 4-mm quartz EPR tubes from Wilmad LabGlass. Non-illuminated samples were flash frozen in liquid N₂ after mixing, while illuminated samples were exposed to light for 10 minutes before freezing. The illumination setup and conditions for EPR samples were identical to those described for photoreduction assays.

C₂H₂ photoreduction action spectrum. The efficiency of α -C158-RuBP C₂H₂ reduction as a function of illumination wavelength was determined by measuring the amount of C₂H₄ produced as a result of irradiation through a set of long-pass filters. (Schott North America) C₂H₂ photoreduction samples were prepared as described above for normal assays and reactions were allowed to proceed for 30 minutes before measuring product formation using GC. The intensity of the incident light was not adjusted to account for decreased photon numbers reaching the sample as a result of the filters. Therefore, the resulting changes in activity represent a qualitative rather than quantitative trend.

Determination of NH₃ produced by N₂ photoreduction. Assays intended to determine the concentration of NH₃ produced during photoreduction of N₂ were based on the method described by Corbin²², which utilizes the reaction of NH₃ with o-phthalaldehyde mercaptoethanol to form a fluorescent product. Photoreduction samples testing N₂ reduction were prepared as described above except that the reaction headspace was exchanged from Ar into N₂ before illumination. The OPT reagent was prepared by dissolving o-phthalaldehyde (TCI

America) and mercaptoethanol in 0.2 M phosphate buffer at pH 7.3 and was then allowed to stand overnight under argon to reduce background fluorescence. Determination of NH_3 was carried out by taking 25 μL samples of photoreduction reactions at regular time intervals during illumination and mixing them with 1.0 mL of the OPT reagent. The reaction was allowed to proceed in the dark for 60 min at which time samples were analyzed for fluorescence. Instead of measuring products on an HPLC, a 250 μL sample of each OPT reacted photoreduction sample was analyzed by separation on a 3-mL reverse phase column (Resource RPC, GE Healthcare) connected to a Shimadzu RF-10AXL fluorescence detector. In addition to measuring NH_3 produced in the presence of N_2 , the amount of H_2 produced under the same conditions was also determined. H_2 measurements were done using the protocol described for standard H^+ photoreduction time courses.

Determination of N_2H_4 produced by N_2 reduction. All N_2 photoreduction assays were also tested for the presence of hydrazine (N_2H_4). N_2H_4 was quantified by reaction with p-dimethylaminobenzaldehyde which forms a yellow product.²³ Total N_2H_4 produced was determined by comparing absorbance at 458 nm with standards prepared by diluting 98% pure solution of N_2H_4 monohydrate (Alfa Aesar) into the Hepes and NaCl photoreduction buffer described above.

Results and Discussion

Light-driven two-electron reduction reactions. In order to investigate light-driven activation of MoFeP, we pursued the reduction of alternative substrates, H^+ and C_2H_2 , which require only two electrons and whose products, H_2 and ethylene (C_2H_4), are readily detected by gas chromatography. A typical reaction included 2.7 mg of RuBP-labeled protein, which is approximately 20 nmol of active sites, in a solution of 200 mM dithionite and 200 mM NaCl at pH 7.75. In nitrogenase turnover assays, dithionite also acts as an O_2 scavenger employed to protect MoFeP clusters from oxidative damage and to maintain the P-cluster in an all-ferrous state (P^{N}). In our reaction mixture, dithionite functions primarily as a sacrificial electron donor, necessitating a much higher concentration than typically used in nitrogenase assays. In deoxygenated vials, the reaction solutions were irradiated in a 20°C water bath with a Hg/Xe lamp using UV- (<300 nm) and IR-cutoff filters under constant stirring, and the headgas was analyzed for products.

Wt-RuBP and α -C196-RuBP MoFeP showed little to no production of H_2 or production of C_2H_4 in the presence of 0.1 atm of C_2H_2 even after 200 min of irradiation. In contrast, irradiation of α -C158-RuBP led to the evolution of both products in equal quantities, with average velocities of 16 nmol C_2H_4 /min and 14 nmol H_2 /min per mg MoFeP over 50 min. Photo-driven C_2H_4 and H_2 production reached a plateau after 50 min despite the presence of excess dithionite, yielding a turnover number of ~110 per active site for both products (Figure 3.3 and Figure 3.4). The EPR spectrum of α -C158-RuBP MoFeP showed no changes in

the characteristic $S = 3/2$ feature of FeMoco after turnover, indicating that the cofactor stays intact (Figure 3.5). On the other hand, the absorption features of α -C158-RuBP attributed to the RuBP label steadily disappeared during turnover, which may be attributable to Ru-ligand dissociation²⁴⁻²⁶ and explain the gradual loss of activity (Figure 3.6).

The characterization of α -C158-RuBP catalyzed two-electron reduction reactions. We next investigated whether C_2H_4 and H_2 production indeed stemmed from our photoreduction scheme and not from an unforeseen reactive site or species. The elimination of any of the reaction components, light, the Ru-photosensitizer, MoFeP, or dithionite, led to the complete abolishment of C_2H_4 and H_2 evolution. When a non-reactive RuBP derivative, NH_2 -RuBP, was included in solution with α -C158 instead of the covalently linked species, no activity was observed, indicating that a surface immobilized Ru-photosensitizer was necessary to ensure efficient electron injection and electronic coupling to the MoFeP clusters (Figure 3.7). Photocatalytic activity was found to be linearly dependent on the concentration of both α -C158-RuBP and dithionite (Figure 3.8 and Figure 3.9).

Alternate electron donors and a possible reaction scheme. As previously mentioned, there are several examples in the literature of light-driven catalysis by enzymes decorated with photosensitizers. These studies utilize a wide variety of molecules as sacrificial reductants depending on their reaction conditions. Interestingly, when dithionite is replaced in our system with other typical sacrificial donors such as TEOA (200 mM), MES (200 mM), or NADH (40 mM), little

photocatalytic activity is observed (Figure 3.9). This specificity may simply be because dithionite is ideally suited for our photoreduction system, with a reduction potential of approximately -300 mV²⁷ and high enough solubility enabling the use of concentrations that result in diffusion limited quenching. A likely reaction scheme describing electron transfer through α -C158-RuBP including dithionite as a sacrificial donor relies on this diffusion-limited quenching of the Ru^{II}BP excited state (*Ru^{II}BP) to generate the reducing Ru^IBP ($E^\circ \approx -1.28$ V)²⁸ species in high yield. The Ru^IBP species then initiates the observed photoreduction activity by donating an electron to the P-cluster (Figure 3.10).

Conversely, generating the Ru^IBP reactive species may not be the only role dithionite plays in MoFeP photoreduction. An action spectrum of C₂H₂ photoreduction activity as a function of irradiation wavelength showed a sharp decrease in activity when filters were used to remove wavelengths less than 395 nm although our RuBP photosensitizer should be excited near 450 nm (Figure 3.11).

In order to test whether this unique ability of dithionite ($\lambda_{\text{max}} = 314$ nm) for supporting catalysis is due to its photochemistry, we carried out activity assays and control experiments using monochromatic 455-nm radiation from an LED source that should only excite the Ru-photosensitizer and not dithionite. These experiments show that photocatalytic activity is maintained at this wavelength, albeit with a lower turnover number, ~35 per active site as compared to ~100 per active site with broad wavelength Hg/Xe lamp illumination, and slower kinetics although MoFeP is considerably more stable under these conditions. α -C158-

RuBP irradiated with 455-nm LED light showed steady activity for at least 300 min, likely due to the elimination of UV-based damage. (Figure 3.12) While these findings confirmed again that the light-driven reactivity stems from the surface immobilized Ru-photosensitizer, they also suggested a possible role for dithionite beyond acting as a sacrificial donor.

Photoreduction inhibition by CO and CN. Having established the catalytic competence of the photosensitized α -C158-RuBP MoFeP system, we sought to determine whether FeMoco is the site of substrate activation. Carbon monoxide (CO) has been shown to interact with FeMoco under turnover conditions and to be a strong inhibitor of all nitrogenase substrates except H^+ .^{29,30} The inclusion of 0.05 atm of CO alongside 0.1 atm of the substrate C_2H_2 abolished light-driven C_2H_4 production (Figure 3.13). On the other hand, H_2 was produced at high levels in the presence of the same amount of CO or CO/ C_2H_2 , matching the inhibition profile for ATP-driven nitrogenase catalysis (Figure 3.13). Similarly, CN^- stalled the photolytic C_2H_2 reduction activity of α -C158-RuBP, with an apparent inhibition midpoint concentration of ~ 0.25 mM CN^- (at 0.05 atm C_2H_2) that agreed well with a previously reported value ($K_i = 0.5$ mM) obtained for wt MoFeP under ATP-driven turnover conditions (Figure 3.13).³¹ These findings strongly imply that FeMoco is the ultimate destination for the photogenerated electrons and the site of catalysis.

Reduction of N_2 . Given that the α -C158-RuBPcatalyzed reduction of H^+ and C_2H_2 appears to occur in a similar, if not identical, manner to catalysis in the native nitrogenase complex, we decided to determine if N_2 could be reduced

under the same conditions. Photoreduction reaction mixtures were thoroughly exchanged into N_2 , however, samples of the reaction mixture taken at predetermined times showed no NH_3 formation. Similarly, N_2H_4 , a possible 4-electron reduction product formed as an intermediate during N_2 reduction, was not detected. It is possible that the photoreduction system produced NH_3 or N_2H_4 in concentrations below the assay detection limit or that another component of the reaction mixture may interfere with the NH_3 detection assay. However, we also compared H^+ reduction in an Ar atmosphere to that in an N_2 atmosphere and saw no change in the amount of H_2 produced (Figure 3.14). Since H_2 formation should be considerably lower if N_2 reduction is occurring, we can conclude that the photoreduction system in its current form cannot reduce N_2 .

Conclusions

Our results characterizing the light-driven, two-electron reduction of substrates by the isolated MoFeP challenge the long-standing belief that FeP and ATP-hydrolysis are absolutely necessary for the reduction of substrates by FeMoco. Forcefully or not, it is possible to inject catalytically productive electrons into FeMoco, and to do this rapidly through light triggering. However, the efficiency of the light-driven, "ATP- and FeP-less" system is only about 1% of the native enzyme complex. This may indicate FeP is not just a simple electron donor to MoFeP but is also involved in coordinating electron transfer, proton transfer,

substrate binding and eventual reduction reactions, as has been suggested but without direct evidence.³²

There are some intriguing explanations for the uniqueness of FeP as a redox activator in nitrogenase catalysis. Various studies have determined that electrons can be transferred between nitrogenase proteins from the FeP [4Fe:4S] cluster to the P-cluster and within the MoFeP from the P-cluster to the FeMoco. However, it is unlikely that inter-protein electron transfer occurs first because the resting state of the P-cluster contains only ferrous iron atoms.^{33,34} Instead, a brief conformational change in the MoFeP may induce initial electron transfer from the P-cluster to the FeMoco, a theory known as the “deficit spending” model²⁰. A subsequent inter-protein transfer from the [4Fe:4S] cluster then may reduce the oxidized P-cluster back to the resting state. This conformational change thereby effectively gates MoFeP electron transfer and may be the result of surface interactions between FeP and MoFeP.²⁰

The possibility of a protein-interaction-dependent electron transfer gate is supported by crystal structures of FeP and MoFeP complexes captured in different nucleotide-bound states in which FeP occupies distinct docking sites on the MoFeP surface. These nucleotide-dependent docking conformations imply a unidirectional motion of FeP on the MoFeP surface over 30-40 Å as it is binding and hydrolyzing MgATP, which is more reminiscent of a motor protein moving on its track rather than the interactions between two redox partners.³⁵ Unidirectional movement through multiple docking geometries may trigger catalytically necessary, but crystallographically undetectable changes in the

MoFeP. In the absence of FeP and ATP hydrolysis, such intermediates would be inaccessible in our photoreduction scheme in its current state and would explain the inefficient electron coupling between the photosensitizer and the MoFeP metal clusters.

The study of any major aspect of nitrogenase catalysis has been hampered due to its dependence on continuous ATP hydrolysis and transient protein interactions, which result in a heterogeneous mixture of redox and nucleotide-bound states of nitrogenase in solution. While recent studies have provided glimpses into substrate interactions on FeMoco, a detailed picture of nitrogenase catalysis is still not available. Through the experiments described in this chapter, nitrogenase catalysis was successfully uncoupled from ATP hydrolysis and protein-protein interactions by introducing a light-triggered electron delivery system, which should enable the study of ET dynamics within MoFeP and the population of discrete redox intermediates for structural investigations. Presently, the quantum yield (ϕ = catalytically useful electrons/photons absorbed by Ru^{II}BP) of our light-driven system is <1% (Appendix 1), which hints at a possible gated electron transfer mechanism in MoFeP and is likely the reason why α -C158-RuBP fails to produce significant levels of NH₃ from N₂. However, these initial findings present a starting point from which to begin optimizing our system, with the intention of both increasing yields of electron injection and uncovering the nature of any conformational gating events that occur within MoFeP during turnover.

Figure 3.1. Cartoon depiction (a) and proposed reaction scheme (b) for eosin-mediated photoinduced nitrogenase reduction of C_2H_2 or N_2 under ATP turnover conditions.

Figure 3.2. Front (a) and side (b) view of the illumination set-up for MoFeP-RuBP photoreduction activity assays.

Figure 3.3. Three MoFeP-RuBP constructs, (a) wt-RuBP, (b) α -C159-RuBP and (c) α -C158-RuBP and their corresponding H⁺ reduction activities under the photoreduction conditions described in the Materials and Methods section. Only the α -C158-RuBP construct produces H₂ above background levels when illuminated.

Figure 3.5. X-band EPR spectra (5 K) of Ru-C158 MoFeP collected before and after 10 minutes of irradiation under the same conditions as photo-driven activity assays, showing that the $S=3/2$ signature of FeMoco does not change. Small features near 1450 and 2100 Gauss are attributed to impurities; they are not observed in other active batches of Ru-C158 MoFeP

Figure 3.6. (a) Specific bleaching of the RuBP absorbance feature, located between 400 to 500 nm, of α -C158-RuBP sample under light-driven reaction conditions. Each sample contained 3 mg MoFeP, 200 mM sodium dithionite, 100 mM Hepes (pH 7.5) and 200 mM NaCl. (b) Close-up detail highlighting the gradual bleaching of RuBP absorbance during illumination. (c) The reduction in α -C158-RuBP absorbance at 450 nm as a function of illumination time. The decay kinetics for RuBP absorbance are monoexponential, with $\tau = 32.9 \pm 1.9$ minutes.

Figure 3.7. α -C158-RuBP H^+ (a) and C_2H_2 (b) photoreduction assays and corresponding controls. Each control has a single component of the assay missing resulting in the abolishment of photoreduction activity. (c) C_2H_2 photoreduction dependence on high intensity light for product formation.

Figure 3.8. (a) C_2H_2 photoreduction with different concentrations of α -C158-RuBP. (b) Linear dependence of light-driven C_2H_4 production on Ru-C158 MoFeP concentration, indicating that reactivity stems from Ru-C158 MoFeP. Points on the graph indicate the total C_2H_4 produced after 90 minutes. These experiments were carried out in a smaller scale compared to those shown in Figure 3.7 a and b. (2.8 mL septum sealed glass vials with 0.17, 0.34, 0.50 and 1.0 mg protein).

Figure 3.9. (a) H^+ photoreduction catalyzed by α -C158-RuBP in the presence of different concentrations of sodium dithionite. (b) Linear dependence of hydrogen production on dithionite concentration, indicating that dithionite may have a second role in light-driven reactivity in addition to acting as a sacrificial electron donor. (c) Hydrogen production from illuminated α -C158-RuBP with alternate electron donors compared to activity in the presence of dithionite.

Figure 3.10. Cartoon depiction (a) and proposed reaction scheme (b) for light induced 2-electron reduction of C_2H_2 and H^+ by α -C158-RuBP in the presence of excess dithionite (DT) and absence of FeP.

Figure 3.11. (a) Action spectrum showing C₂H₄ production after 30 minutes illumination as a function of illumination wavelengths. (b) Absorbance spectra of 0.12 μ M α -C158-RuBP (orange line) and filters (dashed lines) used to adjust illumination wavelengths. (c) Absorbance spectra showing the contribution of individual reaction components to the overall spectrum of α -C158-RuBP, including the strong absorbance of 200 mM dithionite below 400 nm.

Figure 3.12. (a) C_2H_4 production by α -C158-RuBP driven by 455-nm LED excitation and corresponding controls. (b) Close-up view of controls in which each reaction is either missing a necessary component, the protein used is wt-RuBP or TEOA is used instead of dithionite (DT) as an electron source.

Figure 3.13. (a) Carbon monoxide inhibition of light-driven C_2H_2 reduction by α -C158-RuBP. H_2 formation is not affected by the presence of CO, matching the inhibition profile for ATP-driven nitrogenase catalysis. (b) Removing CO from reaction vials restores C_2H_4 production indicating CO inhibition is reversible. (c) Cyanide inhibition of light-driven C_2H_2 reduction by α -C158-RuBP. These experiments were performed with ~ 0.1 mg α -C158-RuBP.

Figure 3.14. (a) α -C158-RuBP does not reduce N_2 by either 6 electrons to NH_3 or 4 electrons to N_2H_4 under the reaction conditions resulting in successful 2 electron transfer. (b) H_2 production by illuminated α -C158-RuBP remains at the same level in both Ar and N_2 atmospheres.

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Chapter 4

Modified Methods for Site-directed Mutagenesis of MoFeP from *A. vinelandii*

Introduction

The creation of genetically altered *Azotobacter vinelandii* strains is a crucial component in most nitrogenase studies, facilitating research into nitrogenase biosynthesis,¹⁻³ cluster assembly⁴⁻⁸, and catalysis⁹. For mechanistic studies in particular, the combination of site directed mutagenesis of the MoFeP and spectroscopy has been the most effective method so far for isolating species that may be reaction intermediates.^{10,11} In addition, studying the reactivity of altered nitrogenase proteins has led to the identification of catalytically important residues in the MoFeP and a potential substrate binding face on FeMoco.^{9,12-15}

Combining site-directed mutagenesis with RuBP-based MoFeP photoreduction studies allows us to expand the utility of our system from simply photo-generating products to studying the mechanism of isolated MoFeP. By observing the effects of amino acid substitutions on photoreduction activity, we hope to identify structural or electronic features that are critical for controlling electron transfer between the P-cluster and the FeMoco. Furthermore, it has been shown that MoFeP with specific mutations near the active site can bind but not fully reduce certain substrates.^{13,16} Incorporating these or similar mutations into RuBP-labeled MoFeP may enable us to photo-generate and trap specific reaction intermediates in high enough yield to study with crystallography or spectroscopic techniques such as EPR or NRVs.

The Nif Gene Cluster

In *A. vinelandii*, the genes involved in nitrogen fixation are organized into two clusters on the bacterial chromosome known as the major and minor *nif* (nitrogen fixation) clusters (Figure 4.1). The major *nif* cluster contains the genes that code for FeP, and the α and β subunits of MoFeP, known as *nifH*, *nifD* and *nifK*, respectively. In addition to these structural components, the formation of active nitrogenase requires the participation of at least eight other gene products, mostly involved in biosynthesis and incorporation of the P-cluster and FeMoco.^{8,17} Like FeP and MoFeP, many of these biosynthetic proteins are easily deactivated by the presence of oxygen. Together, the large number of obligatory gene products and their oxygen sensitivity necessitate the purification of nitrogenase proteins from a native, nitrogen-fixing host strain instead of from more common systems such as over-expression vectors in *Escherichia coli*. Consequently, the generation of mutant nitrogenase proteins in *A. vinelandii* requires directly altering the bacterial chromosome.¹⁸

Genetic Engineering in *Azotobacter vinelandii*

The ability to incorporate exogenous genetic material into the genome of *A. vinelandii* was first discovered in the 1970s, when it was noted that liquid cell cultures starved of iron could take up chromosomal DNA.^{19,20} While the conditions resulting in optimal competency and transformation efficiency have been thoroughly studied, the exact details of DNA binding, uptake and incorporation are still unknown.²¹⁻²³ There is some experimental evidence that

suggests *A. vinelandii* competency may be related to increased siderophore production under iron-limited conditions. Iron deprivation induces the production of a fluorescent green siderophore known as azotobactin and the expression of several large membrane proteins that may serve as siderophore receptors.^{24,25} These changes to the membrane may influence binding of exogenous DNA, however a definitive correlation between azotobactin production and transformation efficiency has yet to be determined.

Current methods for inducing competency in *A. vinelandii* result in transformation frequencies as high as 5% (transformation frequency = number of transformants/ μ g plasmid DNA added).^{23,26} However, this number does not reflect mutation success rate because *A. vinelandii* cells contain approximately 40 copies of their genetic material per cell.²⁷ For the mutant protein to be exclusively expressed, each copy of the chromosome needs to incorporate the new DNA or be somehow inactivated. Since only a small percentage of competent cells will be fully mutated, transformation cultures need to be screened to identify cells containing the desired mutations.

Typically, isolating a successful *A. vinelandii* mutant requires using a combination of multiple screening strategies. A preliminary screening technique such as congression, or the co-transformation of two plasmids, can be used to eliminate any non-competent cells from the transformation pool (Figure 4.2). In this method, the additional plasmid carries a selectable marker such as an antibiotic resistance gene, and therefore only competent cells can survive under the selection conditions.² A second, more specific round of screening can then

be done based on nitrogenase activity. Mutations to the necessary *nif* genes can result in two general phenotypes, N⁺, in which the cells retain their ability to fix nitrogen, and N⁻, in which the cells do not. Mutant strains with N⁻ phenotypes can easily be identified by streaking colonies onto plates with and without a fixed nitrogen source such as NH₄Cl. Colonies that only grow in the presence of NH₄ are then sequenced to confirm the expected mutations.¹⁸

Mutations that result in functional nitrogenase genes can be more difficult to identify because cells may only partially incorporate exogenous DNA. Therefore, N⁺ mutations are typically constructed through a two-step process, utilizing a preliminary transformation to create an appropriate N⁻ strain and then a second 'rescue' transformation to incorporate the desired mutation.¹⁸ The most comprehensive method for this technique involves removing an entire *nif* gene to create a Δnif competent cell strain. A second transformation with a plasmid containing the deleted gene plus the mutation restores nitrogenase activity. Chromosomes that do not undergo homologous recombination cannot express the deleted gene and only mutant copies of the protein are produced.²⁸

While this method does ensure that only nitrogenase proteins containing the desired mutation are produced, we have found that our transformation efficiencies into Δnif competent cells are extremely low. Furthermore, generating the initial Δnif strains was similarly inefficient. Therefore, to create our N⁺ mutants, we have employed a modified version of this deletion-rescue protocol that involves first creating a mutation specific competent cell strain with a 3-5 amino acid deletion centered on the desired mutation site.

Materials and Methods

Plasmids used in A. vinelandii transformations. Initial plasmids used to construct *nifD* and *nifK* mutants in *A. vinelandii* were kindly provided by Dr. Markus W. Ribbe (University of California, Irvine). Plasmid pYM001 contains a *nif* cluster fragment from 1051 base pairs before the start codon of *nifH* to 400 base pairs after the stop codon of *nifD* in a pGem-T Easy vector (Promega) and includes a *nifD* insertion coding for an N-terminal 8 histidine tag between amino acids 3 and 4 of the α -subunit. Plasmid pYM002 contains a *nif* cluster fragment from 500 bases before the start codon of *nifK* to 500 base pairs after the stop codon of *nifK*, also in a pGem-T Easy vector. Plasmid pDB303 contains a 4.5 kbase DNA fragment encoding rifampicin resistance in a pUC8 vector (Sigma). (Figure 4.3)

Creating $\Delta nifD$ and $\Delta nifK$ plasmids. The entire *nifD* gene in plasmid pYM001 and the majority of the *nifK* gene in plasmid pYM002 were incised using a Kpn1 restriction enzyme digest according to the protocol from New England Biolabs. The Kpn1 restriction enzyme was chosen based on previous studies creating deletions in the *nif* gene cluster². There are no Kpn1 cut sites in the pGemT-Easy plasmid and 4 sites in the *nifHDK* gene sequence: *nifH* base pair 204, *nifD* base pairs 308 and 1130 and *nifK* base pair 923. Linearized plasmids were gel purified on a 1% agarose gel with the Promega Wizard SV Gel and PCR Clean-up System and then ligated with T4 DNA Ligase according to the New England Biolabs protocol. The ligation product was transformed into XL1-Blue competent

cells (Agilent) and resulting ampicillin resistant cell colonies were screened for the correct plasmid by sequencing performed at Retrogen Incorporated.

*Transforming $\Delta nifD$ and $\Delta nifK$ plasmids into *A. vinelandii*.* wtAvin cells were made competent as described previously. $\Delta nifD$ and $\Delta nifK$ plasmids were isolated from midiprep cultures and transformed according to the procedure for creating N^- mutations described in the Material and Methods section. Transformation cultures were screened for cells presenting an N^- phenotype by streaking on gridded B^+ and B^- plates containing 5 $\mu\text{g/ml}$ rifampicin. Colonies that could not grow in the absence of a fixed nitrogen source were further screened as previously described.

Transformation of α -C45A-I159C into $\Delta nifD$ cells. Site directed mutagenesis was used to incorporate two mutations into plasmid pYM001, changing α -45^{Cys} to α -45^{Ala} and α -159^{Ile} to α -159^{Cys}. The $\Delta nifD$ cell line (LJ018) was made competent and plasmid was isolated and transformed as previously described. Resulting competent cell cultures were plated onto B^- plates and colonies were further screened using sequencing as already detailed.

*Construction of plasmids with *nif* deletions and amino acid substitutions.* Plasmids containing deletions in either *nifD* or *nifK* and plasmids coding for amino acid substitutions were constructed using site-directed mutagenesis according to the Agilent QuikChange protocol (Agilent Technologies). PCR primers were designed based on the *nifD* and *nifK* gene sequences from the *A. vinelandii* genome and nucleotides for amino-acid substitutions were chosen based on codon usage in the *A. vinelandii nif* gene clusters¹⁷. Mutagenic plasmids were

transformed into XL1-Blue competent cells and mutations were confirmed using sequencing performed by Retrogen Incorporated. Sufficient quantities of mutagenic plasmid for transformations were isolated from 100 to 200 mL LB cultures using a plasmid midi-prep purification kit (Promega). Isolated plasmid dna was the further purified and concentrated using a standard protocol for ethanol precipitation.

A. vinelandii cell culture. *A. vinelandii* cells were grown in Burk's media (B), a sucrose based media enriched with 0.9 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.67 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 36 μM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 2 μM $\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$. Cell cultures without a fixed nitrogen source (B⁻) contained 10 mM KH_2PO_4 , added to the media directly before inoculation. Cultures with a fixed nitrogenase source contained 10 mM KH_2PO_4 and 2 mM NH_4Cl (B⁺), added directly before inoculation. Burk's media for competent cell cultures was prepared without $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (B-Fe⁻).

Induction of competency in A. vinelandii cells. *A. vinelandii* cells containing wild type nif genes (denoted as wtAvin) were made competent using a modified version of the protocol described in the literature. Frozen DMSO stocks of wtAvin cells were streaked on B⁺-Fe⁻ agar plates. Cells were grown at 30°C for two days and were then re-streaked onto a second B⁺-Fe⁻ plate. This process was repeated two more times for a total of 4 growths. Cells from the fourth plate were then used to inoculate a 50 mL B⁺-Fe⁻ culture in a 250 mL Erlenmeyer flask and cultures were grown at 30°C for 20 to 24 hours in a standard laboratory shaker set at 170 rpm. The lower shaking rpm decreases the concentration of O₂ in the wtAvin cultures and further stresses the cells, resulting

in better competency than iron starvation alone. After approximately 20 hours, cell cultures have an intense fluorescent green color due to siderophore production, indicating the cells are competent for transformation. Competent cells were then immediately used for transformation.

A. vinelandii transformations. Transformation cultures contained 50 μ L of competent cells, 30 μ g mutagenic plasmid dna and 1 μ g pDB303, in 100 μ L of 21.5 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffered at pH 7.2 with 17 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Transformation cultures were incubated at 30°C for 30 minutes without shaking. The transformation was then stopped by adding 1.5 mL B^+ - Fe^+ media to the mixture and cultures were grown overnight at 30°C with 200 rpm shaking. Competent cells used in the second step of creating N^+ mutants already contain rifampicin resistance and, therefore, congression cannot be used to initially screen transformation cultures for competency. In this case, the transformation proceeds as described but transformation mixtures do not include the pDB303 plasmid.

Screening transformation cultures for A. vinelandii deletion mutants. All deletion mutations were the result of transformations into wtAvin cells and have clear N^- phenotypes. Transformation cultures grown overnight were plated onto B^+ agar plates containing 5 μ g/mL rifampicin and then incubated at 30°C. Colonies typically appeared after 2 to 4 days. Colonies were then streaked side by side on corresponding, gridded B^+ and B^- agar plates. Gridded plates were incubated at 30°C for 2 to 4 days and any colonies that did not grow on B^- agar plates were further screened. Colony PCR according to the protocol in the

Epicentre® Biotechnologies FailSafe™ PCR PreMix Selection Kit was used to isolate the *nifD* and *nifK* genes from cells with following primers:

<i>nifD</i> residues 1-241	5'-GAGCTCGAAGAGCTGCTGAT-3'
	5'-TAGCAGTGAACCAGGTTTCAGC-3'
<i>nifD</i> residues 238-492	5'-CATCGGCGACTACAACATC-3'
	5'-TCCTGGTATTCCTGGTGG-3'
<i>nifK</i> residues 1-380	5'-ATGCACTCCTGGGATTATCC-3'
	5'-CACTTCTCCGGATCGGAGA-3'
<i>nifK</i> residues 242-524	3'-CGAGACCTACCTGGGCAAC-3'
	5'-TCATGCTCCAGGGACACC-3'

PCR products were purified using the Promega Wizard SV Gel and PCR Clean-up System and were then sent for sequencing by Retrogen Incorporated. Successful mutations contained only the desired substitution or deletions and no additional changes in the *nifD* or *nifK* genes. Transformation efficiency was calculated based on the number of colonies with an apparent N⁻ phenotype divided by the total number of colonies screened, but not all colonies with N⁻ phenotype were sequenced to confirm mutations.

Screening transformation cultures for A. vinelandii N⁺ mutants. All N⁺ mutations were the result of transforming a mutagenic plasmid into a corresponding N⁻ competent cell strain containing a 3 to 5 amino acid deletion centered on the desired point mutations. Transformation cultures were plated directly onto B⁻ agar plates and incubated at 30°C. Colonies appeared after 2 to 4 days and were then streaked onto gridded B⁻ plates to confirm growth in the

absence of NH_4Cl . Colonies that grew in the absence of NH_4Cl were screened for the desired mutations according to the same procedure as described for N⁻ mutations. Successful mutations contained only the desired substitution and no additional changes in the *nifD* or *nifK* genes.

Cell growth and protein purification. Cell growth, protein purification, and activity characterization of labeled protein were performed as previously described in Chapter 2.

$\text{Ru}(\text{bpy})_2(\text{phenIA})$ synthesis and MoFeP protein modification. $\text{Ru}(\text{bpy})_2(\text{PhenIA})$ was synthesized and purified MoFe protein was modified as described in Chapter 2.

Results and Discussion

Construction of Δnif competent strains. A previously described strategy for generating point mutations in the *nifD* and *nifK* genes of *A. vinelandii* employed what is known as a marker-rescue procedure, in which a mutation plasmid is used to rescue the nitrogenase activity of a strain containing a full *nifD* or *nifK* deletion. While this simplifies screening transformation cultures and ensures selected cells will only produce the desired mutant version of MoFeP, we found both the generation of the initial deletion strains and the second rescue transformation had extremely low efficiencies. Nonetheless, we did manage to isolate the $\alpha\text{-C45A-I159C}$ mutation according to a modified version of this protocol. Creation of the linearized $\Delta nifD$ and $\Delta nifK$ plasmids using a KpnI digest was straightforward and efficient with an approximate 75% recovery of the initial

plasmid. Ligation of the plasmid and then transformation resulted in 2 new plasmids, each missing a significant portion of the *nifHDK* gene sequence.

Transformation of the $\Delta nifD$ and $\Delta nifK$ plasmids into wtAvin competent cells proved to be extremely inefficient, with a far lower yield than the 1-2.5% success rate typically reported for *A. vinelandii* transformations. Three attempts at transforming $\Delta nifD$ and $\Delta nifK$ plasmids into wtAvin with subsequent screening of 150 – 200 colonies per transformation resulted in no successful mutations. A fourth attempt to transform $\Delta nifD$ into wtAvin resulted in 1 successful mutation from a pool of 170 colonies, an efficiency of ~0.6 %. We have been unable to successfully transform the $\Delta nifK$ plasmid into *A. vinelandii*.

α -A45-C159 transformation into $\Delta nifD$ cells. Transforming mutagenic plasmids derived from the original pYM001 plasmid into $\Delta nifD$ competent cells has proved similarly inefficient. Two transformations with the plasmid coding for the MoFeP mutation α -C45A-I159C resulted in no rescued growth. A third transformation resulted in colonies with a successful mutation, however this was the only successful transformation we have observed using competent cells from the $\Delta nifD$ cell strain.

*Isolation of N^+ *A. vinelandii* mutant strains.* Incorporating mutations resulting in an N^+ phenotype by direct transformation into wtAvin cells resulted in low transformation efficiencies and cell lines containing both mutated and wild type chromosomes. To increase transformation efficiency and ensure homogeneous expression of mutant MoFeP, we utilized a two-step procedure for creating N^+ mutants that first involved designing a competent cell strain

containing a 3 to 5 amino acid deletion around the desired mutation site. A second round of transformation was then used to repair this deletion with a full sequence containing the site-directed mutation, and successful mutants were identified by the return of an N^+ phenotype (Figure 4.5). While this method requires generating a separate competent cell strain for each point mutation, we have been able to create both the initial deletion mutant and the rescued mutants with up to 50% transformation efficiency after a preliminary screening for competency based on rifampicin resistance. Cells containing deletion mutations were easily isolated by identifying cells with an N^- phenotype (Figure 4.6). Rescued cells were easily identifiable by the return of growth under B^- culture conditions.

MoFeP variants for alternate photoreduction constructs. Using the above protocol we have successfully expressed two additional MoFeP variants with altered labeling sites, α -C45A-L158C and β V157C. A third variant, α -C45A-I159C, was created by transforming a mutagenic plasmid into *A. vinelandii* cells without the *nifD* gen (Figure 4.7). Mutations will hereafter be named without reference to their native amino acids. Standard nitrogenase purification protocols were sufficient to isolate mutated MoFeP with approximately 95% purity as determined by SDS-PAGE. β -C157 MoFeP has an engineered cysteine on the β -subunit, directly across from the original active modification site at α -C158 (Figure 4.7). α -A45-C158 MoFeP is identical to the original active photoreduction construct but lacks the native MoFeP cysteine residue. α -A45-C159 also lacks the native MoFeP cysteine MoFeP and has an exposed cysteine one amino acid closer to the

MoFeP surface than α -C158 (Figure 4.7). Substituting the native α -C45 residue removes any potential interference from a second, non-active RuBP. Labeling MoFeP at the more solvent exposed α -C159 position may better facilitate electron transfer and labeling a residue on the β -subunit allows us to test electron transfer through different α -helices coordinated to the P-cluster.

Labeling and characterization of α -A45-C158-RuBP. Quantitative labeling with IA-RuBP was confirmed by ICP-OES, which indicated the presence of one label per each $\alpha\beta$ -subunit of MoFeP (Table 4.2). Labeling did not significantly affect the protein activity under ATP turnover conditions (Figure 4.8). SDS-PAGE results showed that labeling on Ru-MoFeP was limited to the α -subunit as expected (Figure 4.9). Photoinduced H^+ reduction assays with Ru-MoFeP showed comparable levels of activity to the previously-tested α -C158 mutant (Figure 4.10), which had two RuBP labels per $\alpha\beta$ -subunit.

Labeling and initial characterization of β -C157 and α -A45-C159. We are in the beginning stages of characterizing the photoreduction activity of both β -C157-RuBP and α -A45-C159-RuBP. ICP-OES results confirmed that β -C157 could be quantitatively labeled with IA-RuBP with 2.24 ± 0.63 labels per $\alpha\beta$ -dimer (Table 4.2). Quantification based on UV-vis measurements yielded a comparable ratio of 1.95 ± 0.40 labels per $\alpha\beta$ -dimer. SDS-PAGE results showed that labeling on β -C157-RuBP was present on both the α - and β -subunits which is as expected because this variant retains the native α -C45 residue. (Figure 4.11) Initial quantification of the extent of Ru-BP labeling α -A45-C159 as determined by UV-

vis absorbance indicates 0.92 ± 0.25 labels per $\alpha\beta$ -dimer which correlates well with the expected value of 1 per $\alpha\beta$ -dimer.

While the extent of RuBP-labeling on β -C157 and α -A45-C159 was similar to that on the corresponding α -C158 MoFeP variants (with and without α -C45), preliminary photoreduction experiments revealed significantly lower H^+ and C_2H_2 reduction activity for both β -C157-RuBP and α -A45-C159 MoFeP (Figure 4.12). The distance between RuBP and the P-cluster in α -A45-C159 MoFeP (17 Å) is longer than that in α -A45-C158 MoFeP (15 Å), which may result in less efficient electron transfer and therefore lower photoreduction activity.

An alternative cause for the reduced photocatalytic activity of β -C157-RuBP may be tied to possible ET-gated conformational changes in the P-cluster while relaying electrons to FeMoco. Crystal structures of the 2-electron oxidized (P^{ox}) and dithionite-reduced (P^N) P-cluster reveal very different conformations²⁹ (Figure 4.13). The structure of oxidized P-cluster shows the β -subunit cubane partially detached from the bridging sulfur atom and bound to two additional protein ligands. This shift involves the Fe atom coordinated by the α -helix containing β -C157, which exchanges an interaction with the central sulfur for the γ -O of residue β -S188. While the mechanistic implications of the conformation change are still unclear, exchanging coordination ligands may impose directionality on electron flow through the P-cluster.²⁹ Therefore, directly transferring electrons to the β -subunit rather than α -subunit cubane may be less efficient, hinting at a P-cluster dependent mechanism for coupled proton and electron transfer in MoFeP. While this is an intriguing possibility, a complete

characterization of both constructs under ATP-dependent turnover and photoreduction conditions is required to confirm these initially observed activity differences and determine their cause.

Conclusions

The modified mutagenesis protocol described above has resulted in higher transformation efficiencies for *A. vinelandii* cells and removed the possibility of expressing heterogeneous mutants. The ability to rapidly generate site-directed mutants of the MoFeP now enables us to expand the utility of our various MoFeP constructs used for light-driven catalysis. New MoFeP variants with altered surface exposed cysteine residues will be used to probe electron transfer pathways to both the P-cluster and FeMoco. In addition, creating amino acid substitutions within the MoFeP allows us to perturb proposed electron or proton transfer pathways in the enzyme and identify as of yet undiscovered catalytically necessary residues. Altering the MoFeP interior will also allow us to increase the yield of photoreduced FeMoco, and consequently reaction intermediates, to facilitate spectroscopic or structural studies.

Figure 4.1. Genes in the major *nif* cluster from *A. vinelandii*. Genes are color coded based on the proposed function of their gene products: FeP(structural gene), red; MoFeP α -subunit (structural gene), green; MoFeP β -subunit (structural gene), purple; FeMoco biosynthesis, dark blue; *nif* gene expression, light blue; nitrogenase maturation, orange; electron donation to nitrogenase, light gray; unknown function, dark gray. Adapted from references 8 and 17.

Figure 4.2. Cartoon schematic of congression screening with a mutagenic plasmid containing a *nif* gene and a screening plasmid conveying resistance to the antibiotic Rifampicin (*Rif^R*). All competent cells transformed with the *Rif^R* plasmid can grow under the antibiotic selection pressure.

Figure 4.3. Cartoon representations of mutagenic plasmids pYM001 (a) and pYM002 (b). pYM001 contains the entire *nifH* (FeP) and *nifD* (MoFeP α -subunit) genes with portions of the *nifJ* and *nifK* genes. pYM002 contains the entire *nifK* (MoFeP β -subunit) genes with portions of the *nifD* and *nifT* genes.

Figure 4.4. (a) Cartoon representation of Kpn1 restriction enzyme digest sites in the *nifHDK* genes of the *A. vinelandii* chromosome and mutagenic plasmids pYM001 and pYM002. (b) UV-light imaged agarose gel showing separation of linearized plasmids from digested *nif* gene inserts.

Figure 4.5. Two-step transformation strategy for generating *A. vinelandii* point mutations (boxes) and possible resulting phenotypes. (a) In the first step, a mutation vector containing a deletion is transformed into wtAvin cells. Successful transformations are identified by an N⁻ phenotype. (b) In the second step, a mutation vector containing a point mutation (asterisk) is transformed into the deletion cells. Successful transformations have restored nitrogenase activity.

Figure 4.6. Screening methods used to identify correct *A. vinelandii* mutants. (a) Initial screening for competent cells by congression with plasmid pDB303, a plasmid conferring resistance to the antibiotic Rifampicin. Cells incorporating pDB303 have a resistant phenotype (Rif^R) while those lacking the plasmid (wtAvin) cannot grow in the presence of rifampicin. The yellow-green color of the pDB303 transformed plates is a result of strong *A. vinelandii* growth. (b) Identification of N-phenotype mutations through side-by-side colony streaking on B⁻(left) and B⁺ (right) plates. A red asterisk indicates cells that were unable to grow without NH₄Cl and were further screened for mutations with gene sequencing.

Table 4.1. Mutagenesis scheme and primer pairs used to create the MoFeP variants for alternate photoreduction constructs.

Variant	Mutation (order of addition)	Original Plasmid	Competent Cell Strain	Primer Sequence (5' - 3')
α -A45	$\Delta\alpha$ C45	pYM001	wtAvin	CGGTACCCAGTCCAAAGTCTCCAAACAAGAGTC
				GACTTCTGTTGGAGACTTGGACTGGGTAACCG
	α C45A	pYM001	$\Delta\alpha$ C45	GGTACCCAGTCCAAAGAGGCCATCATCTCCAAACAAGAAG
				CTTCTGTTGGAGATGATGGCCCTTCTTGACTGGGTAACC
α -A45A-C158	$\Delta\alpha$ L158	pYM001 + α 45A	α C45A	CGAGTGCCCGATCGCGGACGACATCG
				CGATGTCGTACGATCGGGCACTCG
	α L158C	pYM001 + α 45A	α A45 $\Delta\alpha$ L158	GAGTGCCCGATCGGGCTGCATCGGGCGACGACATC
				GATGTCGTGCGCCGATGCAGCCGATCGGGCACTC
α -A45-C159	α L159C	pYM001 + α 45A	Δ nifD	CCCGATCGGCCCTGTGCGGGCGACGACATC
				GATGTCGTGCGCCGCACAGCCGATCGGGG
β -C157	$\Delta\beta$ V157	pYM002	wtAvin	CCACCTGCATGGCCGGTGACGACCTCAA
				TTGAGGTCGTACCCGGCCCATGCAGGTGG
	β V157C	pYM002	$\Delta\beta$ V157	CCTGCATGGCCCGAGTGCATCGGTGACGACC
				GGTCGTCACCGATGCACTCGGGCCATGCAGG

Figure 4.7. (a) Proposed alternate labeling sites, α -C159 and β -C157, and their positions relative to the original labeling site α -C158 and the P-cluster. (b) Cartoon representations of labeling sites on α -A45-C158, α -A45-C159 MoFeP and β -C157.

Table 4.2. ICP-OES analysis of the metal content of both unlabeled and RuBP labeled α -A45-C158 MoFeP from *A. vinelandii*.

Protein	Fe/Mo Ratio	Expected Fe/Mo Ratio	Ru/Mo Ratio	Expected Ru/Mo Ratio	Total Points Measured
α -A45-C158	13.13 ± 1.91	15	-----	-----	3
α -A45-C158-RuBP	15.50 ± 0.52	15	1.28 ± 0.47	1	9

Figure 4.8. Activity assays for H^+ reduction (a) and C_2H_2 reduction (b) by α -A45-C158 MoFeP and α -A45-C158-RuBP MoFeP under ATP turnover conditions. Labeling MoFeP with RuBP does not have a significant effect on for both substrates.

Figure 4.9. SDS-PAGE gel of unmodified and RuBP-labeled α -A45-C158 MoFeP imaged under UV-light (a) or stained with Coomassie Blue (b), illustrating that the RuBP label is located on the α -subunit. The first lane in each gel is a molecular weight marker.

Figure 4.10. α -A45-C158-RuBP H^+ reduction activity under photoreduction conditions. These reactions were carried out in a solution of 100 mM Hepes, 200 mM NaCl buffered at pH 6.5.

Table 4.3. ICP-OES analysis of the metal content of RuBP labeled β C157 MoFeP variant.

Protein	Fe/Mo Ratio	Expected Fe/Mo Ratio	Ru/Mo Ratio	Expected Ru/Mo Ratio	Total Points Measured
B-C157	14.53 ± 0.37	15	-----	-----	10
β -C157-RuBP	14.79 ± 1.63	15	2.24 ± 0.63	2	6

Figure 4.11. SDS-PAGE gel of unmodified and RuBP-labeled β -C157 MoFeP imaged under UV-light (a)) or stained with Coomassie Blue (b), illustrating that the protein has a RuBP label located on both the β -subunit and the α -subunit (α -C45).

Figure 4.12. Initial tests of β -C157-RuBP C_2H_2 (a) and H^+ (b) and α -A45-C159-RuBP H^+ (c) photoreduction activity. The reactions were carried out at pH 6.5.

Figure 4.13. Conformational changes in the P-cluster seen in crystal structures of (a) oxidized (PDB ID: 2MIN) and (b) dithionite reduced (PDB ID: 1M1N) MoFeP. α -helices anchoring the P-cluster and FeMoco in the α - and β -subunits of FeMoco are highlighted in black and gray, respectively.

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Chapter 5

The Light-Driven, Six-Electron Reduction of HCN to CH₄ by Photosensitized MoFeP

Introduction

As we described in Chapter 3, the α -C158 MoFeP variant labeled on its surface with a Ru-polypyridine photosensitizer (α -C158-RuBP) could be photo-activated to catalyze the two-electron reduction of H^+ and C_2H_2 into H_2 and C_2H_4 , respectively. However, the quantum yield (ϕ = catalytically useful electrons/photons absorbed) of this particular light-driven system was low (ca. 1%) and the rates of product formation were only about 0.5% of those observed with the native nitrogenase complex under ATP-dependent turnover conditions.¹ Despite this lower efficiency, the photo-activation of MoFeP catalysis offers the important advantage that the reduction of MoFeP is unimolecular and can be initiated rapidly, which is a prerequisite to interrogating the fleeting redox intermediates populated during substrate reduction.

Another important step toward completely bypassing ATP/FeP-dependence and driving the native 8-electron catalytic reaction with light is the demonstration that multiples of two electrons can be efficiently delivered to FeMoco. The observation that α -C158-RuBP MoFeP could reduce the 2-electron reductions of H^+ and C_2H_2 but not the 6-electron reduction of N_2 to NH_3 raises interesting questions about conformationally-gated ET in MoFeP and the ability of the photoactivated FeMoco to achieve highly reduced states that could bind and activate N_2 .² It is generally accepted that nitrogenase substrates and inhibitors bind to different reduction states of FeMoco. These states are commonly referred to by their position in the Thorneley-Lowe cycle, a widely accepted kinetic scheme describing nitrogenase catalyzed N_2 reduction (Figure

5.1). In this scheme FeMoco is reduced from the resting state (E_0) by 8 successive, discrete electron and proton transfers steps. In the absence of all other substrates, the FeMoco will cycle between the E_0 and E_1 or E_2 states as it catalyzes H^+ reduction. Conversely, N_2 reduction requires the FeMoco accumulate enough electrons to achieve the E_3 or E_4 state before N_2 binding can occur.³ Therefore, the absence of observable NH_3 production by α -C158-RuBP may indicate that E_1 and E_2 states are within reach by our photo-reduction scheme and the E_3 or E_4 states of FeMoco necessary for N_2 reduction are not. Examining the light-driven reduction of alternate nitrogenase substrates may help to further elucidate the electron transfer capabilities of the isolated MoFeP.

Nitrogenase can catalyze the reduction of numerous multiply bonded molecules besides N_2 , including CO ,^{4,5} CO_2 ,⁶ and HCN ⁷⁻⁹. HCN is a particularly intriguing substrate because it is isoelectronic with N_2 and features a similarly strong (887 kJ/mol) triple bond. HCN is reduced and protonated six-fold through a pathway that is likely similar to the alternating pathway that has been suggested for N_2 .⁸⁻¹¹ Importantly, while N_2 binding requires FeMoco reduction under high electron flux, HCN or its conjugate base CN^- (proposed to be an inhibitor) can bind FeMoco under low electron flux conditions, where they can compete with H^+ reduction.^{8,12} Indeed, as previously mentioned in Chapter 3, HCN/CN^- effectively suppressed photoinduced H^+ and C_2H_2 reduction by Ru-labeled MoFeP. This observation supports the possibility that HCN may be reduced with our light-activated system to generate CH_4 or methylamine (CH_3NH_2):

In this Chapter, we describe the catalytic reduction of HCN to CH₄ by a modified version of the original Ru-labeled MoFeP, α -A45-C158-RuBP. Our findings support the presence of an ET-gating process within MoFeP and suggest that once FeMoco attains one- or two-electron-reduced states (E₁ or E₂), the subsequent reduction of the bound substrates proceed with high efficiency.

Materials and Methods

Construction of the α -C45A-L158C MoFeP strain was done as described in Materials and Methods in Chapter 4. Unless otherwise noted, all chemicals and reagents were purchased from Fisher Scientific, VWR International or Sigma-Aldrich. Na¹³CN (99%) was obtained from Icon Isotopes, and D₂O (99.9%) was purchased from Cambridge Isotope Laboratories. All work requiring active nitrogenase proteins was preformed either in an anaerobic tent (Coy Laboratories) with an oxygen level less than 5 ppm or on the bench top using standard Schlenk line techniques. Cell growth, protein purification, RuBP modification and characterization of labeled protein were all done as previously described in Chapter 2.

Sample Preparation for Photoreduction Assays. Photoreduction assays were performed as described in Chapter 3 with the following modifications. Reaction mixtures contained 2.7 mg of MoFeP, 200 mM dithionite, 10 mM NaCN,

100 HEPES at pH 7.5, and 200 mM NaCl in a final volume of 9 mL. To study the effect of substrate concentration on activity, concentrations of NaCN were varied by adding appropriate volumes of a 50-mM NaCN stock solutions prepared in 100 mM HEPES at pH 7.75 and 200 mM NaCl. To study the effect of pH on activity, the final solution pH was adjusted using a series of 100 mM Hepes and 200 mM NaCl buffer solutions with pH values varying between 6.5 to 10. The assay vials were submerged in a temperature controlled water bath for the duration of the assay and the temperature was maintained at 20°C with a Neslab water circulator.

Reaction progress was monitored continuously by taking either 0.5 mL or 50 μ L samples of the reaction vial headgas at predetermined times to measure H₂ or CH₄ produced, respectively. For H⁺-photoreduction experiments, 0.5 mL of Ar was back-added to the sample vials after removing each headgas aliquot. CH₄ was separated on an Alumina column (5'x1/8"x0.85" SS) (Alltech) connected to a flame ionization detector. The carrier gas was He, and the oven temperature was 150°C. The gas chromatograph was calibrated using known amounts of either 10% H₂ in Ar or 1 atm C₂H₂. The detectors had a consistent linear response to products in the concentration ranges relevant for the photoreduction experiments.

GC-MS Analysis of Photoreduction Products. GC-MS samples were prepared as described above for the photoreduction experiments, except that each sample contained 8.1 mg of RuBP-labeled MoFeP, and the gas phase was switched from Ar to He prior to illumination. Samples were exposed to 400-W light

for 3 hours prior to analysis. GC-MS measurements were performed using a Hewlett Packard G1800A GCD system connected to a quadrupole mass analyzer. For each sample, 1.0 mL of headgas was injected through splitless injector equilibrated to 30°C onto a HP-PLOT/Q capillary column (30 m x 0.320 mm x 20.00 μ m) (Agilent Technologies). The carrier gas was He and the oven temperature was maintained at 30°C. A 1.65-minute detection delay was used to avoid interference from any residual Ar in the sample atmosphere, which eluted at the column void volume. To investigate the proton source for photogenerated CH₄, MoFeP was exchanged into a buffer prepared using a $\leq 90\%$ D₂O stock solution using a 10-DG column (Bio-Rad), and all buffers and substrates were prepared in D₂O. The GC-MS data were analyzed using OpenChrom software (www.openchrom.net).

Attempted NH₃ Detection in Photoreduction Products. Assays intended to determine the concentration of NH₃ produced during HCN photoreduction are based on the reaction of NH₃ with o-phthalaldehyde mercaptoethanol to form a fluorescent product, as described by Corbin *et al.*¹³ The OPT reagent was prepared by dissolving o-Phthalaldehyde (TCI America) and mercaptoethanol in 0.2 M phosphate buffer. The mixture was then allowed to react overnight under Ar. Quantification of NH₃ was carried by taking 25 μ L aliquots from photoreduction assay samples at regular time intervals during illumination and mixing them with 1.0 mL of the OPT reagent. The reaction was allowed to proceed in the dark for 60 min, and samples were then analyzed for fluorescence with a Shimadzu RF-10AXL fluorescence detector upon separation

on a 3-mL reverse phase column (Resource RPC, GE Healthcare) connected to DuoFlow workstation (BioRad). Elution profiles for NH_4Cl calibration standards show drastic differences when the samples were prepared in the presence and absence of 200 mM dithionite, indicating that the OPT reagent reacts with dithionite, and its dynamic range for NH_3 detection is severely suppressed. (Figure 5.3) It is also possible that the dithionite quenches the fluorescence from the product formed by reacting the OPT reagent with NH_3 . Adjusting the ratio of the photoreduction sample to OPT reagent, or changing sample volumes loaded onto the reverse phase column did not improve the fluorescence response of the assay.

Results and Discussion

Identification of the Products of HCN Photoreduction. All of the experiments characterizing the light-driven reduction of HCN by MoFeP, were done using the α -C45A-L158C mutant of *Azotobacter vinelandii* MoFeP (see Chapter 4 for details on mutagenesis), which features only a single accessible surface cysteine (α -C158) for modification with the iodoacetamido-derivative of $[\text{Ru}(\text{bpy})_2(\text{phen})]^{2+}$ (IA-RuBP).¹⁴ GC analysis of products formed during C_2H_2 photoreduction in the presence of 10 mM NaCN revealed a previously undetected peak in the spectrum. Since HCN is a well-characterized nitrogenase substrate, we decided to examine if Ru-MoFeP could directly catalyze the reduction of HCN.

A typical reaction solution for photoinduced HCN reduction included 2.7 mg of Ru-MoFeP (≈ 20 nmol of active sites) in a solution of 200 mM dithionite as the reductive quencher, 10 mM NaCN, 200 mM NaCl and 100 mM HEPES at pH 6.5-7.5. Reaction solutions were irradiated in a 20°C water bath with a Hg/Xe lamp using UV- and IR-cutoff filters under constant stirring, and the headgas was analyzed for products by gas chromatography-mass spectrometry (GC-MS). Irradiating the reaction solutions that contained all of the above components led to the appearance of a prominent peak that was determined through MS to be CH_4 (Figure 5.2). Substitution of NaCN with the isotopically labeled substrate, Na^{13}CN , led to a 1-amu shift (to 17 amu) in the mass of the product peak (Figure 5.2), confirming that CH_4 results from the reduction of HCN/CN^- . Reactions performed in 90% D_2O produced the 20-amu product, CD_4 , as well as other partially deuterated CH_4 species, indicating that the hydrogens in the product originated from protons in the bulk solution (Figure 5.2). In addition to CH_4 , nitrogenase catalyzed HCN/CN^- reduction should also produce an equimolar amount of NH_3 and possibly, CH_3NH_2 . Yet, the high concentrations of dithionite needed for photocatalytic turnover precluded the quantification of NH_3 or CH_3NH_2 , as dithionite reacts with the fluorescent indicators used for the detection of amine-containing species.¹³ (Figure 5.3)

Characterization of light-driven CH_4 production. Under constant irradiation at pH 7.0 and 10 mM NaCN, the production of CH_4 proceeded with an initial velocity (measured between 0 and 15 min) of 0.4 nmol CH_4 /min per mg of MoFeP. In the absence of any one component from the complete reaction

system (light, RuBP, MoFeP, dithionite or NaCN), no CH₄ was produced. (Figure 5.4) The proposed photocatalytic scheme should be similar to that for 2-electron reduction reactions where catalytically competent electrons are transferred to the P-cluster after the generation of a strongly reducing Ru(I) species. (Figure 5.5) As we previously observed with photoinduced H₂ and C₂H₄ formation, CH₄ production eventually reached a plateau after approximately 90 minutes. This loss in activity appears likewise to be due to ligand substitution on the RuBP functionality, whereby the MLCT bands of RuBP steadily disappear during turnover ($\tau = 26.4 \pm 0.9$ min) (Figure 5.6), paralleling the tapering of CH₄ production.

Determination of electron transfer efficiency. In addition to producing 130 nmol CH₄ at pH 7.0 and 10 mM NaCN, the Ru-MoFeP system also generated 450 nmol H₂ from simultaneous H⁺ reduction. Not taking into account possible 2- or 4-electron reduction products, this translates into a total of 1680 nmol of electrons transferred during catalysis. In comparison, under the same reaction conditions but without NaCN, the system was able to transfer a total of 3200 nmol of electrons when only catalyzing the reduction of H⁺ (Figure 5.7). Studies have shown that the CN⁻ ion is a strong inhibitor of total electron flow during nitrogenase turnover,^{7,9} which may explain the reduction in the total amount of electrons transferred to substrates (HCN and H⁺).

The quantum yield for CH₄ formation at pH 7.0 (based on the initial 15 min of activity) is calculated to be ca. 0.07%, compared with 0.86% for H₂ production in the absence of NaCN. If each successive transfer of 2 electrons from RuBP to

FeMoco and the substrates was governed by the same mechanism and proceeded with the same efficiency, the theoretical yield of CH₄ formation would be $(0.86\%)^3 = 6.3 \times 10^{-7}$. The fact that the observed yield is more than 1100-fold higher than the theoretical yield indicates that the ET steps from RuBP into FeMoco during the 6-electron HCN reduction are not equivalent. It is commonly accepted that there must be a FeP-induced conformational change within MoFeP, which controls the initial reduction of FeMoco and the subsequent binding of substrates.¹⁵ We propose that, in the absence of activation of this gate by ATP-dependent FeP binding, the probability of reaching the E₁ or E₂ states of FeMoco is low, leading to diminished yields of H⁺ and HCN binding and reduction relative to the ATP-driven system. Nevertheless, as implied by the disproportionally high yields of CH₄, once HCN is committed to the catalytic cycle upon reaching the E₁ or E₂ states of FeMoco, the subsequent reduction steps proceed with relatively high efficiency.

CH₄ production and reaction pH. The extent of photoinduced CH₄ production by Ru-MoFeP strongly depends on the solution pH. The maximal activity occurs at ca. pH 6.5 with a quantum yield for CH₄ production that is nearly twice as much as that at pH 7.0 and five times as much as that at pH 7.5. This increase in activity between pH 7.5 and pH 6.5 does not scale with a decrease in the concentration of the proposed inhibitor, CN⁻ (pK_a = 9.2) (Figure 5.8). The activity for photoinduced H₂ formation catalyzed by our original construct, α -C158-RuBP, also peaks at pH 6.5 (Figure 5.9). Burgess and colleagues observed similar, bell-shaped pH vs. activity profiles with *A. vinelandii* nitrogenase

for various substrates (N_2 , H^+ , C_2H_2) under ATP/FeP-dependent turnover conditions.¹⁶

A distinction between the profiles for photoinduced and ATP/FeP-driven reduction processes is that the latter shows maximal activity at a full pH unit higher (ca. pH 7.5) than the former. This shift may well stem from a mechanistic difference between the photoinduced and ATP/FeP-driven reactions, where the rate-determining steps for substrate reduction may involve different protonatable residues. In the case of the photoinduced reaction, a transition in activity occurs with a midpoint at ca. pH=7, invoking the participation of a histidine residue. Alternatively, the shift may simply be a manifestation of the fact that the ATP/FeP-driven reaction involves complex, pH-dependent steps upstream from substrate reduction that are bypassed in the photoinduced reaction (e.g., ATP-binding/hydrolysis and FeP-MoFeP interactions).

Conclusions

Clearly, new experimental strategies are needed to uncouple biological N_2 fixation from ATP hydrolysis and FeP-mediated ET, which in turn should pave the way to in-depth studies of the complex ET, PT and substrate activation steps within MoFeP. The above described experiments characterize the light-driven, 6-electron reduction of HCN – a triply-bonded, diatomic molecule, isoelectronic with N_2 – by MoFeP. Our results suggest that the efficiency of our ATP-uncoupled system is primarily limited by the initial reduction of FeMoco, which is believed to be activated by an ATP/FeP-dependent conformational change in the native

system. Based on recent evidence that this conformational change may be mimicked by simple amino acid substitutions, it should be possible to drive the full 8-electron catalytic cycle of nitrogenase by light. Furthermore, we have already observed differences between MoFeP reactivity under turnover and photoreduction conditions, demonstrating that uncoupling MoFeP catalysis from FeP will enable the exploration of previously masked details of its mechanism.

Figure 5.1. A modified version of the Thorneley-Lowe scheme adapted from reference 8 and 12. E_N represents different reduction states of the FeMoco. N₂ binds to the 4 electron reduced FeMoco and precipitates H₂ evolution. The pathway includes an alternate H₂ evolution from the E₂ step which returns the FeMoco to the resting state.

a) 10 mM NaCN

b) 10 mM Na¹³CN c) 10 mM NaCN in D₂O

Figure 5.2. GC-MS analysis for CH₄ production during photoreduction experiments with Ru-MoFeP. The GC traces were acquired before (blue) or after 1-hour illumination (cyan) of the samples that contain (a) NaCN, (b) Na¹³CN, or (c) NaCN dissolved in deuterated buffer solution.

Figure 5.3. (a) Reverse-phase FPLC elution profiles of solutions containing the NH_3 indicator o-phthalaldehyde (OPT) and standard solutions of NH_4Cl in the presence (red) and absence (blue) of 200 mM dithionite (DT). A new fluorescent product is formed in the presence of dithionite. (b) Dependence of OPT-based product fluorescence on NH_4Cl concentration. The presence of dithionite (DT) significantly suppresses the dynamic range of OPT necessary for quantifying the small amounts NH_3 produced during photoreduction assays.

Figure 5.4. Cyanide reduction assays (orange circles) and corresponding controls, each of which is missing the indicated component of the complete reaction system.

Figure 5.5. Proposed photocatalytic scheme for the 6-electron reduction of HCN to CH₄ and NH₃. Dithionite (DT) acts as a reductive quencher for the high-yield generation of Ru(I) species.

Figure 5.6. (a) Changes in the absorbance at 450 nm for RuBP-labeled α -C45A/L158C-MoFeP under conditions used for photoreduction activity assays. (b) Close-up detailing the gradual bleaching of RuBP absorbance during illumination. Samples contained 3 mg MoFeP, 200 mM sodium dithionite, 100 mM Hepes (pH 7.5) and 200 mM NaCl. The decay kinetics for RuBP absorbance are monoexponential, with $\tau = 26.4 \pm 0.9$ minutes for RuBP-labeled α -C45A/L158C-MoFeP.

Figure 5.7. H₂ production during photoreduction assays in the presence (blue circles) or absence (open circles) of NaCN.

Figure 5.8. (a) pH dependence of CH_4 production. (b) Changes in the rate of CH_4 formation (based on the first 45 minutes of irradiation) as a function of pH and [HCN].

Figure 5.9. (a) Changes in photo-induced H_2 formation catalyzed by for RuBP-labeled α -C45A/L158C-MoFeP at different pH's. (b) The rate of H_2 formation after 45 minutes of illumination as a function of pH.

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Chapter 6

Dissertation Conclusions

The quest to effect and better understand nitrogen fixation has been a century-long endeavor, first motivated by the agricultural demands of a rapidly expanding global population, and now by the need to sustain such a population with a less damaging environmental impact, as well as, by the fundamental challenge posed by the difficulty of this chemical reaction. The latter two motivations have both prompted a closer look at the enzyme nitrogenase, which can catalyze nitrogen fixation under ambient conditions. Despite decades of research, however, the mechanistic details of nitrogenase catalysis are still not well understood, largely because the enzyme's mechanism depends on ATP hydrolysis and protein-protein interactions to orchestrate electron transfer and substrate reduction. Although a preliminary mechanism based on spectroscopic results details possible reaction intermediates¹, it is still unclear why and how ATP hydrolysis is ultimately utilized for the reduction of N_2 , how electrons and protons are transferred through the MoFeP and how substrate reduction itself is carried out by FeMoco.

It is clear that alternative experimental approaches are needed to make any further progress toward a better understanding of the nitrogenase mechanism. Along these lines, our approach of driving nitrogenase catalysis by light provides the clear advantage of circumventing the need for FeP-dependent ATP hydrolysis by converting nitrogenase catalysis into a unimolecular, easily-triggerable reaction. By uncoupling MoFeP catalysis from ATP hydrolysis, we have developed a new way to study nitrogenase that could eventually lead to a detailed understanding of its catalytic mechanism.

As with many new methodologies, there is quite a bit of room for improvement in light-driven MoFeP catalysis. Currently, the maximum yield of our system is only about 1% that of the wild type enzyme and the catalytic activity is limited to roughly 100 turnovers. Furthermore, we believe that the flux of photogenerated electrons into FeMoco is either not sufficiently high or properly timed to support the reduction of N_2 to NH_3 although it is adequate to catalyze the similar reduction of HCN. While the low yield of the system is a problem that will need to be addressed in order to facilitate structural or spectroscopic studies, our initial findings are already enough to suggest a revised theory on electron gating in MoFeP and begin to describe differences between light- and ATP-driven catalytic pathways.

It is commonly believed that there must be a FeP-induced conformational change within MoFeP, which controls the initial reduction of FeMoco and the subsequent binding of substrates.^{2,3} However, there is currently very little direct experimental evidence to support this theory.^{4,5} The results presented herein, detailing the efficiency of light-driven 2 and 6-electron reduction in MoFeP, are strongly indicative of such a gating mechanism. As would be expected if gating is necessary for efficient electron transfer, the catalytic efficiency of light-driven substrate reduction is only a fraction of that under turnover conditions. Nevertheless, as implied by the disproportionately high yield of CH_4 formation when compared to 2-electron reduction products H_2 and C_2H_4 , electron transfer steps during substrate binding and substrate reduction may not be equivalently regulated. Therefore, an ATP/FeP-dependent conformational gating even may

be necessary for the initial reduction of FeMoco to allow substrate binding, but not for the subsequent electron transfer reactions needed for substrate activation.

What remains a challenging but exciting goal is to uncover the nature of any conformational gating events that occur within MoFeP during turnover. Any future studies on light-driven MoFeP catalysis will certainly involve producing new mutants that mimic a conformationally-activated state of the enzyme which allows efficient reduction of FeMoco in the absence of ATP/FeP. In addition, similar structure/function studies in the context of our light-activation scheme may help identify critical residues in MoFeP involved in electron transfer, proton transfer and substrate binding. Importantly, such studies may ultimately lead to the structural characterization of catalytic intermediates for the first time.

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Appendix 1

Calculating the quantum yield of α -C158-RuBP photoreduction at pH 7.75

Measured wavelength range: $\lambda = 395 - 550$ nm (395 nm long pass filter, 550 nm short pass filter)

Measured wattage with lamp set to 400watts: 0.69 watts = 0.69 J/sec

Energy of a photon at 475 nm (average energy over wavelength range):

$$E = \frac{hc}{\lambda} = \frac{(6.626 \times 10^{-34} \text{ J} \cdot \text{s})(3 \times 10^8 \text{ m/s})}{(475 \times 10^{-9} \text{ m})} = 4.182 \times 10^{-19} \text{ J/photon}$$

Number of photons reaching detector:

$$\# \text{ of photons} = \frac{\text{watts}}{\text{energy of photon}} = \frac{0.69 \text{ J/second}}{4.182 \times 10^{-19} \text{ J/photon}} = 1.650 \times 10^{18} \text{ photons/second}$$

Total number of photons in 15 minutes:

$$\text{total photons} = 15 \text{ minutes} \times \left(\frac{60 \text{ seconds}}{\text{minute}}\right) \times \left(\frac{1.650 \times 10^{18} \text{ photons}}{\text{second}}\right) = 1.485 \times 10^{21} \text{ photons}$$

Correct for the area difference between reaction vial and wattage meter:

$$\text{area of reaction vial} = l \times w = 2.3 \text{ cm} \times 1.5 \text{ cm} = 3.45 \text{ cm}^2$$

$$\text{area of detector} = \pi r^2 = \pi \times (0.9 \text{ cm})^2 = 2.54 \text{ cm}^2$$

$$\text{total photons to reaction} = 1.485 \times 10^{21} \text{ photons} \times \left(\frac{3.45 \text{ cm}^2}{2.54 \text{ cm}^2}\right) = 1.485 \times 10^{21} \text{ photons}$$

Correct for photons lost to scattering (~4%/surface):

- 4% for beaker

$$\text{total photons} = 1.485 \times 10^{21} \text{ photons} - (0.04 \times 1.485 \times 10^{21}) \text{ photons} = 1.426 \times 10^{21} \text{ photons}$$

- 4% for reaction vial

$$\text{total photons} = 1.426 \times 10^{21} \text{ photons} - (0.04 \times 1.426 \times 10^{21}) \text{ photons} = 1.369 \times 10^{21} \text{ photons}$$

Subtract photons absorbed by MoFe protein:

- average absorbance of 1.29×10^{-6} M MoFe protein from 395 to 500 nm ~ 0.1375

$$A = -\log\left(\frac{\%T}{100}\right)$$

$$\%T = 100 \times 10^{-A} = 100 \times 10^{-0.1375} = 72.86 \%$$

- 27.14 % of total photons are absorbed by MoFe protein

$$\text{total photons} = 1.369 \times 10^{21} \text{ photons} - (0.2714 \times 1.369 \times 10^{21}) \text{ photons} = 9.974 \times 10^{20} \text{ photons}$$

This leaves 9.974×10^{20} incident photons available to excite Ru(bpy)₃(phen)A

Maximum number of photons absorbed by Ru(bpy)₂(phenA)

- 4 Ru/1 MoFeP = 5.16×10^{-6} M Ru(bpy)₂(phenA)
- average absorbance of 5.16×10^{-6} M Ru(bpy)₂(phenA) from 395 to 500 nm ~ 0.08

$$A = -\log\left(\frac{\%T}{100}\right)$$

$$\%T = 100 \times 10^{-A} = 100 \times 10^{-0.08} = 83.18 \%$$

- 16.82 % of total photons are absorbed by Ru(bpy)₂(phenA)

$$\text{total photons absorbed} = 0.1682 \times 9.974 \times 10^{20} \text{ photons} = 1.678 \times 10^{20} \text{ photons}$$

Correct for Cys45 Ru(bpy)₂(PhenA):

- assume absorbs 50% of incident photons

$$\text{total photons absorbed} = 0.5 \times 1.678 \times 10^{20} \text{ photons} = 8.388 \times 10^{19} \text{ photons}$$

Total successful electron transfers:

- total C₂H₄ produced in 15 minutes = 383.04 nmol

$$383.04 \text{ nmol C}_2\text{H}_4 \times \left(\frac{2 \text{ nmol e}^-}{1 \text{ nmol C}_2\text{H}_4}\right) \times \left(\frac{1 \text{ mol}}{1 \times 10^9 \text{ nmol}}\right) \times \left(\frac{6.626 \times 10^{23} \text{ e}^-}{1 \text{ mol}}\right) = 5.076 \times 10^{17} \text{ e}^-$$

Quantum Yield:

- quantum yield = total electrons transferred/total photons absorbed

$$\text{quantum yield} = \left(\frac{\text{total e}^- \text{ transferred}}{\text{total photons absorbed}}\right) \times 100 = \left(\frac{5.076 \times 10^{17} \text{ e}^-}{8.388 \times 10^{19} \text{ photons}}\right) \times 100 = 0.605\%$$

Overall quantum yield = 0.61%

Appendix 2

In addition to the mutations described in the text, we successfully transformed several other mutagenic plasmids into *A. vinelandii* cells. All mutations were confirmed through sequencing as described in the Materials and Methods section of Chapter 4.

MoFeP mutations α -A45-C158 (Table 1 and Figure 1) and α -A45-C158-Q195 (Table 2 and Figure 2) were further confirmed through protein crystallography and structure determination. α -A45-C158 was crystallized through sitting-drop vapor diffusion with a well solution consisting of 13% PEG8000, 1.1 M NaCl, 0.1 M sodium cacodylate at pH 6.5 and 10 mM sodium dithionite. α -A45-C158-Q195 MoFeP was crystallized through sitting-drop vapor diffusion with a well solution consisting of 18% PEG2000, 0.7 M NaCl, 0.1 M Tris at pH 8.0 and 10 mM sodium dithionite. For both mutants, the drop consisted of 2 μ L of protein (buffered in a 100 mM Tris and 200 mM NaCl solution at pH 7.75) and 2 μ L of the well solution. Crystals typically grew in one to two weeks. Crystals used for diffraction collection were cryoprotected by soaking in the well solution supplemented with 20% glycerol and were then frozen in liquid nitrogen.

X-ray diffraction data were collected at 100 K at the Stanford Synchrotron Radiation Laboratory (BL 9-2) using 0.98-Å radiation. The data were integrated with MOSFLM¹ and scaled with the program SCALA². The structures of α -A45-C158 and α -A45-C158-Q195 MoFeP were determined to 2.1 Å and 2.2 Å, respectively, by molecular replacement with the program MOLREP^{2,3} using the highest resolution wild type MoFeP structure (PDB ID: 1M1N) as the search model.

Rigid-body, positional and thermal refinement with REFMAC^{2,4} along with manual rebuilding, and water placement produced the final model. All figures were produced with PYMOL⁵.

The following appendix describes the mutagenesis scheme for all successfully transformed *A. vinelandii* mutants. The primer pairs listed below the description of the mutation plasmid were used to incorporate additional mutations not present in the original plasmid. The *A. vinelandii* strain used to generate competent cells for a particular transformation is listed after the description of the corresponding mutation plasmid.

Variant: α -A45

Mutation order: wt $\rightarrow\Delta\alpha$ -C45 $\rightarrow$ α -C45A

Phenotype: wild type MoFeP with no surface exposed cysteine residues

Original Plasmid: pYM001

Mutation Plasmid 1: $\Delta\alpha$ -C45

Forward Primer (5'-3'): CGGTACCCAGTCCAAGTCTCCAACAAGAAGTC

Reverse Primer (5'-3'): GACTTCTGTGGAGACTTGGACTGGGTAACCG

Competent Cell Strain 1: wtAvin

Mutation Plasmid 2: α 45^{C $\rightarrow$ A}

Forward Primer (5'-3'): GGTACCCAGTCCAAGAAGGCCATCATCTCCAACAAGAAG

Reverse Primer (5'-3'): CTTCTGTGGAGATGATGGCCTTCTGGACTGGGTAACC

Competent Cell Strain 2: $\Delta\alpha$ -C45

Variant: β -H98

Mutation order: $\Delta\beta$ -Y98 $\rightarrow$ β -Y98H

Phenotype: wild type MoFeP that catalyzes N₂H₄ reduction in the presence of Eu^{III}-EGTA or Eu^{III}-DTPA⁶

Original Plasmid: pYM002

Mutation Plasmid 1: $\Delta\beta$ -Y98

Forward Primer (5'-3'): TCCCAGGGTTCGTCCTCCTACTTCAAC

Reverse Primer (5'-3'): GTTGAAGTAGGAGCGGACGCAACCCTGGGA

Competent Cell Strain: wtAvin

Mutation Plasmid 2: β -98^{Y $\rightarrow$ H}

Forward Primer (5'-3'): GGGTTGCGTCGCCCACTTCCGCTCCTA

Reverse Primer (5'-3'): TAGGAGCGGAAGTGGGCGACGCAACCC

Competent Cell Strain: $\Delta\beta$ -Y98

Variant: α -A45-C158

Mutation order: α A-45 $\rightarrow$ α -A45- Δ L158 $\rightarrow$ α -A45-L158C

Phenotype: active photoreduction construct

Original Plasmid: α 45^{C $\rightarrow$ A}

Mutation Plasmid 1: α 45^{C $\rightarrow$ A} Δ L158

Forward Primer (5'-3'): CGAGTGCCCGATCGCGACGACATCG

Reverse Primer (5'-3'): CGATCTCGTCACGATCGGGCACTCG

Competent Cell Strain: α -A45

Mutation Plasmid 2: α 45^{C $\rightarrow$ A} 158^{L $\rightarrow$ C}

Forward Primer (5'-3'): GAGTGCCCGATCGGGCTGCATCGGGCGACGACATC

Reverse Primer (5'-3'): GATGTCGTCGCCGATGCAGCCGATCGGGCACTC

Competent Cell Strain: α -A45- Δ L158

Variant: α -A45-C158 β -H98

Mutation order: α -A45-C158 $\rightarrow$ α -A45-C158 $\Delta\beta$ Y98 $\rightarrow$ α -A45-C158 β Y98H

Phenotype: active photoreduction construct

Original Plasmid: α 45^{C $\rightarrow$ A} 158^{L $\rightarrow$ C}

Mutation Plasmid 1: $\Delta\beta$ Y98

Forward Primer (5'-3'): TCCCAGGGTTGCGTCCGCTCCTACTTCAAC

Reverse Primer (5'-3'): GTTGAAGTAGGAGCGGACGCAACCCTGGGA

Competent Cell Strain: α -A45-C158

Mutation Plasmid 2: β 98^{Y $\rightarrow$ H}

Forward Primer (5'-3'): GGGTTGCGTCGCCCCTCCGCTCCTA

Reverse Primer (5'-3'): TAGGAGCGGAAGTGGGCGACGCAACCC

Competent Cell Strain: α -A45-C158 $\Delta\beta$ Y98

Variant: α -A45-C158-A70

Mutation order: α -A45-C158 $\rightarrow$ α -A45-C158 Δ V70 $\rightarrow$ α -A45-C158 V70A

Phenotype: active photoreduction construct with larger binding pocket^{7,8}

Original Plasmid: α 45^{C $\rightarrow$ A} 158^{L $\rightarrow$ C}

Mutation Plasmid 1: α 45^{C $\rightarrow$ A} 158^{L $\rightarrow$ C} Δ V70

Forward Primer (5'-3'): CCTACGCCGGTTCCAAATGGGGCCCC

Reverse Primer (5'-3'): GGGGCCCCATTGGAACCGGCGTAGG

Competent Cell Strain: α -A45-C158

Mutation Plasmid 2: α 45^{C $\rightarrow$ A} 158^{L $\rightarrow$ C} α 70^{V $\rightarrow$ A}

Forward Primer (5'-3'): GTTCCAAAGGCGCGGTCTGGGGCCCC

Reverse Primer (5'-3'): GGGCCCCAGACCGCGCCTTTGGAAC

Competent Cell Strain: α -A45-C158 Δ V70

Variant: α -A45-C158-Q195

Mutation order: wt $\rightarrow$ α -C45A-L158C-H196Q

Phenotype: N⁻ with impaired proton transfer ability⁹

Original Plasmid: α 45^{C $\rightarrow$ A} 158^{L $\rightarrow$ C}

Mutation Plasmid 1: $\alpha 45^{C \rightarrow A}$ $158^{L \rightarrow C}$ $196^{H \rightarrow Q}$

Forward Primer (5'-3'): GTGCCTGGGCCAGCACATCGCCAAC

Reverse Primer (5'-3'): GTTGGCGATGTGCTGGCCCAGGGAC

Competent Cell Strain: wtAvin

Variant: α -A45-C189

Mutation order: $\alpha A45 \rightarrow \alpha A45\text{-}\Delta V189 \rightarrow \alpha A45\text{-}V158C$

Phenotype: alternate labeling location for photoreduction

Original Plasmid: $\alpha 45^{C \rightarrow A}$

Mutation Plasmid 1: $\alpha 45^{C \rightarrow A}$ $\Delta V189$

Forward Primer (5'-3'): CGAAGGCTCCGCCAGTCCCTGGGCC

Reverse Primer (5'-3'): GGCCCAGGGACTGGCGGAAGCCTTCG

Competent Cell Strain: $\alpha A45$

Mutation Plasmid 2: $\alpha 45^{C \rightarrow A}$ $189^{V \rightarrow A}$

Forward Primer (5'-3'): AAGGCTCCGCGGCTGTCCAGTCCCTGG

Reverse Primer (5'-3'): CCAGGGACTGGGAACAGCCGCGGAAGCCTT

Competent Cell Strain: α -A45 $\Delta V189$

Variant: β -C157

Mutation order: wtAvin $\rightarrow \Delta \beta$ -V157 $\rightarrow \beta$ -V157C

Phenotype: alternate labeling location for photoreduction

Original Plasmid: YM002

Mutation Plasmid 1: $\Delta \beta V157$

Forward Primer (5'-3'): CCACCTGCATGGCCGGTGACGACCTCAA

Reverse Primer (5'-3'): TTGAGGTCGTCACCGGCCATGCAGGTGG

Competent Cell Strain: wtAvin

Mutation Plasmid 2: $\beta 157^{V \rightarrow C}$

Forward Primer (5'-3'): CCTGCATGGCCGAGTGCATCGGTGACGACC

Reverse Primer (5'-3'): GGTCGTCACCGATGCACTCGGCCATGCAGG

Competent Cell Strain: $\Delta \beta V157$

Variant: β -H98-C157

Mutation order: β -H98 $\rightarrow \beta$ -H98 $\Delta V157 \rightarrow \beta$ -H98 V157C

Phenotype: alternate labeling location for photoreduction

Original Plasmid: β -98^{Y→H}

Mutation Plasmid 1: β -98^{Y→H} $\Delta \beta V157$

Forward Primer (5'-3'): CCACCTGCATGGCCGGTGACGACCTCAA

Reverse Primer (5'-3'): TTGAGGTCGTCACCGGCCATGCAGGTGG

Competent Cell Strain: β -H98

Mutation Plasmid 2: β -98^{Y→H} $157^{V \rightarrow C}$

Forward Primer (5'-3'): CCTGCATGGCCGAGTGCATCGGTGACGACC

Reverse Primer (5'-3'): GGTCGTCACCGATGCACTCGGCCATGCAGG

Competent Cell Strain: β -H98 $\Delta V157$

Variant: α -A45-C159

Mutation order: wtAvin $\rightarrow\Delta nifD\rightarrow\alpha$ -C45A-I159C

Phenotype: alternate labeling location for photoreduction

Original Plasmid: YM001

Mutation Plasmid 1: $\Delta nifD$

From Kpn1 digest (Chapter 4)

Competent Cell Strain: wtAvin

Mutation Plasmid 2: α -45^{C $\rightarrow$ A} 159^{I $\rightarrow$ C}

Forward Primer (5'-3'): GGGCATCGGCCTGTGCGGGCGACGACATC

Reverse Primer (5'-3'): GATGTCGTCGCCGCACAGGCCGATCGGG

Competent Cell Strain: $\Delta nifD$

Table A2.1. X-ray data collection and refinement statistics for α -A45-C158 MoFeP.

$$\ddagger R_{\text{sym}} = \sum_j |I_j - \langle I \rangle| / \sum_j |I_j|$$

$$\S R = \sum | |F_{\text{obs}}| - |F_{\text{calc}}| | / \sum |F_{\text{obs}}| \quad (2\sigma \text{ cutoff})$$

|| Free R calculated against 5% of the reflections removed at random.

¶ Root mean square deviations from bond and angle restraints.

Residues in complex	1996
No. of complexes/asymmetric unit	1 $\alpha_2\beta_2$
Metal ions in asymmetric unit	2 x (15 Fe + 1 Mo)
Waters in asymmetric unit	1203
Unit cell dimensions (Å)	70.08 x 151.07 x 107.15
	$\alpha=\gamma=90^\circ$, $\beta=101.82^\circ$
Symmetry Group	P2 ₁
Resolution range (Å)	48.68 – 2.10
X-ray wavelength (Å)	0.98
Number of unique reflections	148120
Completeness (%)	87.0
$\langle I/\sigma \rangle$	2.7
$R_{\text{sym}}^\ddagger$ (%)	11.4
$R^\S$ (%)	22.4
Free $R^{ }$ (%)	29.4
Rms Bond $^\¶$ (Å)	0.017
Rms Angle $^\¶$ (°)	3.5
Ramachandran plot (%)	
Residues in most favored regions	93.5
Residues in allowed regions	5.5
Residues in disallowed regions	1.0

Figure A2.1. Electron density map around the mutated residues of α -A45-C158 MoFeP.

Table A2.2. X-ray data collection and refinement statistics for α -A45-C158-Q195 MoFeP.

$$\ddagger R_{\text{sym}} = \sum_j |I_j - \langle I \rangle| / \sum_j |I_j|$$

$$\S R = \sum | |F_{\text{obs}}| - |F_{\text{calc}}| | / \sum |F_{\text{obs}}| \quad (2\sigma \text{ cutoff})$$

|| Free R calculated against 5% of the reflections removed at random.

¶ Root mean square deviations from bond and angle restraints.

Residues in complex	1996
No. of complexes/asymmetric unit	1 $\alpha_2\beta_2$
Metal ions in asymmetric unit	2 x (15 Fe + 1 Mo)
Waters in asymmetric unit	517
Unit cell dimensions (Å)	79.71 x 129.98 x 107.02
	$\alpha=\gamma=90^\circ$, $\beta=110.88^\circ$
Symmetry Group	P ₂₁
Resolution range (Å)	51.57 – 2.20
X-ray wavelength (Å)	0.98
Number of unique reflections	159652
Completeness (%)	97.6
$\langle I/\sigma I \rangle$	3.2
$R_{\text{sym}}^\ddagger$ (%)	15.3
$R^\S$ (%)	21.5
Free $R^{ }$ (%)	27.6
Rms Bond $^\¶$ (Å)	0.019
Rms Angle $^\¶$ (°)	3.5
Ramachandran plot (%)	
Residues in most favored regions	93.0
Residues in allowed regions	6.2
Residues in disallowed regions	0.8

Figure A2.2. Electron density map around the mutated residues of α -A45-C158-Q195.

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