Pacific Northwest
NATIONAL LABORATORY

PNNL-33326

Understanding Proton Movement in [Fe-Fe] Hydrogenases

September 2022

Joseph A. Laureanti
Garry W. Buchko
Marjolein T. Oostrom
Bojana Ginovska
Wendy J. Shaw

U.S. DEPARTMENT OF ENERGY

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

DISCLAIMER

PACIFIC NORTHWEST NATIONAL LABORATORY
operated by
BATTELLE
for the
UNITED STATES DEPARTMENT OF ENERGY
under Contract DE-AC05-76RL01830

Printed in the United States of America

Available to DOE and DOE contractors from the Office of Scientific and Technical Information,
P.O. Box 62, Oak Ridge, TN 37831-0062
www.osti.gov
ph: (865) 576-8401
fax: (865) 576-5728
email: reports@osti.gov

Available to the public from the National Technical Information Service
5301 Shawnee Rd., Alexandria, VA 22312
ph: (800) 553-NTIS (6847)
or (703) 605-6000
email: info@ntis.gov
Online ordering: http://www.ntis.gov

PNNL-33326

Understanding Proton Movement in [Fe-Fe] Hydrogenases

September 2022

Joseph A. Laureanti
Garry W. Buchko
Marjolein T. Oostrom
Bojana Ginovska
Wendy J. Shaw

Prepared for
the U.S. Department of Energy
under Contract DE-AC05-76RL01830

Pacific Northwest National Laboratory
Richland, Washington 99354

Abstract

Nature uses specialized metalloenzymes to carry out small molecule activation reactions, including CO_2 fixation, O_2 activation, and proton reduction, with unparalleled efficiency, rates, and selectivity. The latter reactions are performed by hydrogenases, protein metallo-complexes that interconverts H_2 to protons and electrons (H_2 oxidation) and the reverse reaction (H_2 production) with incredibly low energy input and amazingly fast kinetics. Reproducing both the activity and efficiency of metalloenzymes in sustainable anthropogenic systems remains one of the “holy grails” of inorganic chemistry. However, identifying the precise molecular components responsible for these desirable properties has been challenging in the natural metalloenzymes, hindering efforts to develop analogous processes in synthetic compounds. Considering the inherent complexity of a metalloenzyme and the many interactions, both strong and weak, that contribute to the function of an enzyme, we have elected to model natural metalloenzymes on a biochemical platform. Towards this end, we have a developed structural, functional, and mechanistic mimic of the [Ni-Fe] hydrogenases within a robust protein scaffold, rubredoxin, to understand the influence of the secondary coordination environment on the metal center. This involved preparing a series of three rubredoxin constructs containing a single point mutation at Val positions and making the NMR chemical shift assignments for the paramagnetic (nickel-substituted) and non-paramagnetic (zinc-substituted) form. These physical studies were complemented with *in silico* molecular dynamic studies on proteins with [Fe-S] clusters to engineer new proton channels to test *in vitro*. To assist our search for new natural metalloprotein scaffolds within the vast number of sequenced genomes, we created a neural network-based program to identify proteins with specific metal-binding sites. These computational and physical studies with metalloenzymes provide direct insight into the fundamental chemical principles driving the natural systems and offer design principles for developing catalysts that utilize analogous principles.

Summary

NMR backbone-assignment data were collected and the chemical shifts tabulated for a wild type rubredoxin and three constructs containing a single point mutation. The NMR data for both the paramagnetic (nickel-substituted) and non-paramagnetic (zinc-substituted) form (eight data sets in all) are being used for various physical calculations, including the fitting of the magnetic susceptibility tensor using the program ***Paramagpy*** (still in progress).

A neural network-based program was created to identify proteins in sequenced genomes with the ability to bind specific metals and metal clusters. A manuscript describing this program has cleared information release (PNNL-SA-177378) and will be submitted to the journal ***Protein Science***.

Molecular dynamics studies are being performed to evaluate the feasibility of constructing new secondary proton transfer channels by creating point mutations at select residues in identified hydrogen bonding networks (still in progress).

Acknowledgments

This work is supported by the PNNL Laboratory Directed Research and Development (LDRD) program and in part by the U.S. Department of Energy (DOE), Office of Science, Office of Basic Energy Sciences (BES), Division of Chemical Sciences, Geosciences & Biosciences. It was performed at the Pacific Northwest National Laboratory (PNNL), a facility operated by Battelle for the U.S. Department of Energy, including access to the W.R. Wiley Environmental Molecular Sciences Laboratory (EMSL), a national scientific user facility sponsored by the U.S. DOE Biological and Environmental Research program. Battelle operates PNNL for the U.S. Department of Energy under contract DE-AC05-76RL01830.

Acronyms and Abbreviations

H_2ase, hydrogenase.
NiRd, nickel-substituted rubredoxin.
NiV08D, nickel-substituted rubredoxin with Val-08 to Asp substitution.
NiV34H, nickel-substituted rubredoxin with Val-34 to His substitution.
NiV37N, nickel-substituted rubredoxin with Val-37 to Asn substitution.
NMR, nuclear magnetic resonance.
PTP, proton transfer pathway.
ZnRd, zinc-substituted rubredoxin.
ZnV08D, zinc-substituted rubredoxin with Val-08 to Asp substitution.
ZnV34H, zinc-substituted rubredoxin with Val-34 to His substitution.
ZnV37N, zinc-substituted rubredoxin with Val-37 to Asn substitution.

Contents

Figures

1.0 Introduction

The high level goal of this proposed work is to ultimately understand the principles governing proton transport, an ubiquitous and fundamental component of biological systems.[1,2] Furthermore, efficient shuttling of protons has been shown to be incredibly important for reactions relevant to renewable energy such as the conversions of small molecules like H_2, CO_2, N_2, or O_2. Unfortunately, there is a disparity of published work directly applied to investigating proton movement in electrocatalytic enzymatic systems. This is most likely due to the difficulties inherent to this task as we discovered in this LDRD. For this venture, we initially aimed to employ a thoroughly understood electrocatalytic enzymatic system, CpI [Fe-Fe] hydrogenase (H_2ase), Figure 1A.[3] The H_2ase machinery interconverts H_2 to protons and electrons (H_2 oxidation) and the reverse reaction (H_2 production) with incredibly low energy input and amazingly fast kinetics. Although there is a great deal of structural homology between H_2ase systems, the preference for catalytic activity for a given system has been finely tuned by evolutionary pressure, thus creating a catalytic preference for H_2ase's from various organisms where H_2 oxidation or H_2 production may be more essential to ensure environmental fitness. We aimed to design mutant protein scaffolds of H_2ase to destroy the native proton pathway and introduce new proton pathways, thereby elucidating important design principles for proton movement, with the goal of transferring these design principles to systems for CO_2 related chemistry. The scientific premise is that proton transport in biological systems has evolved to regulate proton delivery and removal, and these principles are transferable between systems. Our original goal was to use NMR spectroscopy and electrochemical techniques to intimately follow proton and electron movement in CpI [Fe-Fe] H_2ase. This adventure required assigning the ^{1}H-^{15}N HSQC spectrum for CpI [Fe-Fe] H_2ase, which initially looked promising for this 452-residue protein as shown in Figure 1B. However, even with a fully deuterated sample and the latest TROSY-based NMR pulse programs, we were not able to collect backbone assignment data with sufficient sensitivity to make unambiguous chemical shift assignments. Consequently, our approach was scaled back to study a simpler, nickel-substituted rubredoxin as a mimic for a [Ni-Fe] hydrogenase[4] with a focus on the influence of the secondary coordination sphere around the nickel center. The secondary coordination sphere are atoms of amino acid residues not directly bound to the metal that interact with the primary ligand, fine tuning its activity by modulating the reactivity and properties of the active site.[5]

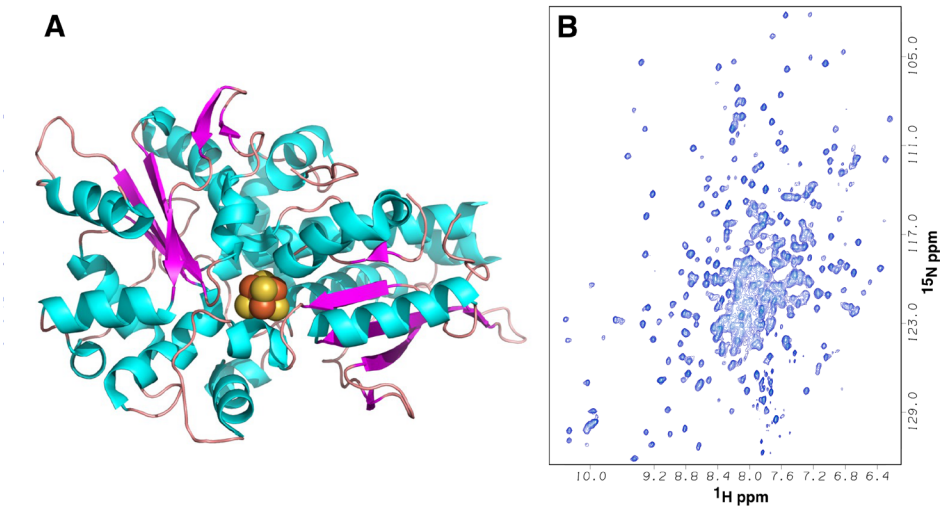

Figure 1. [Fe-Fe] hydrogenase NMR. (A) Cartoon representation of the crystal structure for the CpI [Fe-Fe] hydrogenase. (B) The ^{1}H-^{15}N HSQC spectrum for ^{2}H-, ^{13}C-, ^{15}N-labeled CpI [Fe-Fe] hydrogenase. The good resolution and chemical shift dispersion in the proton and nitrogen dimensions show the protein is structured and that chemical shift assignments were potentially possible.

1.1 Rubredoxin model for a metal center.

Desulfovibrio desulfuricans rubredoxin is a 45-residue protein with four cysteine residues that hold the metal in place as shown in Figure 2. Traditional viologen assays show that this protein, when substituted with nickel, is active for H_2 evolution.[6] Moreover, various studies show that the reaction mechanism for NiRd is similar to bacterial [Ni-Fe] H_2ases,[7] making this rubredoxin a good minimal enzyme model.[8] Other advantages in using NiRd to explore the second coordination sphere around the metal is that it is one tenth the size of CpI [FeFe] (Figure 1), 45 versus 452 residues, it does not require difficult to synthesis metal complexes, and it is not oxygen sensitive. We chose V08D, V34H, and V37N mutations because earlies studies showed they altered the electrochemistry most significantly.[4]

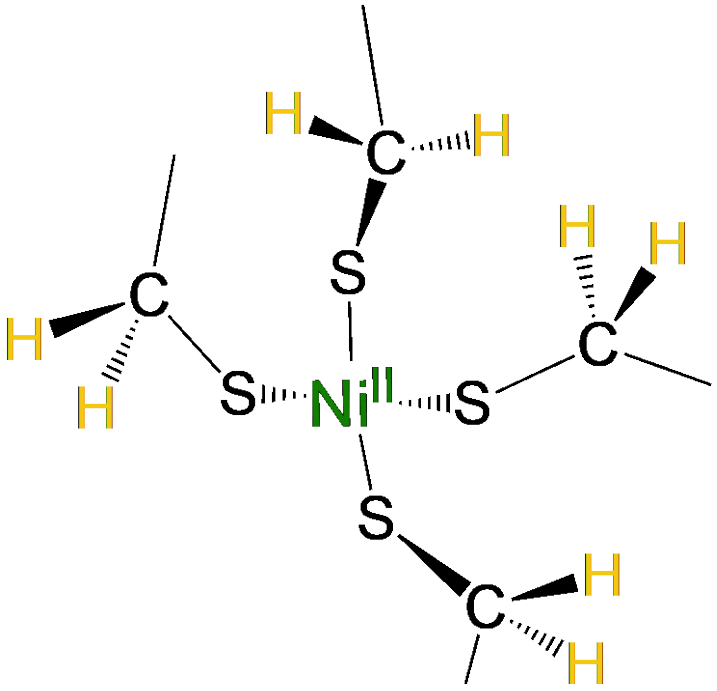

Figure 2. The metal site. The sulfur atoms from four cysteine residues hold the metal in place.

The proton spectra of both nickel- and zinc-substituted rubredoxin in Figure 3 highlight the primary feature of spectra containing a paramagnetic species - large "paramagnetic" shifts of nuclei chemical shifts. While the ^{1}H chemical shifts are largely between 12 to -1 ppm with diamagnetic zinc, with paramagnetic nickel some nuclei shift nearly 400 ppm. Chemical shifts are very dependent of the local electronic environment around the nuclei, and hence, provide valuable information regarding the position of nuclei around the metal center (eg: the secondary coordination sphere). The observation that the spectra for three single-mutant constructs are significantly different than wild-type NiRd strongly indicate these mutations effect perturbations to the secondary coordination sphere that may be responsible for the differences in activity for H_2 evolution.

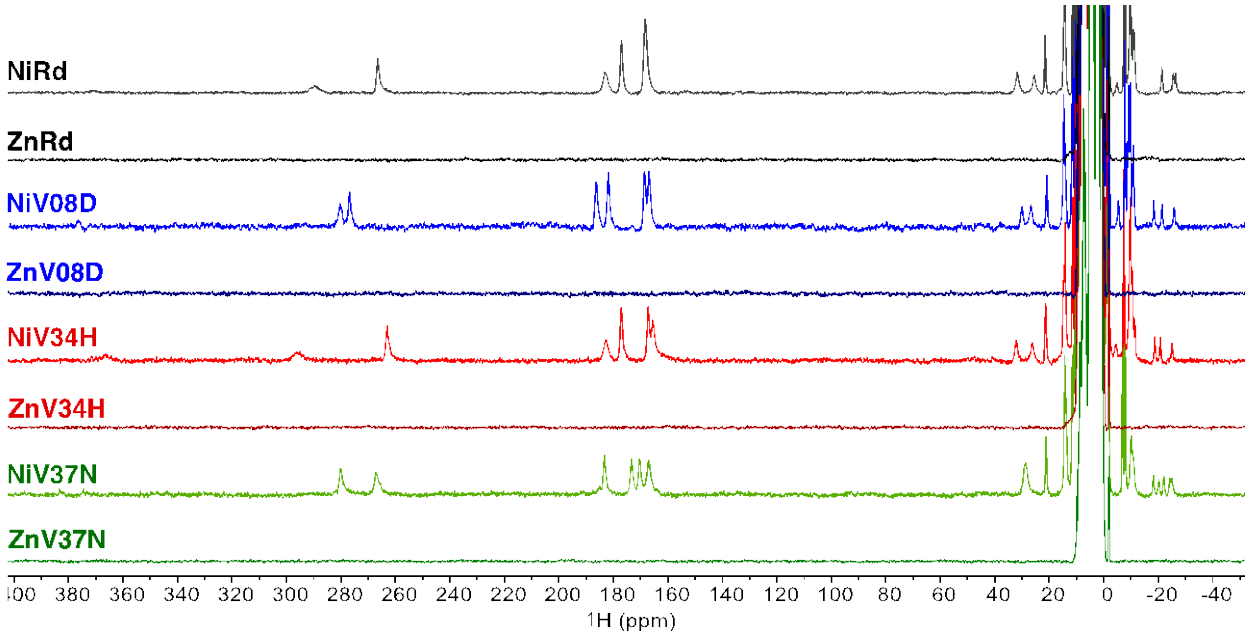

Figure 3. Rubredoxin proton spectra. Overlay of the one-dimensional ^{1}H spectra for the nickel- and zinc-substituted rubredoxins. Differences in the downfield-shifted proton chemical shifts are indicative of structural and electronic differences about the nickel center.

While the large paramagnetic shifts provide valuable information for physical calculations, the downside is that paramagnetic species also markedly shorten the relaxation times of effected nuclei. This property, together with the large chemical shift ranges, make two- and especially three-dimensional NMR experiments typically used to make protein chemical shift assignments unsuitable primarily because the signal decays before the pulse-sequence is finished. Methods have been established to get around this problem, however, many of these "work-arounds" require modifying, and then optimizing, pulse-programs.[9,10] While our success was mixed using these new methods, we discovered it helped to acquire one-dimensional spectra for carbon and nitrogen nuclei as well because this enabled us to know "where" nuclei were located, eliminating "fishing" for the right chemical shift range. Figure 4 shows the one-dimensional ^{15}N spectra for ZnRd, NiRd, and the three nickel-substituted mutants. As per the proton spectra in Figure 3, the spectra for the nickel-substituted proteins are different, further evidence that the mutations are affecting the secondary coordination sphere. Note that a problem with the direct detection of ^{15}N is that this nuclei is not very sensitive. Fortunately, it was possible to prepare very concentrated samples, in the 3 mM range, to counter this insensitivity.

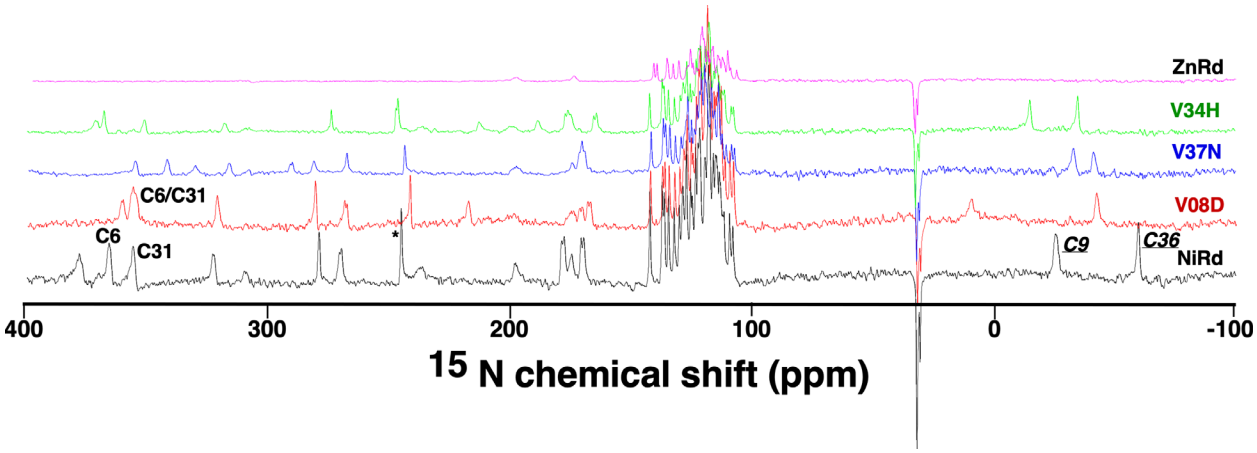

Figure 4. Rubredoxin nitrogen-15 spectra. Overlay of the one-dimensional ^{15}N spectra for NiRd, ZnRd, and three nickel-substituted constructs containing single point mutations. In the zinc-substituted versions of these proteins the nitrogen chemical shifts are all within an ~50 ppm window while the window for the nickel-substituted rubredoxins span almost 500 ppm.

The NMR data collected for the paramagnetic (nickel-substituted) and diamagnetic (zinc-substituted) form of rubredoxin (eight data sets in all) will be used for various physical calculations, including the fitting of the magnetic susceptibility tensor using the program ***Paramagpy***. As of the writing of this report the tabulations and calculations are still in progress.

1.2. Classifying Metal-Binding Sites with Neural Networks

To advance our ability to predict the impacts of the protein scaffold on catalysis, robust classification schemes to define features of protein structures that will influence reactivity are needed. One of these features is a protein's metal-binding ability, as metals are critical to the metalloenzymes catalytic conversion. As a step toward realizing this goal, in this work we used convolutional neural networks (CNN)'s to enable the classification of metal cofactor binding to a protein scaffold. Convolutional neural networks enable images to be classified based on multiple levels of detail in the image, from edges and corners to entire objects, and can provide rapid classification. First, six CNN models were fine-tuned to classify the 20 standard amino acids to choose the best performing CNN model. This model was then trained in two parallel efforts: to classify a 2D image of the environment within a given radius of the central metal-binding site, either an Fe ion or a [2Fe-2S] cofactor, with the metal visible or hidden. The two sub-classifications of the [2Fe-2S] cofactors were a standard [2Fe-2S] cofactor and a Rieske [2Fe-2S] cofactor. The average recall or success for the model correctly identifying a binding site based on images of the amino acid or the amino acid environment around a metal cofactor, both with and without the metal cofactor present, was >95%, in spite of our perception of the increased challenge of the metalloenzyme identification. This demonstrates that machine learning methodology to classify and distinguish similar metal-binding sites, even in the absence of a visible cofactor, is indeed possible and offers an accelerated approach to metal binding site identification in proteins.

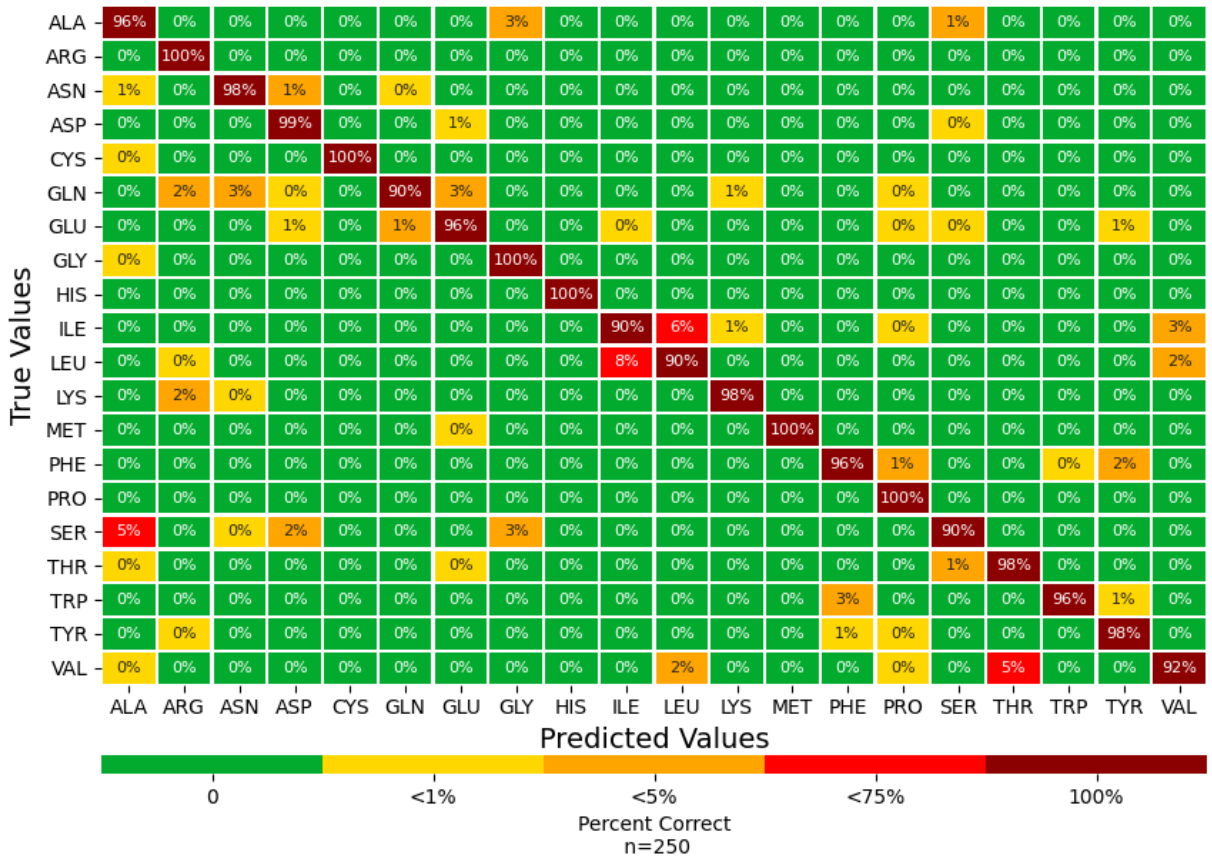

Figure 5. Predicted versus true values. Confusion matrix from the ResNet model with the test dataset as input. Each amino acid employed 250 rotations.

1.2. Molecular dynamic studies.

Molecular dynamics studies were performed to evaluate feasibility of constructing a secondary (new) proton transfer channel, by creating point mutations at select residues that were identified to be able to create a hydrogen bond network if replaced by polar residues. In order to accomplish this, we worked on refining the available force field parameters for the [Fe-S] clusters including the H-cluster, and generalizing the parameters to be complementary to additional force fields (Amber14) and transferable to more efficient code (Gromacs). The improved parameters resulted in better stability in the simulations and are transferable to other FeS containing proteins, such as *Clostridium acidurici* ferredoxin. As of the writing of this report the calculations were still in progress and required analysis.

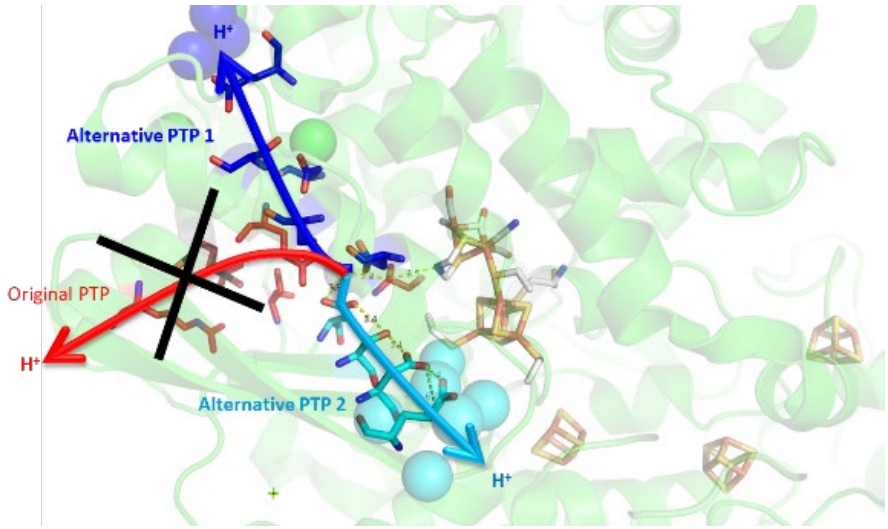

Figure 6. Designing new proton transfer pathways. By changing one amino acid at a time, *in silico* molecular dynamics simulations are being used to identify alternative proton transfer pathways (PTP) in CpI [Fe-Fe] and other H_2ases.

2.0 References

(1) Hosseinzadeh, P.; Lu, Y. Design and fine-tuning redox potentials of metalloproteins involved in electron transfer in bioenergetics, *BBA-Bioenergetics* ***2016***, *1857*, 557-581.

(2) Laureanti, J. A.; O'Hagan, M.; Shaw, W. J. Chicken fat for catalysis: A scaffold is as important for molecular complexes for energy transformations as it is for enzymes in catalytic function, *Sustain Energ. Fuels* ***2019***, *3*, 3260-3278.

(3) Lampret, O.; Duan, J. F.; Hofmann, E.; Winkler, M.; Armstrong, F. A.; Happe, T. The roles of long-range proton-coupled electron transfer in the directionality and efficiency of [FeFe]-hydrogenases, *Proc. Natl. Acad. Sci. U.S.A.* ***2020***, *117*, 20520-20529.

(4) Slater, J. W.; Marguet, S. C.; Gray, M. E.; Monaco, H. A.; Sotomayor, M.; Shafaat, H. S. Power of the secondary sphere: Modulating hydrogenase activity in nickel-substituted rubredoxin, *Acs. Catal.* ***2019***, *9*, 8928-8942.

(5) Laureanti, J. A.; Ginovska, B.; Buchko, G. W.; Schenter, G. K.; Hebert, M.; Zadvornyy, O. A.; Peters, J. W.; Shaw, W. J. A positive charge in the outer coordination sphere of an artificial enzyme increases CO_2 hydrogenation, *Organometallics* ***2020***, *39*, 1532-1544.

(6) Saint-Martin, P.; Lespinat, P. A.; Fauque, G.; Berlier, Y.; Legall, J.; Moura, I.; Teixeira, M.; Xavier, A. V.; Moura, J. J. Hydrogen production and deuterium-proton exchange reactions catalyzed by desulfovibrio nickel(II)-substituted rubredoxins, *Proc. Natl. Acad. Sci. U.S.A.* ***1988***, *85*, 9378-9380.

(7) Fichtner, C.; Laurich, C.; Bothe, E.; Lubitz, W. Spectroelectrochemical characterization of the [nife] hydrogenase of *Desulfovibrio vulgaris Miyazaki F*, *Biochemistry* ***2006***, *45*, 9706-9716.

(8) Slater, J. W.; Shafaat, H. S. Nickel-substituted rubredoxin as a minimal enzyme model for hydrogenase, *J Phys Chem Lett* ***2015***, *6*, 3731-3736.

(9) Lehr, M.; Paschelke, T.; Trumpf, E.; Vogt, A. M.; Nather, C.; Sonnichsen, F. D.; McConnell, A. J. A paramagnetic NMR spectroscopy toolbox for the characterisation of paramagnetic/spin-crossover coordination complexes and metal-organic cages, *Angew. Chem. Int. Edit.* ***2020***, *59*, 19344-19351.

(10) Piccioli, M. Paramagnetic NMR spectroscopy is a tool to address reactivity, structure, and protein-protein interactions of metalloproteins: The case of iron-sulfur proteins, *Magnetochemistry* ***2020***, *6*.

Pacific Northwest National Laboratory

902 Battelle Boulevard
P.O. Box 999
Richland, WA 99354

1-888-375-PNNL (7665)

www.pnnl.gov