

The Importance of the Active Site Canopy in [NiFe]-Hydrogenases from *Escherichia coli*



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The Importance of the Active Site Canopy in [NiFe]-Hydrogenases from *Escherichia coli*

Hydrogenase-2 from *E. coli* is a multi-faceted [NiFe]-hydrogenase that is important because of its dual role as a model enzyme capable of reversible proton reduction and its prominence in microbe metabolism, yet previous investigations of Hyd-2 have been limited by low yield. In this investigation, a novel overexpression system for the catalytic core of Hyd-2 (termed Hyd-2 NOP) was designed and used to increase the yield of Hyd-2. Hyd-2 NOP was thoroughly characterized to ensure it remained nearly identical to Hyd-2 purified by previous strategies. The increased yield enabled structural characterization of the enzyme, and the structure allowed for a more accurate prediction of the full quaternary structure of Hyd-2. The structure also confirmed the positioning of three amino acid residues that are theorized to play an important, yet ambiguous, role in catalysis.

These residues were mutated, and the resulting protein variants were electrochemically and structurally characterized to better understand the role of these amino acid residues. Two of these residues, Asp103 and Arg479, are located directly above the bimetallic cofactor in the active site, and when mutated, confirmed the significance of the secondary coordination sphere in H₂ activation. Fortunately, the R479K mutation trapped intermediates in the inactivation pathway that have never been isolated. The third residue, Glu14, has been shown to be important for the proton pathway in [NiFe]-hydrogenase, yet recent evidence from Hyd-1 challenged that finding. Comparative studies between the Hyd-1 and Hyd-2 variants with a Glu14 mutation showed the unique role of the proximal Fe-S cluster in Hyd-1 of repairing the proton pathway.

Collaborations with Guangyu Liu (Sir William Dunn School of Pathology, University of Oxford) and Dr. Liyun Zhang (Armstrong Group, Inorganic Chemistry Laboratory, University of Oxford) also explored the role of Hyd-2 in pathology and as a crucial component of biotechnology, respectively.

Collaborations

Alexander Finney and Professor Frank Sargent measured the expression levels of overproduced Hyd-2 in various strains and a gel of the rocket immunoelectrophoresis assay performed by them is presented in Chapter 3. Crystal structures and homology modeling of Hyd-2 were done by Dr. Steve Carr (Research Complex at Harwell). Also, Dr. Gemma Harris (Research Complex at Harwell) performed the SEC-MALS on Hyd-2 NOP shown in Chapter 4. Dr. Will Myers also performed all EPR spectroscopy on Hyd-2 R479K-OP after I prepared the samples. His results are presented in Figure 7.11. Experiments with *Shigella sonnei* in Chapter 5 were done in collaboration with Guangyu Liu (DPhil student, Sir William Dunn School of Pathology, University of Oxford), and he began all experiments (plating, inoculation, starter cultures), and measured cell death for experiments with *Shigella sonnei*. We collaboratively designed and executed the experiments to probe the effect of GlpG on growth on H₂, H₂ uptake, and H₂ production.

For work in Chapter 10, I collaborated with Dr. Liyun Zhang on strategies to covalently bind Hyd-2 NOP to electrodes (Armstrong Group, University of Oxford). We collaboratively designed the variants, primers, and molecular biology strategy for mutating Hyd-2 NOP to alter the surface cysteines. Dr. Liyun Zhang performed all the molecular biology and purification under my direction. I advised Dr. Liyun Zhang on technical portions of the experiment relating to overexpressed Hyd-2 such as the activation procedure required before assembling the photosynthetic H₂ production systems and helped interpret results. Dr. Liyun Zhang carried out all assembly of AgNCs-PMAA/TiO₂, electrochemical and gas chromatography measurements.

Work presented concurrently in another thesis

All of the experiments of Chapter 5 were done collaboratively with Guangyu Liu (Tang Group, Sir Dunn School of Pathology, University of Oxford). In his thesis, he is submitting the results of the H₂ uptake in *Shigella sonnei* which is the focus of section 5.4.2 which includes Figure 5.6 and Figure 5.7 in my thesis.

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Publications

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Abbreviations

<i>A. vinosum</i>	<i>Allochromatium vinosum</i>
<i>D. fructosovorans</i>	<i>Desulfovibrio fructosovorans</i>
<i>D. gigas</i>	<i>Desulfovibrio gigas</i>
<i>D. vulgaris</i>	<i>Desulfovibrio vulgaris</i>
<i>E. coli</i>	<i>Escherichia coli</i>
<i>H. marinus</i>	<i>Hydrogenovibrio marinus</i>
<i>R. eutropha</i>	<i>Ralstonia eutropha</i>
Å	angstrom
MBH	membrane bound hydrogenase
CODH	carbon monoxide dehydrogenase
CE	counter electrode
DFT	Density functional theory
ENDOR	electron-nuclear double resonance
EPR	electron paramagnetic resonance
IR	infrared
KIE	kinetic isotope effect
MHB	mixed hydrogenase buffer
MWNT	multiwalled carbon nanotubes
PFE	protein film electrochemistry
PGE	pyrolytic graphite electrode
RE	reference electrode
QM/MM	Quantum Mechanics/Molecular Mechanics
SCE	saturated calomel electrode
SHE	Standard Hydrogen Electrode
WE	working electrode
TM	transmembrane
Tat	twin-arginine translocation

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Chapter 1

Introduction

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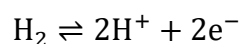
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1 Introduction

1.1 Hydrogen as a fuel

Most recent advances in personal electronics have been driven by the miniaturization of lithium-ion batteries. These batteries that power cell phones, laptops, and cars, take electric potential generated elsewhere, store it as chemical bonds, and release it upon demand. Unsurprisingly, this process is conceptually similar to what happens within biological systems. Microbes generate reduction potential and use that reduction potential for a variety of metabolic processes. At times, when there is an excess of reduction potential and a lack of electron acceptors within a cell's environment, the biological system must discharge and ideally store the reduction potential for later use. It is this ancient mechanism of energy conservation that scientists and engineers are working to mimic to meet the ever-increasing energy demand.

One way that cells carry out energy conversions is through the interconversion of molecular hydrogen (H_2) and protons (H^+) according to the following equation:



Hydrogenases are enzymes that catalyse both the reduction of protons into H_2 and the oxidation of H_2 into protons at their metallic cofactor active sites.¹ These enzymes are of great interest because of their ability to catalyse the reversible reaction at rates similar to platinum while only containing common metals that are cheap and abundant like Ni and Fe.² The production of hydrogen is of increasing interest because it can be used as a fuel or in reactions to synthesize hydrocarbons or ammonium.³ Unfortunately, clean production of hydrogen has proved elusive.⁴ Because platinum is so costly and rare, various scientific groups^{5,6} are trying to create synthetic catalysts with equal

efficiency as hydrogenases, using the same non-precious metals: nickel and iron. In order to better design these catalysts, more must be understood about the mechanism employed in nature that has been optimized by millions of years of evolution.⁷

1.2 Hydrogenases

Hydrogenases are widespread appearing across bacteria, archaea, and some eukaryotic phyla in terrestrial, aquatic, and host-associated organisms.⁸ This ubiquity could be indicative of their role in energy metabolism of some of Earth's earliest organisms.⁷ The diverse array of hydrogenases can broadly be divided into three categories based on the metal contained in the active site: [FeFe]-hydrogenases, [NiFe]-hydrogenases, and [Fe]-hydrogenases.¹

[FeFe]-hydrogenases produce H₂ at high turnover rates, but they are extremely sensitive to O₂.⁹ By contrast, [NiFe]-hydrogenases are characterized as uptake hydrogenases that are typically more O₂-tolerant as they are only reversibly inactivated upon transient O₂ exposure.¹⁰ [NiFe]-hydrogenases have therefore been the subject of extensive investigation for both their ability to withstand attack by O₂ and their potential application in biotechnological applications to replace Pt as hydrogen oxidizers.⁴

1.3 [NiFe]-hydrogenase

1.3.1 Structure

In general, the catalytic core of [NiFe]-hydrogenases consist of a large subunit (α) that contains the bimetallic active site cofactor and small subunit (β) that contains at least one, but usually three, Fe-S clusters that act as electron relays.¹¹ [NiFe]-hydrogenases operate in various compartments of the cell with a range of redox

partners including menaquinones, nicotinamide containing cofactors, and F_{420} .¹ These characteristics, as well as structure, subunit composition, and active site composition, help divide [NiFe]-hydrogenases into four main groups and 22 subgroups.^{8,12}

The most well studied [NiFe]-hydrogenases are the membrane-bound hydrogenases that make up Group 1. This group is then further divided into 8 subgroups based on the role of the enzyme and its redox partner.⁸ For ease of reference, these subgroups are placed in, and frequently referred to, by two larger categories: standard hydrogenases and O_2 -tolerant hydrogenases. Standard [NiFe]-hydrogenases are reversibly inactivated in the presence of small amounts of O_2 and are typically capable of significant quantities of both H_2 oxidation and production at neutral pH under anaerobic conditions. By contrast, O_2 -tolerant hydrogenases are capable of H_2 oxidation in a mildly aerobic environment but usually are incapable of H_2 production at neutral pH. Among the standard Group 1 [NiFe]-hydrogenases that have been thoroughly characterized are the [NiFe]-hydrogenases from *Desulfovibrio vulgaris* (group 1, subgroup a),¹³ *Desulfovibrio gigas* (1b),¹⁴ and *Allochromatium vinosum* (1e).¹⁵ While structural characterization of O_2 -tolerant hydrogenases came nearly 15 years after the first structural characterization of standard [NiFe]-hydrogenase, the structures of the O_2 -tolerant membrane-bound hydrogenases (all 1d) from *Ralstonia eutropha*,¹⁶ *Hymenobacter marinus*,¹⁷ and *Escherichia coli* (Hyd-1)¹⁸ have been solved and the enzymes have been well characterized.

The structures of both standard and O_2 -tolerant [NiFe]-hydrogenases have shown that the structure of the active site is highly conserved across all [NiFe]-hydrogenase groups. The Ni of the bimetallic active site of [NiFe]-hydrogenases is coordinated by sulphurs from four cysteines, two sulphurs as bridging ligands to Fe and

two sulphurs terminally coordinating the Ni (Figure 1.1). A subset of [NiFe]-hydrogenases contain a selenocysteine in place of one of the cysteines (C546, see Figure 4.9 for detailed figure of active site) that terminally coordinates the Ni atom at the active site.¹⁹ These enzymes, termed [NiFeSe]-hydrogenases, are also reversible hydrogenases and are known to be more O₂-tolerant than standard reversible [NiFe]-hydrogenases and belong to Group 1a.

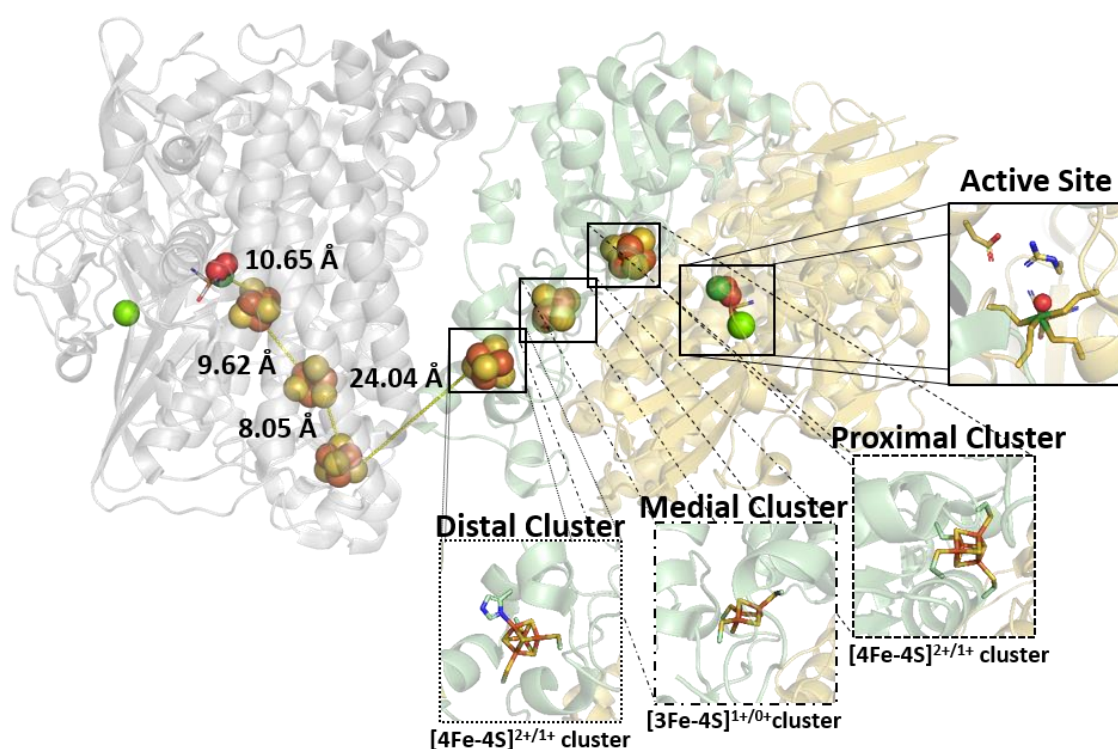


Figure 1.1: Structure of standard membrane-bound [NiFe]-hydrogenase from *D. gigas* (PDB 1YQ9). In one dimer the small subunit is coloured green and the large subunit gold; in the second both subunits are grey to accentuate the position of the metal centres. One of the dimers is coloured grey for clarity of the Fe-S clusters. The distance (Å) between the Fe-S clusters and their redox partner is shown as well as the distance between the distal clusters of the two small subunits of the dimer. The redox transition of each of the clusters is also listed.²⁰

In addition to the cysteines that coordinate with the Ni, the Fe atom is coordinated by three inorganic ligands: two CN and one CO. Another bridging ligand between Ni and Fe is present depending on the state of the hydrogenase.^{21,22} Given the dependence on biologically rare ligands at the active site, a complex set of chaperone

and assembly proteins are required to properly assemble the active site.²³ Much research in the past decade has elucidated this process that has been proposed to occur in four steps: CN synthesis, CO-synthesis, Fe-CO-2CN synthesis, and Ni insertion.^{24–27} Although there are ongoing investigations to determine the exact order of Fe-CO-2CN synthesis, it is important to note the length and complexity of the process to incorporate biologically rare ligands at the active site. If at any point one of these ligands becomes limiting, hydrogenase synthesis is stopped, and the immature protein is disassembled. After the large subunit is fully assembled and checked, a peptide tag on the C-terminal is cleaved by a specific endopeptidase to signal a properly assembled large subunit.²⁸

The Fe-S clusters of the small subunit primarily serve as a wire: shuttling electrons from outside the protein to the active site where the reaction occurs. Typically, Fe-S clusters exist in one of two redox states. For example, the distal cluster in Hyd-2 is a [4Fe-4S] cluster that has an oxidation number of +1 in its reduced state and +2 in its oxidized state (Figure 1.1). These redox transitions facilitate the transport of electrons along the Fe-S cluster chain between the surface of the protein and the active site. However, the cluster that occupies the proximal position in O₂-tolerant [NiFe]-hydrogenases has been shown to be capable of spanning an additional redox state allowing it to deliver two electrons in a rapid, sequential manner.^{16–18} This property allows the special Fe-S cluster to serve as a reservoir of electrons for rapid electron delivery that helps O₂-tolerant [NiFe]-hydrogenases reduce attacking O₂ to harmless water.

The Fe-S clusters of the small subunit are synthesized and inserted into the small subunit via pathways that are not specific to hydrogenase. Although the exact mechanism of Fe-S cluster synthesis is still undefined, it has been shown that the

membrane-bound [NiFe]-hydrogenases depend on the 'ISC' pathway encoded by the *isc* operon.²⁹ The Fe-S clusters of [NiFe]-hydrogenase are distinguished by their distance from the active site: proximal, medial, and distal in order of increasing distance from the active site (Figure 1.1). The distal cluster serves as the entry/exit point for electrons as they are relayed by the Fe-S clusters to and from the active site. The Fe-S clusters are typically within 14 Å of their redox partners which is the range of rapid electron tunnelling (Figure 1.1).³⁰

For some membrane-bound [NiFe]-hydrogenases, once the small subunit is fully formed and loaded with Fe-S clusters, it associates with the large subunit and is processed and transported to the cytoplasmic membrane by the twin arginine translocation (Tat) protein export system. The Tat system derives its name from the signal peptide motif (SRRxFLK). In the case of the catalytic core, the Tat signal is located on the N-terminal of the small subunit and cleaved by a signal peptidase after proper export.¹¹

1.3.2 Hydrogenase states

The active site of [NiFe]-hydrogenases exist in a variety of states in which Ni changes oxidation states, whereas the Fe(II) atom of the active site remains unchanged in all states.³¹ Upon reversible oxidative inactivation, a hydroxide tightly coordinates the metal cofactor forming two states (Figure 1.2).³² In a state termed Ni-A, the active site experiences another oxidation to one of the cysteines bound to the metal cofactor, whereas in Ni-B the active site simply coordinates a hydroxide.^{32,33} While Ni-B takes seconds to reactivate upon the application of reduction potential, Ni-A takes several minutes to hours to reactivate.

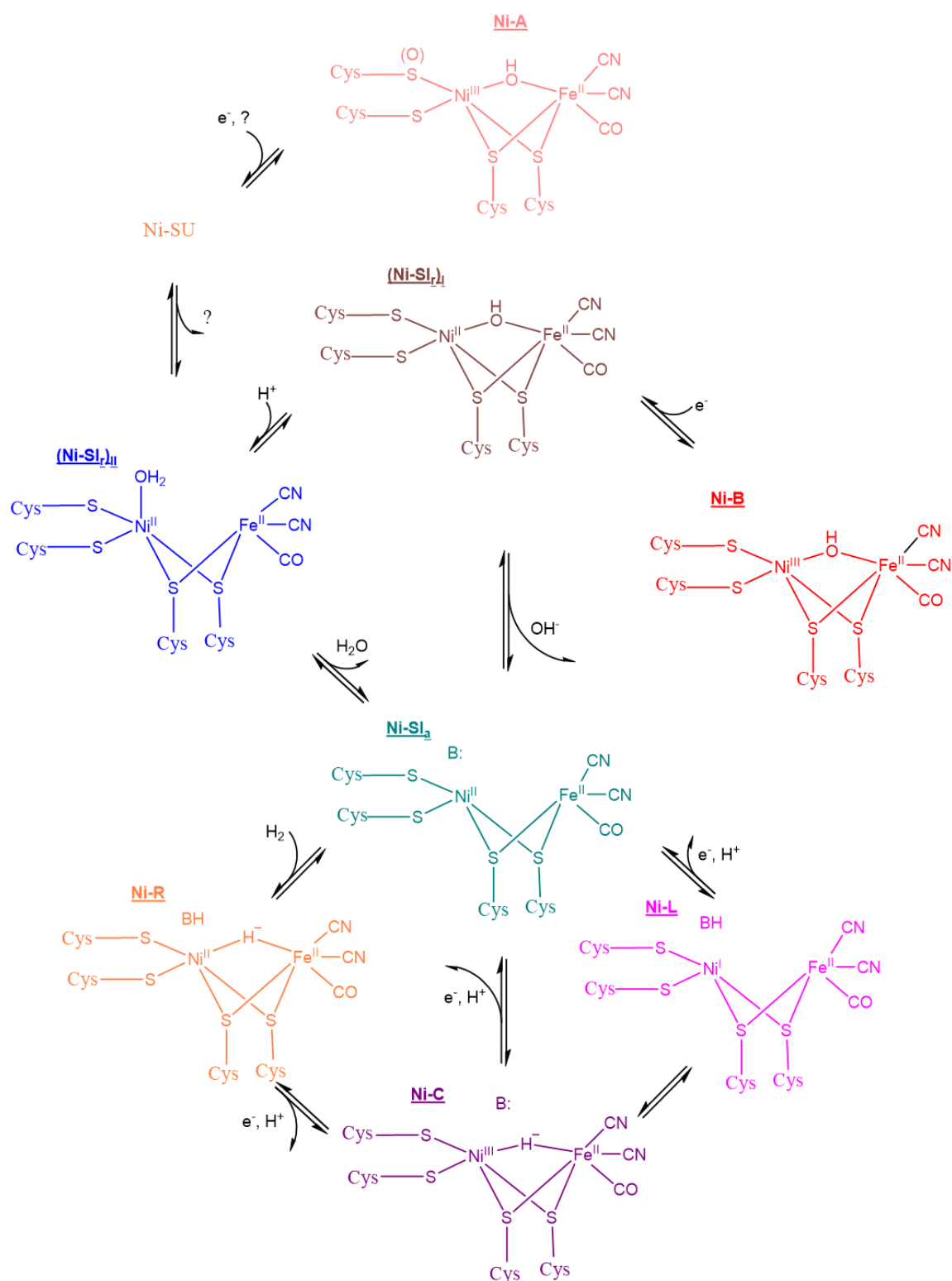


Figure 1.2: Proposed reaction scheme for hydrogen activation in [NiFe]-hydrogenases. Ni-R, Ni-C, Ni-A, and Ni-B have been well-characterized (though there is still debate concerning the nature of the oxidation on the terminal sulphur observed in Ni-A and that ambiguity is reflected in the scheme) whereas Ni-SU, (Ni-SI_r)_I, (Ni-SI_r)_{II}, Ni-SI_a, and Ni-L have only been observed spectroscopically and their role and structure is still under investigation.^{34–36} Thus, the proposed structures of species only observed spectroscopically are shown in the scheme with the exception of Ni-SU for which there is little agreement in the literature about the structure of Ni-SU. The base responsible for activating H₂ is still under debate. Here the B denotes an unspecified base at the active site that could also be one of the terminal S atoms bound to the Ni.

While the chemical meaning of the mechanism of hydrogen oxidation and production by [NiFe]-hydrogenases is still the subject of widespread research, a general picture of the catalytic mechanism has emerged over the past decades.¹ This picture has been enhanced by understanding how inhibitors interact with the active site.^{37,38} Upon one-electron reduction of Ni-B, Ni-SI_r is formed (Figure 1.2).³⁴ In this state, a hydroxide continues coordinating the [NiFe] cofactor, and the active site is therefore unreactive to CO attack.³⁴ In some [NiFe]-hydrogenases a second (Ni-SI_r)_{II} state has been characterized that is in acid-base equilibrium with (Ni-SI_r)_I suggesting that the two states differ by a protonation event.³⁹ It has been theorized that the protonation event occurs to either the sulphur of a terminal cysteine (C546) or the bridging hydroxide, converting it to a water molecule that coordinates the Ni atom of the [NiFe] cofactor.^{34,35,40} Less is known about the structures of the reactivation pathway from Ni-A. A one electron reduction of Ni-A leads to a spectroscopically unique Ni-SU with a structure that is undefined. Further reduction of Ni-SU leads to the most oxidized state of the catalytic cycle, Ni-SI_a, but it has been shown that (Ni-SI_r)_{II} could also be an intermediate in the pathway from Ni-SU to Ni-SI_a.³⁴

Further reduction of Ni-SI_r leads to Ni-SI_a that is reactive to CO attack and subsequently forms Ni-SCO.⁴¹ If, instead, the active site is free of inhibitors and in the presence of hydrogen, the active site metals will act in conjunction with a base and heterolytically cleave hydrogen to form Ni-R. In this state, a hydride occupies the bridging position between Ni and Fe, and it is the most reduced state of [NiFe]-hydrogenases.⁴² Oxidation of the Ni leads to Ni(III) with a bridging hydride still present—a state termed Ni-C.⁴³ If Ni-C is illuminated at low temperatures, the hydride

leaves as a proton reducing the Ni atom to Ni(I) in a state termed Ni-L.¹ It has also been shown that Ni-L could be a true catalytic intermediate for some [NiFe]-hydrogenases as it has been observed under dark turnover conditions for *E. coli* Hyd-1.³⁶ It is worth noting that most of the work elucidating the mechanism of [NiFe]-hydrogenases has been done on membrane-bound uptake hydrogenases and therefore the experiments have been focused on various states formed during hydrogen oxidation.^{42,44} The law of microscopic reversibility dictates that the processes for hydrogen oxidation are fully reversible, and therefore hydrogen could be produced by the reverse mechanism of hydrogen oxidation. Notwithstanding, there is evidence of multiple spectroscopically unique configurations of catalytically active states (3 Ni-R states and 3 Ni-L states).¹ The significance, role, and physical identity of these states are still being investigated, yet their existence may provide evidence of hydrogenase's ability to fine tune the reaction mechanism depending on the conditions and direction of the reaction.

1.3.3 Oxygen attack

From the original discovery of hydrogenases, they have been known to exhibit extreme sensitivity towards O₂.⁴⁵ The effect of oxygen on hydrogenases is one of the most important and widely studied interactions with hydrogenases because of its wide-ranging implications.^{46–49} Any practical future application of hydrogenase enzymes or, more likely, hydrogenase mimics, whether in a fuel cell or as an industrially important

¹ While the nomenclature of the states in the [NiFe]-hydrogenases vary, the name for Ni-L is the most controversial as investigators disagree on the role Ni-L plays, if any, in the mechanism and name it accordingly. In this thesis, the naming convention will follow the most widely used nomenclature in the literature.

catalyst to produce hydrogen, depends on the ability to withstand exposure to O₂ because creating large-scale, robust anaerobic environments is prohibitively expensive.

Typically, the active site is converted to a mixture of two states upon exposure to oxygen: Ni-A and Ni-B. While this inactivation is reversible, other conversions upon O₂ attack cause irreversible damage by creation and subsequent reaction of reactive oxygen species.^{50,51} These form when oxygen attacks a reduced active site and scavenges the electrons to form a singly or doubly reduced oxygen species. Protein film electrochemistry coupled with site-directed mutagenesis have served to investigate and measure the oxygen tolerance of certain hydrogenases.^{50,52} Significant work has been carried out on O₂-tolerant hydrogenases from various organisms to understand the characteristics of the enzyme that confer oxygen tolerance.^{32,49,53}

Two notable pathways to oxygen tolerance should be mentioned. The first, simpler pathway to oxygen tolerance is the ability of the protein matrix to exclude harmful O₂ molecules from the active site by the size of the gas channels that allow gaseous access to the active site.⁵⁴ This approach has been studied with [NiFe]-hydrogenase variants that replace a valine that lies at the bottleneck of the gaseous channel with bulkier amino acids.⁵⁵ These variants have exhibited greater tolerance and activity during prolonged exposure to O₂ and have provided compelling evidence about the importance of gaseous channels.⁵⁵ The second theorized group of O₂-tolerant characteristics is an enhanced mechanism for the hydrogenase's active site to be rescued by a sufficient supply of electrons which makes the hydrogenase act as an oxidase, reducing O₂ to water in a four-electron reduction. Because of this reaction, the [NiFe]-hydrogenase is converted almost exclusively to the Ni-B state which facilitates easy reactivation and continued activity.

1.3.4 Protein film electrochemistry for investigation of hydrogenase

Early investigations into hydrogenase's reaction to O_2 provided limited results as oxygen oxidizes any redox mediators used in standard electrochemical techniques. Many advances in the understanding of O_2 -tolerant hydrogenases have come from a suite of techniques pioneered by Professor Fraser Armstrong at the University of Oxford^{56,57} known as protein film electrochemistry (PFE). In this technique, small amounts of redox active enzymes, such as hydrogenase, are adsorbed onto an electrode in an electroactive configuration. The protein forms a near monolayer which can be swept through a range of potentials, and activity is measured directly as current (for a more detailed description of PFE see section 2.10).

This technique has allowed for detailed measurements of small quantities of enzyme in which the conditions in the cell (temperature, gas concentration, etc.) are fully controlled. Using this technique, it was revealed that not only do O_2 -tolerant hydrogenases reactivate from oxygen attack quickly but also can maintain hydrogen oxidation activity in the presence of O_2 .⁵⁰ To better understand the mechanism of oxygen-tolerance, two model hydrogenases of *E. coli* are the subject of intense research because they exemplify the divide between standard and O_2 -tolerant hydrogenases.

1.4 Hydrogenases in *E. coli*

E. coli is known to produce four hydrogenases, but the two hydrogenase isotypes that are the most thoroughly investigated within *E. coli* (and the focus of this work) are Hydrogenase-1 (Hyd-1) and Hydrogenase-2 (Hyd-2). Hyd-1 is encoded by the *hya* operon whereas Hyd-2 is encoded by the *hyb* operon. Both enzymes are membrane-bound, periplasmically oriented [NiFe]-hydrogenases. Like all Group 1 [NiFe]-hydrogenases, both enzymes are made up of a large subunit (HybC in Hyd-2 and

HyaB in Hyd-1) that contains a [NiFe] cofactor and a small subunit (HybO in Hyd-2 and HyaA in Hyd-1) that contains three Fe-S clusters that act as electron relays.

Notwithstanding the similarities and shared environment in the periplasm of *E. coli*, Hyd-1 is an O₂-tolerant enzyme and Hyd-2 is an O₂-sensitive enzyme. The ability of Hyd-1 to maintain a significant portion of activity during exposure to oxygen and then quickly and fully recover after exposure to air has drawn significant investigation.⁵⁰ Hyd-1 has been shown to be particularly good at operating as an oxidase when exposed to oxygen,⁵³ converting the attacking O₂ to water as described above. Upon investigation, two key characteristics have emerged as important to O₂ tolerance. The first characteristic is the existence of a unique [4Fe-3S]6cys proximal cluster found in Hyd-1 (and indeed other O₂-tolerant [NiFe]-hydrogenases)¹ that accesses the 5+/4+/3+ oxidation states compared to the more standard [4Fe-4S] proximal cluster in Hyd-2 that only transitions between the 2+/1+ oxidation states.^{16–18} When Hyd-1 is exposed to oxygen, the [4Fe-3S]6cys cluster releases an electron and then undergoes a structural change that enables the release of a second electron via a proton coupled electron transfer resulting in an Fe(III) subsite and a deprotonated amide.⁵⁸ Thus, the proximal cluster in Hyd-1 is able to quickly and sequentially release two electrons to reduce oxygen in the active site.⁴⁸ Indeed, variants that have replaced cysteines in the proteinaceous shell that stabilizes the superoxidized [4Fe-3S]6cys⁵⁺ cluster with the residues found surrounding a standard [4Fe-4S] cubane, like one that is found in Hyd-2, have lacked the signature O₂ tolerance of Hyd-1 (see Section 4.1.3 for a detailed explanation of the [4Fe-3S]6cys cluster).^{47,53}

The second characteristic that is theorized to provide a greater degree of oxygen tolerance in Hyd-1 is the quaternary structure. Although in its simplest configuration,

Hyd-1 is a heterodimer made up of 1 small and 1 large subunit ($\alpha\beta$), it typically exists as a dimer of heterodimers ($\alpha\beta$)₂, *i.e.* tetramer. Investigations comparing the oxygen tolerance of the heterodimeric to tetrameric configurations of Hyd-1 have revealed that the heterodimer alone is less O₂ tolerant.⁴⁹ This discovery built on the work that Volbeda et al. did to show that, in its tetramer configuration, the distal clusters of the two small subunits of certain hydrogenases are within 14 Å of each other and therefore capable of rapid electron transfer from one dimer to another. The proximity of the two dimers could allow one dimer to reduce attacking O₂ with the products of H₂ oxidation from the sister site.^{30,59} The tetrameric conformation is common to most membrane-bound hydrogenases, yet standard hydrogenases are typically arranged with the distal clusters too far apart for rapid electron tunneling.⁴⁹ For example, the two distal clusters of *D. gigas* are 24.0 Å apart (Figure 1.1). However, Hyd-2 is also theorized to exist in this tetrameric conformation with a high level of structural similarity to Hyd-1. Structural models of Hyd-2 based on Hyd-1 have even suggested that the distal clusters could remain within 14 Å of each other. Solving the crystal structure of Hyd-2 could therefore explain more about this characteristic and to what degree this conformation plays a role in oxygen tolerance. The differences between the two enzymes, though, go beyond oxygen sensitivity.

Although enzymes cannot have a catalytic bias other than that dictated by thermodynamics, these two enzymes exhibit very different apparent biases due to a variety of factors.⁵² Hyd-1 is biased towards hydrogen oxidation. With a relatively large over-potential (50 mV) required for hydrogen oxidation when operating at pH 6, Hyd-1 performs no measurable proton reduction under these conditions.⁵² Conversely, Hyd-2 is capable of significant hydrogen evolution at pH 6, yet it still exhibits bias towards

hydrogen oxidation.⁵² These two hydrogenases, though, are thought to have identical active sites, and therefore serve as excellent hydrogenases to study to understand the nuances of the mechanism of hydrogen oxidation and proton reduction as well as oxygen tolerance.

Recent work has predominantly focused on Hyd-1 for two reasons. First, Hyd-1 is stable under a wide range of pH, high temperature, and prolonged exposure to O₂. The resiliency of the enzyme makes it more suited to different investigative techniques. Second, the purification of Hyd-1 yields a greater amount of enzyme (around ten-fold greater compared to Hyd-2)⁵² which typically makes variant expression more assured as decreases in expression that sometimes accompany genetic manipulation will still yield significant quantities of enzyme. This emphasis on a single class of hydrogenase limits the utility of observations. Moreover, Hyd-2 is a truly reversible catalyst at neutral pH, and variants of Hyd-2 would have greater implications for the mechanism for hydrogen evolution, which is of utmost interest.

1.5 Hyd-2

The role of Hyd-2 came to light when Ballantine et al identified Hyd-2 as the major respiratory uptake hydrogenase in *E. coli*.⁶⁰ Hyd-2 was shown to be maximally expressed during exponential phase under anaerobic conditions with glycerol as the carbon source and fumarate as the terminal electron acceptor.⁶¹ Their assays relied on tryptic digest of Hyd-2 that cleaved the transmembrane (TM) domain of the small subunit freeing the catalytic core (Figure 1.3).⁶⁰ These initial results confirmed the ability of the catalytic core to catalyse hydrogen oxidation with a truncated TM domain, and through the use of analytical ultracentrifugation, the catalytic core of Hyd-2 was predicted to exist as a tetramer (HybOC)₂.⁶⁰

Although the original genetic assignment of Hyd-2 mistook the predicted Fe-S clusters of partner ferredoxin (HybA) as the Fe-S clusters in the small subunit, the operon of Hyd-2 has been mapped as *hybOABCDEFG*.^{62,63} The catalytic core is formed by HybC, the large subunit containing the [NiFe] active site cofactor, and HybO, the small subunit that contains three Fe-S clusters (Figure 1.3). HybO and HybA are both synthesized with N-terminal Tat signals that assist in maturation and targeting to the periplasmic side of the cytoplasmic membrane where both subunits are anchored by a single C-terminal TM domain.^{23,64} These two subunits are predicted to form a molecular wire that connects electrons from the interconversion of H₂ and protons with reduction/oxidation of menaquinones at HybB. Although HybC and HybO have close homologues in Hyd-1, HybA and HybB are unique to Hyd-2.

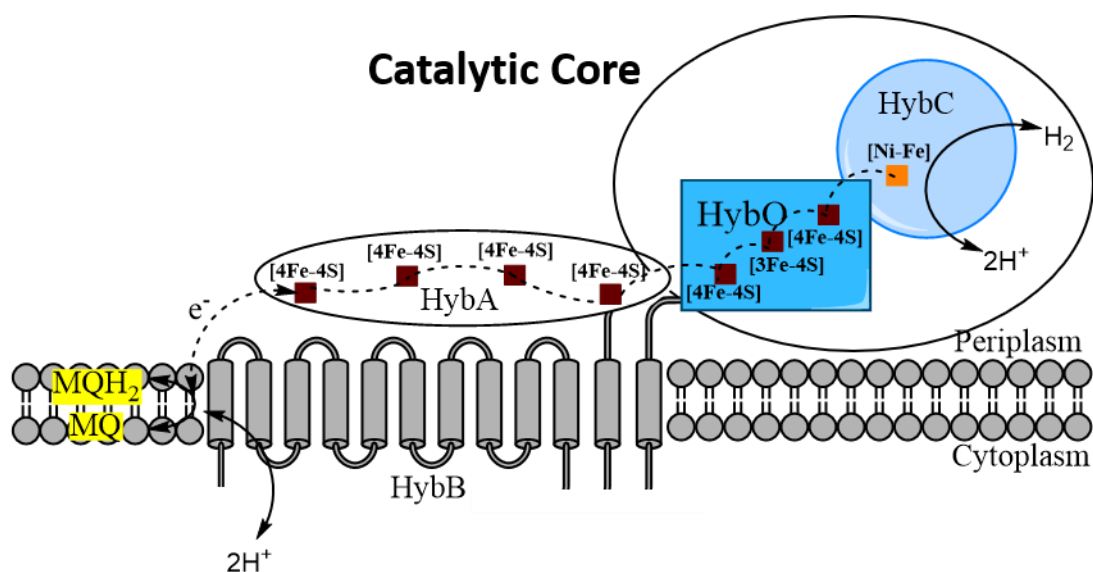


Figure 1.3: Minimal schematic of the position of different subunits of Hyd-2 in relation to the cytoplasmic membrane illustrating the reduction and oxidation occurring in the quinone pool at HybB and a pathway for electron relay through the Fe-S clusters HybA and HybO to the [NiFe] cofactor of HybC.

Hyd-2 has been shown to act as an in vivo bidirectional redox valve allowing excess reduction potential during fermentation to be released as H₂ and, under

different conditions, oxidizing H_2 to generate energy for the cell.⁶⁵ It has also been found that a proton uncoupler (carbonyl cyanide m-chlorophenyl hydrazine, CCCP) can totally inhibit hydrogen production activity suggesting that hydrogen production is driven by proton motive force.⁶⁵ This evidence implies that the reverse reaction, hydrogen oxidation, generates a proton gradient. It is therefore likely that HybB is responsible for coupling hydrogen oxidation to both menaquinone reduction and proton translocation. The mechanism by which it transports protons is still unknown, as it lacks classical haem ligands typically found in 'cytochrome B' proteins like the subunit associated with Hyd-1 (HyaC). HybB is more like the cofactor-less PsrC-type proteins that act as true conformational ion pumps.⁶⁶ The structure of Hyd-2 would therefore also elucidate important, novel bioenergetic mechanisms.

The physiological importance of Hyd-2 is exemplified by its importance in animal pathogens such as *Salmonella enterica*^{67–69} as well as plant pathogens like *Pectobacterium atropsecticum*.⁷⁰ Interest in Hyd-2 therefore extends beyond its capability to efficiently produce hydrogen, to understanding its unique mechanism of linking hydrogen production and oxidation to proton translocation with implications in the pathogenicity of *E. coli* and other microorganisms.

1.6 Scope of this thesis

This thesis details efforts to develop Hyd-2 into a model system that can be used to gain insight into the mechanism and importance of hydrogen activation. Chapter 2 details the materials and methods used for the investigation as well as electrochemical theory. Chapter 3 discusses the efforts to increase the production of Hyd-2 ultimately leading to the design and creation of a novel overexpression system that yields a Hyd-2 variant with nearly identical enzymatic characteristics. Chapter 4 presents a crystal

structure of HybOC enabled by the increased yield of Hyd-2 from the overexpression system. Building on the insight gained from the predicted full quaternary structure of Hyd-2 and, in collaboration with the Dunn School of Pathology, University of Oxford, Chapter 5 explores the role of Hyd-2 and other proteins that could process Hyd-2 in pathogens. Chapters 6 and 7 present the results of site directed mutagenesis at the active site and insight gained about the mechanism of hydrogen activation and aerobic inactivation. Chapters 8 and 9 discuss the results of site directed mutagenesis of amino acids that are important in proton transfer and electron transfer. Importantly, for chapters 6-9, comparisons with the homologous mutations in Hyd-1 serve to guide the research and gain further insight into the similarities and differences between the two enzymes. Finally, concluding remarks processing the resulting insight about Hyd-2 holistically are included in Chapter 10.

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Chapter 2

Materials, methods, and theory

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2.1 Molecular biology techniques

2.1.1 Purification of plasmid DNA

Lysogeny broth (LB) (5 mL) with the corresponding antibiotic (100 µg/mL ampicillin or 25 µg/mL chloramphenicol) was inoculated with a single colony of *E. coli* DH5α containing the plasmid and was incubated overnight (30°C for pMAK plasmids, 37°C for pQE-80L plasmids) shaking at 200 rpm. Plasmid DNA was purified from the culture using QIAprep Spin Miniprep Kit according to the manufacturer's instructions.

2.1.2 Agarose gel electrophoresis

Agarose gels were prepared by mixing 0.7 % agarose (Melford) with TAE buffer (40 mM Tris-acetate, 50 mM EDTA, 1M acetic acid, pH 8.0). The resulting mixture was microwaved for 1 min, appropriate quantity (3 µL for 60 mL gel) of 20,000x RedSafe Nucleic Acid Staining Solution (Intron Biotechnology) was added, and the gel was allowed to set in the appropriate mould. Samples of DNA were prepared for electrophoresis by the addition of an appropriate volume of 6x DNA loading dye (NEB B7024). Gels were submerged in TAE buffer and a voltage of 100 V was applied until the dye front reached the end of the gel. The 1 kb DNA ladder (NEB 3232) was used as a marker. DNA was visualized via the fluorescence of the RedSafe staining solution upon illumination with UV light. For DNA extraction from the agarose gel, the resulting bands of DNA were excised from the gel with a clean scalpel. The DNA was then extracted from the agarose gel using the QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.1.3 Preparation of competent *E. coli* cells

Non-selective LB (5 mL) was inoculated with a single colony of *E. coli* DH5 α from the non-selective plate and incubated at 37°C overnight, shaking. The following morning, this culture was subcultured (50 μ L) into non-selective LB (50 mL) and similarly incubated until an optical density of approximately 0.6 was reached after which, the cells were placed on ice for 30 min. Cells were then harvested in a Beckman GS-6R centrifuge at 1462 RCF, 4°C for 10 min, and the supernatant discarded. Cells were gently resuspended in TFB1 solution (30 mL) and remained on ice (20 min) after which the centrifugation step was repeated. The cells were resuspended in TFB2 (2 mL) and aliquoted (50 μ L) into autoclaved Eppendorf tubes and flash frozen in liquid nitrogen for storage at -80°C.

TFB1 buffer contained potassium acetate (30 mM), rubidium chloride (100 mM), calcium chloride (10 mM), manganese chloride (50 mM), hexamminecobalt (III) chloride (3 mM), glycerol (15% v/v) and was adjusted to pH 5.8 with acetic acid. TFB2 buffer contained MOPS (10 mM), calcium chloride (75 mM), rubidium chloride (10 mM), glycerol (15% v/v) and was adjusted to pH 6.5 with potassium hydroxide. The resulting solutions were filter sterilized (0.22 μ m) and stored in the dark at 4°C.

2.1.4 Transformation of *E. coli* cells with plasmid DNA

Cells were thawed on ice before addition of 0.5-2.0 μ L of plasmid DNA. The resulting mixture remained on ice for 30 min before being heat shocked (37°C for pMAK plasmids and 42°C for pQE-80L plasmids) for 30 s. The cells were placed on ice for 2 min. Non-selective LB (1 mL) was then added and the cells were incubated shaking (30°C for pMAK plasmids and 37°C for pQE-80L) for 1-4 h before being plated onto selective LB agar plates and incubated overnight (30°C for pMAK plasmids and 37°C for pQE-80L).

2.1.5 DNA sequencing

Sanger Sequencing was performed by Source Bioscience to confirm mutations and desired molecular biology constructs. Plasmid DNA was sequenced as is, whereas the portion of interest in the genomic DNA was amplified via colony PCR (see 2.2.1) and sequenced.

2.2 Polymerase chain reaction (PCR) technique

PCR for cloning was carried out using high-precision Q5 polymerase (NEB). PCR for amplification of a region of interest in chromosomal DNA (which would subsequently be sequenced to check for a mutation) was carried out with the economical, yet more error-prone, Taq polymerase. All PCR conditions are listed in the following section.

2.2.1 PCR with Taq Polymerase

For colony PCR, a colony suspension was made with 100 μL of MQ H_2O and the amount of template depended on the approximate concentration of the suspension. For suspensions with a large colony, 1-2 μL were used and that amount was doubled for suspensions with smaller colonies.

Table 2.1: PCR mix for PCR with Taq polymerase.

Reagent	Volume (μL)	Supplier
MQ H_2O	Up to 50	Sigma
10x Taq Buffer	5	Invitrogen
MgCl_2 (50 mM)	1.5	Invitrogen
W1 detergent	2.5	Invitrogen
dNTPs (10 mM)	1	Thermo Fisher
Forward Primer (100 μM)	0.5	Thermo Fisher
Reverse Primer (100 μM)	0.5	Thermo Fisher
Taq Polymerase	0.5	Invitrogen
Template DNA	2-4	N/A

Table 2.2: PCR cycling parameters for Taq polymerase.

Temp (°C)	Time (s)	Repeat
95	300	1
95	30	30
58	58	
72	60 per kB	
72	420	1

2.2.2 PCR with Q5 polymerase

Table 2.3: PCR mix for PCR using Q5 polymerase.

Reagent	Volume (μL)	Supplier
MQ H ₂ O	Up to 50	Sigma
5x Q5 Buffer	10	NEB
GC Enhancer	10	NEB
dNTPs (10 mM)	1	Thermo Fisher
Forward Primer (10 μM)	2.5	Thermo Fisher
Reverse Primer (10 μM)	2.5	Thermo Fisher
Q5 Polymerase	0.5	NEB
Template DNA	1-2	N/A

Table 2.4: PCR cycling parameters for PCR with Q5 polymerase.

Temp (°C)	Time (s)	Repeat
98	300	1
98	30	30
58	30	
72	30 per kB	
72	420	1

2.3 Site-Directed Mutagenesis

2.3.1 Gibson Assembly

Genetic engineering in the present work relied on the methodology in which two or more fragments of DNA containing overlapping portions can be assembled with a mixture of exonuclease and polymerase. This technique is termed Gibson Assembly.¹ The exonuclease removes 5' to 3' DNA creating sections of single-stranded DNA on both ends of each fragment. These single-stranded DNA sections are complementary and therefore will anneal to create the desired plasmid. The product of Gibson Assembly was

transformed into competent cells to verify the successful assembly of the fragments. Primers were designed for Gibson assembly to allow for 25-40 bp overlap between the two fragments (Figure 2.1), and when necessary, these fragments were analysed with online tools (<https://www.idtdna.com/calc/analyzer>) to ensure secondary ssDNA structures would not impede the assembly. The fragments in this thesis were amplified using PCR in separate reactions and the PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen). DpnI (1 μ L), a restriction endonuclease that targets methylated DNA, was then added to the purified PCR products to digest any residual template DNA for 8 h at 37°C. Incubation at 80°C for 20 minutes was then used to inactivate the DpnI. Each amplicon was then separated by gel electrophoresis to ensure that the amplicon was the correct size and was the sole product of the PCR amplification.

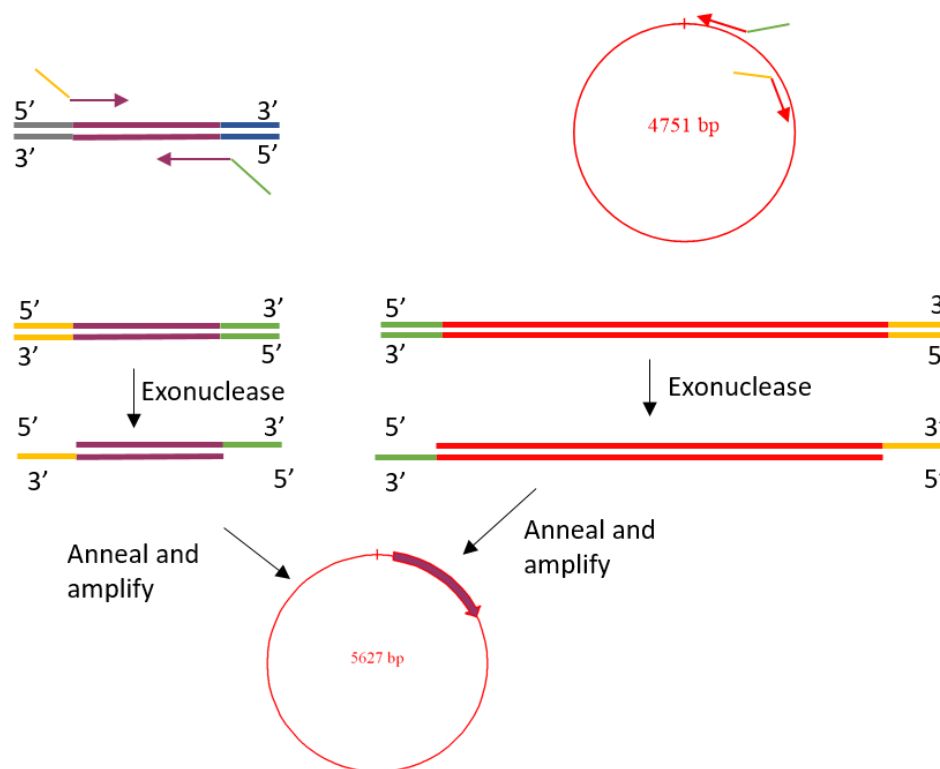


Figure 2.1: Diagram of two-fragment Gibson Assembly including amplification of the gene (left) and linearization and amplification by PCR of the plasmid (right). The genes are then treated with exonuclease that removes DNA bases from 5' to 3' followed by annealing and amplification.

The concentration of the amplified fragment was then measured using a GE Nanovue Plus Spectrophotometer, and its concentration adjusted so that a mixture of equal volumes of the two fragments would result in a 1:5 molar ratio of large fragment:small fragment. This mixture (1.5 μ L of each fragment) was combined with Gibson Assembly Master Mix (3 μ L, NEB) and incubated at 50°C for 1h-4h. The product was then transformed into commercially competent *E.coli* cells (DH5 α , NEB).

2.3.2 Primer design for site-directed mutants

Primers for site-directed mutagenesis were designed by replacing the nucleic acids in the wild-type codon for the desired mutated codon based on codon usage in *E. coli*.² Two mutagenic primers were designed to anneal to the plasmid to amplify the gene in different directions in coordination with the general primer pair for the plasmid.

2.4 pMAK plasmid

For mutations to the bacterial chromosome a methodology based on Hamilton *et al* was used.³ In this methodology, transferring a mutation from a pMAK plasmid to the chromosome takes advantage of the plasmid's temperature sensitive replicon (pSC101), which replicates the plasmid at the permissive temperature (30°C) but not at the non-permissive temperature (44°C), and the plasmid's chloramphenicol resistant cassette (Cm^r). Using these two features in tandem, it is possible to distinguish cells that in different steps have taken up the plasmid, integrated the plasmid into the chromosome, and been cured of the plasmid and then rely on the statistical probability of two homologous crossover events occurring between the gene on the plasmid and in the chromosome (in this instance *hybC*). The portion of the chromosome in question is

amplified and sequenced to verify the mutation. This process, commonly referred to as the 'pMAK cycle,' was carried out according to the following protocol (Figure 2.2).

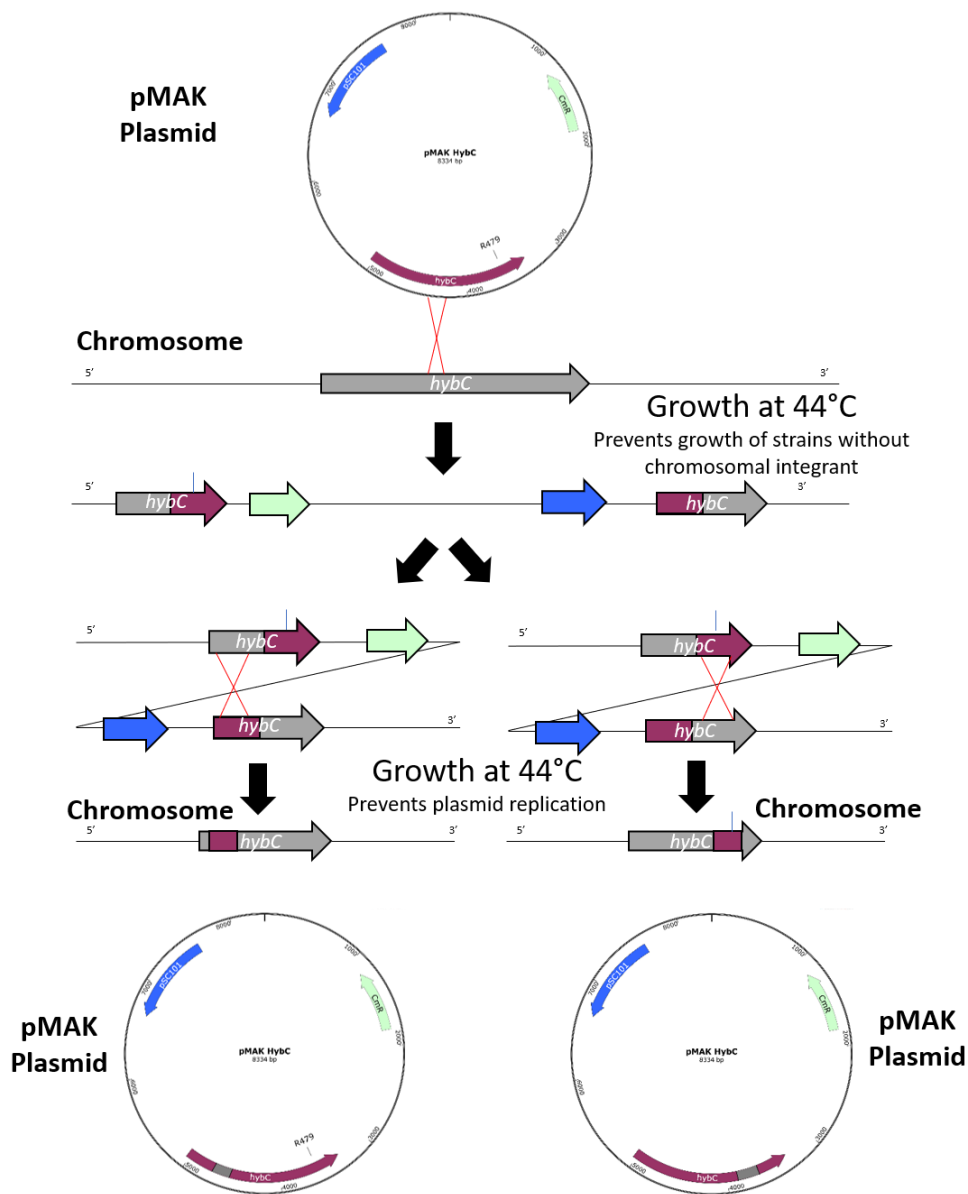


Figure 2.2: Diagram of the stages and possible outcomes of the pMAK cycle. The pMAK plasmid with the gene of interest containing a mutation (labelled R479) forms a cointegrate with the chromosome (first homologous crossover event). The cointegrate will then undergo a second homologous crossover event expelling the plasmid while maintaining a portion of the gene of interest that either contains (right) or does not contain (left) the mutation.

Cells of the desired strain were made chemically competent and were transformed with the appropriate pMAK plasmid (see section 2.1.4) and grown in the presence of chloramphenicol at a concentration of 25 µg/mL (abbreviated CAM).

Selective LB (5 mL, CAM) was then inoculated with the transformant and grown overnight at 30°C, shaking. Ten-fold serial dilutions of the culture were made by combining 900 µL of LB with 100 µL of culture and the 1^{-3} , 1^{-4} , 1^{-5} serial dilutions were plated onto selective agar (CAM) and grown overnight at 44°C while control plates were grown on selective agar (CAM) at 30°C. Three separate batches of selective LB (CAM) were inoculated with four *large* colonies of co-integrates from each of the test plates and then grown overnight at 30°C. A 10 µL aliquot was then subcultured into 10 mL of selective LB (CAM) and grown overnight at 30°C, shaking. That culture was then streaked onto selective LB (CAM) and grown overnight at 30°C. Nonselective LB was then inoculated with a single colony in duplicate (i.e. 6 cultures) and then grown up overnight at 44°C, shaking. The cultures were then streaked onto nonselective LB agar and grown overnight at 44°C. Seven colonies from each plate were suspended in 50 µL of DI H₂O for colony PCR and sequencing to confirm the chromosomal mutation.

2.5 Protein production and purification

2.5.1 Purification of Hyd-2-N

Hyd-2-N was expressed in *E. coli* strain FTH013 by growing quasi-anaerobically at 37°C in Luria broth supplemented with glycerol (0.5 % w/v) and sodium fumarate (0.4 % w/v). Cells were maintained quasi-anaerobic by completely filling growth vessels with inoculated medium and loosening the cap to allow gas produced by the cells to displace dissolved O₂. Cells were harvested in late exponential phase (9 hours following inoculation, 1% inoculum used) at 3000 RCF, resuspended in resuspension buffer [Tris 100 mM, pH 7.5, EDTA 1 mM, NaCl 50 mM, with added DNase (0.003% w/v) and lysozyme

(0.012% w/v)], and frozen at -80°C. Purification of Hyd-2-N relied on the chromosomally-encoded C-terminal his-tag on *hybO*: cells were thawed at room temperature and lysed by passing twice through a cell disruptor system (Constant Systems) at 20 kPSI. Unlysed cells were pelleted by centrifugation 2284 RCF and passed through the cell disruptor at 25 kPSI. Cell membranes were pelleted from the lysed cells by ultracentrifugation at 150,000 RCF and 4°C for 60 mins and resuspended in Tris buffer (100 mM Tris, 350 mM NaCl, pH 7.5), before being mechanically homogenized by hand. The homogenized membranes were solubilized in Triton X-100 (3% v/v) stirring for 40 mins at 4 °C, and debris pelleted at 150,000 RCF for 60 mins at 4 °C. Imidazole (40 mM) and DTT (1 mM) were added to the resulting supernatant solution before being applied to a Ni-affinity column (5 mL CV, His-trap HP, GE Healthcare), pre-equilibrated with Tris (20 mM, pH 7.2), NaCl (350 mM), imidazole (60 mM), Triton X-100 (0.02% v/v), DTT (1 mM).

For electrochemical analyses, the concentrated Hyd-2-N was buffer exchanged (PD-10 desalting columns, GE Healthcare) into Tris (50 mM, pH 7.2), NaCl (350 mM), Triton X-100 (0.02% v/v), glycerol (10% v/v), DTT (1 mM) and snap-frozen in N₂(l).

2.5.2 Purification of Hyd-2-NOP

Recombinant, overproduced Hyd-2 (Hyd-2-NOP) was expressed in and purified from *E. coli* HJ001-hyp transformed with pO^C plasmid. Cells were grown in Luria Broth with added glycerol and fumarate as described above, supplemented with ampicillin (to a final concentration of 100 µg/mL). Cells were maintained quasi-anaerobic as described above. Transcription from the plasmid was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM when an n OD₆₀₀ of 0.25 was attained. Cells were harvested 9 hours after induction in the late exponential phase,

resuspended and frozen as outlined above. Purification of Hyd-2-NOP followed the previously published procedure for Δ TM hydrogenases⁴ : frozen cell pellets were thawed at room temperature and protease inhibitor tablets were added (cOmplete, mini EDTA-free protease inhibitor cocktail, Roche, 1 tablet per 30 L culture) and allowed to stir into solution for 20 mins at 4 °C. Cells were lysed by passing twice through a cell disruptor system (Constant Systems) at 20 kPSI. Debris was pelleted by ultracentrifugation at 150,000 RCF for 30 mins at 4°C. Imidazole (50 mM) and DTT (1 mM) were added to the resulting supernatant solution before it was applied to a Ni-affinity column (5 mL CV, His-trap FF Crude, GE Healthcare) pre-equilibrated with Tris (50 mM, pH 7.2), NaCl (350 mM), imidazole (50 mM), DTT (1 mM). Unbound proteins were washed from the column over 20 column volumes. A 16 CV imidazole concentration gradient (50 to 500 mM) was used to elute Hyd-2-NOP. Fractions were assessed for content and purity by their absorbances at 280 nm and 420 nm and by SDS-PAGE. Positive fractions were pooled and concentrated using centrifugal concentrators with a 50,000 molecular weight cut-off (Vivaspin).

For electrochemical analyses, the concentrated Hyd-2-NOP was buffer exchanged (PD-10 desalting columns, GE Healthcare) into Tris (50 mM, pH7.2), NaCl (350 mM), glycerol (10% v/v), DTT (1 mM) and snap-frozen in N₂(l).

For structural analyses, the concentrated Hyd-2-NOP was buffer exchanged (PD-10 desalting columns, GE Healthcare) into Tris (20 mM, pH7.2), NaCl (150 mM), DDM (0.02% w/v), DTT (1 mM) before further purification by size exclusion chromatography (Superdex 200 Increase 10/300, GE Healthcare). Fractions were assessed for content and purity by their absorbance at 280 nm and 420 nm and by SDS-PAGE. Positive fractions were pooled and concentrated using centrifugal concentrators with a 50,000 molecular weight cut-off

(Vivaspin) to a concentration of 5 mgmL⁻¹, which was determined by the method of Bradford.⁵

2.6 Instrumentation

2.6.1 Spectroscopy

The Parkin Elmer Lambda 19 spectrometer was used for all aerobic spectroscopy in the UV/visible range. This included measuring the optical density of cultures at 600 nm and Bradford assay samples. An Oceans Optics S2000 spectrometer was used for anaerobic spectroscopy.

2.6.2 Electron paramagnetic resonance (EPR) spectroscopy

Continuous wave measurements were carried out using an X-band (9.1-9.9 GHz) Bruker EMX spectrometer equipped with a low temperature helium flow cryostat (Oxford Instruments CF935). In an anaerobic glovebox (Belle Technologies, O₂ <2 ppm), the enzyme was stirred in a gas tight cell at 20 °C under a flow of H₂, Ar, or N₂. The potential of the solution was monitored using a two-electrode system which comprised a Pt working electrode and a Ag/AgCl reference electrode. The EPR samples were prepared using as-isolated enzyme in MES buffer (100 mM MES, 100 mM NaCl, 10% glycerol, pH 6).

For EPR samples at which the potential was controlled, the samples contained mediators (1,2-naphthoquinone, phenazinemethosulfate, indigo tetrasulfonate, 2-hydroxy-1,4-naphthoquinone, benzyl viologen, methyl viologen all at a final concentration of 40 µM) and the enzyme was kept under a constant flow of N₂. Small aliquots of Na₂S₂O₄

(200 mM and 400 mM diluted in pH8 borate buffer) and $K_3[Fe(CN)_6]$ (200 mM and 400 mM diluted in MES buffer) were added whilst continuously monitoring the potential of the sample. As-isolated enzyme was fully chemically oxidized (steady at +380mV) before returning to +150 mV, and finally -230 mV. Samples were taken (250 μ L) when the desired potential (+150 mV = oxidized, -230 mV = reduced) was steady. The enzyme was transferred from the cell to the EPR tube (W707-SQ-250, 4 mm low precision, Fluorochem) with a gas tight syringe and flash frozen in an isopropanol slush.

2.6.2.1 Borate buffer

Separate solutions of H_3BO_3 50 mM and $Na_2B_4O_7$ 12.5 mM were prepared in MQ water, and then the $Na_2B_4O_7$ solution was titrated into the H_3BO_3 solution until pH 8 was attained.

2.6.3 Spectrophotometric assays of hydrogenase activity

For native enzyme and canopy variants, a micro-Eppendorf tube of the enzyme was placed under 100% H_2 for 12-24 hours to fully activate the enzyme. To measure H_2 oxidation activity, the reduction of 2 mM benzyl viologen ($E=-358$, $\epsilon=8.4 \text{ mM}^{-1} \text{ cm}^{-1}$ at 604 nm) was monitored at 604 nm under anaerobic conditions in a sealed cuvette containing 1 mL of K_iPO_4 buffer at 6.0 pH saturated with H_2 . Enzyme (1-10 μ g) was injected to initiate the reaction and the solution was stirred throughout. For the reverse reaction, the enzyme was then placed under 100% Ar for between 12 h and 18 h. To measure the H_2 production, the oxidation of 0.40 mM fully reduced methyl viologen ($E=-446$, $\epsilon=13.9 \text{ mM}^{-1} \text{ cm}^{-1}$ at 604 nm, reduced with equimolar concentration of sodium dithionite)⁶ was

monitored at 604 nm under anaerobic conditions in a sealed cuvette containing 1 mL of K_2PO_4 buffer at pH 6.0 saturated with Ar.

For comparison between E14Q-OP and native Hyd-2, the enzymes were activated under a 100% H_2 environment for 24-36 hours. The K_2PO_4 buffer ranged from pH 6.0 to pH 9.0 and contained 100 mM NaCl. All other conditions were the same.

2.7 Protein biochemistry techniques

2.7.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out on 10% NuPAGE gels (Invitrogen) with running buffer (50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7) at 200 V for 45 min according to the manufacturer's instructions. Pre-stained protein markers (SeeBlue Plus 2, Invitrogen) were used. Prior to loading, samples (10 μL) were mixed with sample buffer (106 mM tris HCl, 141 mM tris base, 2% SDS, 10% glycerol v/v, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM Universal Indicator, pH 8.5).

2.7.2 Coomassie Brilliant Blue staining of polyacrylamide gels

Coomassie Brilliant Blue staining was carried out using the SimplyBlue SafeStain (Invitrogen) according to the manufacturer's instructions.

2.7.3 Determination of protein concentration

Protein concentrations were determined by the method of Bradford.⁵ The Coomassie (Bradford) Protein Assay Kit (Pierce) was used following the manufacturer's instructions.

2.7.4 Western Blot

Typical SDS-PAGE was carried out, but rather than subsequent staining and gel visualization by Coomassie Brilliant Blue, the SDS-PAGE gel was trimmed to the appropriate size for Western Blot and soaked in Western Blot Transfer Buffer (25 mM Tris, 192 mM glycine) for 5 min. A membrane of nitrocellulose and two pieces of Whitman paper were cut to the dimensions of the SDS-PAGE gel and were pre-soaked in transfer buffer. The transfer cassette was then set up in the following order: Whitman paper, nitrocellulose membrane, SDS-PAGE gel, Whatman Paper. The transfer was electrophoresed for 90 min at 200 mA. The membrane was then incubated for 1 h at 4°C in powdered skim milk PBS-T solution (5% w/v skim milk, phosphate buffer saline, 0.1% tween-20). The membrane was then treated with 1:5,000 dilution (diluted with PBS-T buffer) of anti-Hyd-2 serum (raised against rabbit, provided by Prof. Frank Sargent, University of Dundee) for 5 h, treated with anti-rabbit serum (raised against goats, DyLight 650 conjugated) for 1 h at room temperature, and washed three more times with PBS-T.

2.8 Crystallography

Following purification, Hyd-2 was concentrated to 5 mg ml⁻¹ using centrifugal concentrators with a 50,000 molecular weight cut-off (Vivaspin). Protein concentration was assessed by Bradford assay (see 2.7.3). The protein was either purged under a flow of the gas or treated in a pressure vessel (Tynclave Steel, Büchiglasuster). Initially, crystals of as-isolated Hyd-2-NOP were obtained using the sitting drop vapour diffusion

technique, whereby 100 nL of protein solution was mixed with an equal volume of crystallisation solution using a Mosquito robot (TTP labtech, UK) followed by incubation at 293 K. Long rod-shaped crystals typically appeared within 24 hours from a mixture of 100 mM Bis-tris pH 5.5, 200 mM MgCl₂ and 25% w/v PEG 3350. Prior to X-ray data collection, crystals were manually transferred to a cryoprotecting solution of 100 mM Bis-tris pH 5.5, 200 mM MgCl₂, 27% w/v PEG 3350, and 15% v/v glycerol for 30-60 s before flash-cooling by immersion in liquid nitrogen.

Diffraction data for the as-isolated native Hyd-2 was collected at beamline P14, PETRA III (Hamburg) at a wavelength of 0.976 Å using a Pilatus 6M hybrid pixel array detector. All other data were collected at various beamlines (Diamond Light Source, UK) indicated in the text. Initial as-isolated Hyd-2 crystals were plated aerobically but allowed to grow in an anaerobic glove box. Subsequent crystals were both plated and grown in an anaerobic glove box. All data sets were collected at 100 K using a helical line scan to minimise photo-reduction during exposure to X-rays. All data reduction was performed using DIALS⁷ and Aimless.⁸ Initial phase estimates for the as-isolated enzyme were generated using Phaser⁹ with PDB file 5A4M as a search model. Since initial electron density maps were fragmented and difficult to interpret, manual addition of [4Fe4S] clusters in the proximal position and resetting the occupancy of the other metal centres using COOT¹⁰ followed 50 cycles of all atom refinement in REFMAC5¹¹ greatly improved the electron density allowing automated model building using BUCCANEER¹². Subsequent rounds of modelling and refinement were performed manually using COOT and REFMAC5 using TLS restraints and isotropic B-factors. All structure superpositions were performed using LSQKAB¹³ and analysis of protein interfaces using PISA¹⁴, occupancy of the SD oxygen atom at Cys546 was estimated by deletion of the carbonyl

oxygen atom of Arg428, then recalculation and comparison of the size of the peaks in the resulting Fo-Fc difference map. The refinement statistics of each structure are presented in the corresponding chapter and four of the parameters presented in the table are calculated using the following standard equations:

$$R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$

$$R_{\text{meas}} = \frac{\sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{\frac{1}{2}} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$

$$R_{\text{pim}} = \frac{\sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$$

$$R_{\text{work}} = \frac{\sum_{hkl} ||F_{\text{obs}}| - F_{\text{calc}}||}{\sum_{hkl} |F_{\text{obs}}|}$$

where $I_i(hkl)$ is the intensity of reflection hkl , $\sum_i$ is the sum over all i measurements of reflection hkl , $N(hkl)$ is the multiplicity of reflection hkl , and F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. R_{free} is calculated in the same manner as R_{work} but using a random subset (5%) of reflections that are excluded from refinement. $CC_{1/2}$ is Pearson's correlation coefficient between random half data sets. All crystallographic data collection and refinement were carried out by Dr. Steve Carr, Research Complex at Harwell.

2.8.1 Gas treatment

Reduced structures of Hyd-2-NOP were obtained from protein solutions that were reduced by subjection to a H_2 flow (50 scc min⁻¹) for at least 8 h at 4°C before crystallization. Protein was transported to the beamline under anaerobic conditions in sealed vessels; crystallization trays were also assembled under anaerobic conditions. Hyd-2 variants were reduced in a similar way or by treatment in a pressure vessel (4 bar, H_2 , Tinyclave Steel, Büchiglasuster) at 4°C for 12 h. Protein was then subsequently purged

with the gas of choice (CO, H₂, O₂, or ¹⁸O₂) for 20 min or placed in the pressure vessel with that gas.

2.9 Atmospheric control

2.9.1 Glove box

All electrochemical work and most crystallographic work (except initial as-isolated structures of Hyd-2 NOP and Hyd-2 R479K-OP) in this study was carried out in anaerobic glove boxes (MBRAUN 150B-G or Belle Technologies with a N₂ atmosphere (O₂ < 3 ppm)).

2.9.2 Gasses

The rate of gas flow was controlled using mass flow controllers (Sierra Instruments) and these instruments also allowed the precise mixing of gasses. All gasses used were of high purity and supplied by BOC or Air Products.

2.10 Protein film electrochemistry

Protein film electrochemistry (PFE) is a suite of techniques in which a thin layer of protein is adsorbed onto an electrode, namely the working electrode (WE). A range of potentials can then be applied to the protein and its catalytic activity is directly measured as current.^{15,16} In this case the working electrode performs the interconversion of protons and molecular hydrogen (H₂). When the WE is at potentials lower than the potential of the H₂/H⁺ couple, proton reduction occurs. By contrast, when the WE is at potentials higher than the potential of the H₂/H⁺ couple, H₂ oxidation occurs. Whereas traditional

protein electrochemistry relies on mediators to set the potential of the solution, protein film electrochemistry allows direct control of the potential applied to the protein. This technique is superior mainly because it requires minimal amounts of protein and ensures accurate and homogenous potential and environmental control over the entire sample. These advantages allow PFE to provide insight into slight shifts in the activity and steady-state kinetics of the protein.

In PFE, the current can be limited by either (1) interfacial electron transfer between the electrode and enzyme, (2) intramolecular electron transfer within the enzyme, (3) the inherent activity or kinetics of the enzyme, or (4) the diffusion of substrate to the electrode. These factors are partially controlled for by controlling the environment through a water-jacketed cell and fixed gas flow rates as well as rotating the electrode to increase diffusion at the electrode (Figure 2.3). For hydrogenase, electron transfer is extremely efficient and at relatively slow scan rates (0.1 mV/sec to 30 mV/sec) is unlikely to limit activity.

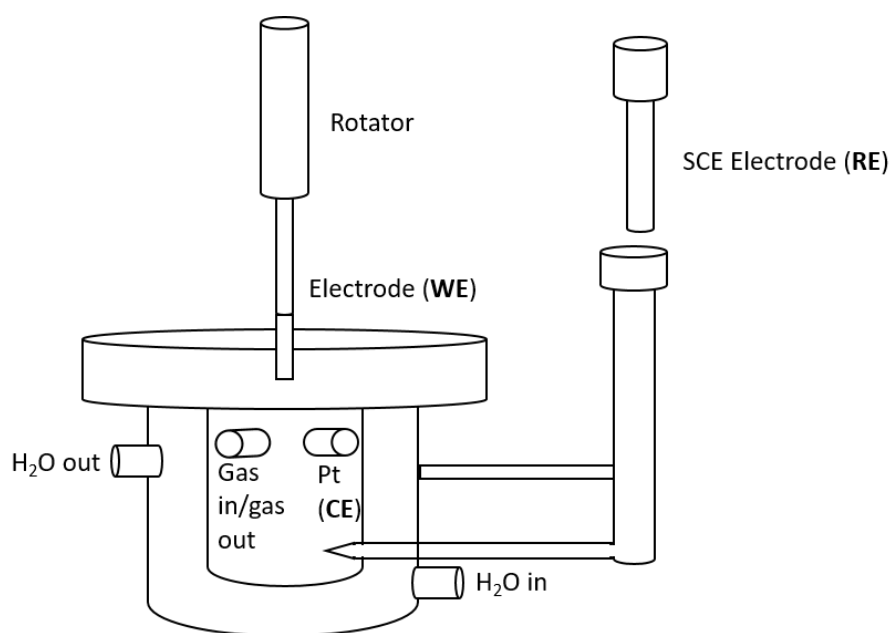


Figure 2.3: Diagram of standard electrochemical cell.

In its simplest configuration, protein film electrochemistry can be carried out with a two-electrode configuration in which the counter electrode (CE) doubles as the reference electrode (RE). Due to the material demands of the CE, this approach provides limited control over the potential of the working electrode. Instead, this thesis mainly relies on a three-electrode setup in which the reference electrode is parallel to the cell and separated by a reference arm that limits the current flow through the reference electrode (Figure 2.4). Moreover, without the demands of the counter electrode, the reference electrode can consist of non-reactive materials that are at a known potential. In this case, the potential applied to the WE is described as

$$E = E_{WE} - E_{RE} - iR$$

where E_{WE} is the potential across the WE/solution interface, E_{RE} is the potential across the RE/solution interface, R is the resistance of the electrolyte solution, and i is the current between the WE and the RE (different than the measured current i between the WE and CE) which should be minimal given a sufficiently large R .

The material for the counter electrode is chosen based on its ability to perform the necessary reactions to balance reaction at the working electrode. Platinum is ideal given its ability to catalyse proton reduction and water oxidation to balance hydrogen oxidation and proton reduction, respectively.

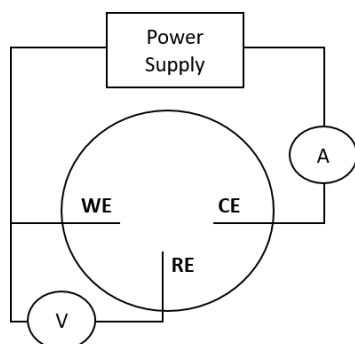


Figure 2.4: Electrical schematic of the circuit formed by a three-electrode electrochemical setup where V is a voltmeter and A is an ammeter.

2.10.1 Non-faradaic current

Non-faradaic current describes the current that arises from charge that never leaves the electrode, i.e. charge accumulation on the electrode. As the potential is swept, charge at the electrode changes causing ions in the solution to rearrange to accommodate the charged electrode, i.e. to maintain neutrality the charge in solution must be equal and opposite to the charge on the electrode, so ions in the solution dynamically align at an opposite and equal intensity as the charge on the electrode. This double layer of charge creates a capacitance effect that charges and discharges according to the direction and rate of the potential sweep (Figure 2.5). Non-faradaic current depends on electrode size, scan rate, and solution composition, and because defects in the electrode (cracked graphite, inadequate seal between resin and graphite, etc.) can complicate the non-faradaic current, the non-faradaic current of the electrode is checked before applying protein.

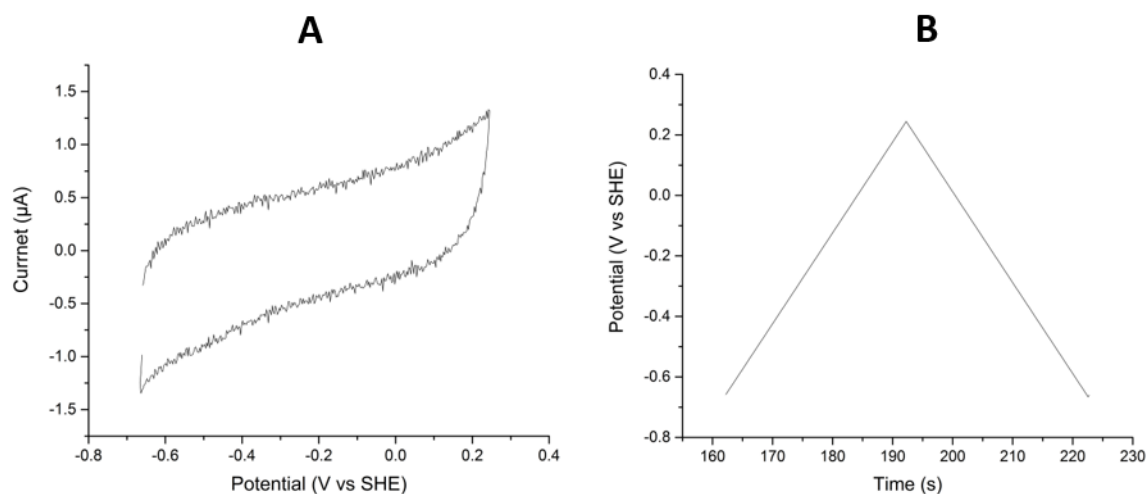
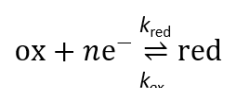


Figure 2.5 Electrochemical plots. Cyclic voltammogram (CV) at 30 mV/sec of a large PGE electrode (Panel A). Trace of the potential against time of a typical CV (Panel B).

2.10.2 Faradaic current

Faradaic current describes charge that leaves the electrode that can typically be categorized either as non-turnover current, i.e. current that changes the oxidation state of the enzyme adsorbed on the electrode, or turnover current, i.e. current that results from the turnover/catalysis by the enzyme in the consumption of substrate to make product. Ideally, non-turnover peaks can be used to calculate the coverage of the enzyme on the electrode and in turn the rate of catalysis, but not all enzyme/electrode combinations have discernible non-turnover peaks.

In this investigation, pyrolytic graphite edge (PGE) electrodes were used for all electrochemical experiments. Hydrogenase on PGE does not give discernible non-turnover peaks, and negative current indicates the rate of proton reduction at the electrode whereas positive current indicates the rate of hydrogen oxidation. Extensive modelling of protein-electrode interactions have been published,^{16–20} but for this thesis it is sufficient to model the reaction as a process in which two chemical species are interconverted described by the following equation:



where “ox” is the oxidized species, “red” is the reduced species, n is the number of electrons, and k_{red} and k_{ox} are rate constants for oxidation and reduction, respectively. For such a situation, the current (i) is described by the following equation:

$$i = AFj$$

where A is the electrode surface area, F is the Faraday constant, and j is the electron flux in moles (unit area)^{−1} s^{−1}. Depending on the direction of the flux, j is then described by the following equations:

$$j_{ox} = nk_{ox}\Gamma_{red}$$

$$j_{red} = nk_{red}\Gamma_{ox}$$

where Γ_{red} and Γ_{ox} are the electroactive coverage of the reduced and oxidized species, respectively. The two equations can therefore be reduced to the following simplified equation:

$$i_{cat} = AFnk_{cat}\Gamma$$

where i_{cat} and k_{cat} correspond to correct terms for reduction or oxidation. Because the electroactive coverage of a protein film depends on factors including film history, concentration of protein applied to the film, and electrode size, and the electroactive coverage cannot be measured, two separate films cannot be compared directly. This limitation also prevents a determination of the turnover rate for hydrogenase on PGE electrodes. In this thesis, PFE was used to examine the effect of altering parameters (i.e. H_2 concentration, temperature, inhibitor concentration, etc.) using established procedures to determine enzymatic characteristics.

2.10.3 Electrochemistry experiments

The two main PFE methods used in this investigation are cyclic voltammetry and chronoamperometry. In cyclic voltammetry, the potential is cycled over time and both the current and potential are measured. In chronoamperometry, a constant potential is applied to the electrode and only the resulting current is measured.

2.10.3.1 Growth of Film

The PGE electrode (geometric surface area 0.03-0.05 cm²) was sanded (Hookit P400 sandpaper) and rinsed. Immediately following abrasion, enzyme solution (0.5–3 μ L at

10–400 μM) was applied to the electrode for 30 s by pipetting the enzyme off and back on to the electrode. The electrode was then rinsed with ultrapure H_2O (Millipore, 18 $\text{M}\Omega$) for 30 s to remove unadsorbed enzyme. Between 3 and 10 CVs (30 mV/sec, 100% H_2) were performed to approximate the size of the film, and enzyme was scraped off by dabbing with a paper towel if the film was too large. The film then underwent an activation procedure which typically consisted of a cycle of holding the film at low potential for 60 s (-0.600 V vs SHE) followed by a high potential hold for 15 s (-0.17 or 0 V vs SHE depending on the enzyme variant). The low potential hold reduces the oxidized species at the active site allowing the enzyme to become catalytically active while the high potential holds verify the progress of the activation protocol. After three cycles of alternating potential, a CV was performed. This activation procedure was performed until maximum current was achieved.

2.10.3.2 Film loss

Protein films typically suffer film loss which is understood as desorption of the protein from the electrode or permanent unfolding of the protein at the electrode. Film loss typically can be modelled as exponential decay of the film, though the rate of film loss depends on the film conditions including scan rate and temperature. To account for film loss, most PFE experiments were returned to the original conditions periodically throughout the experiment to verify the stability of the film and plot the exponential decay. Experiments that alter the conditions of the film therefore suffered a greater amount of error due to the complications of film loss correction.

2.10.3.3 Energy of activation

The ΔH of the formation of the transition state for hydrogen oxidation was calculated by measuring activity of the hydrogenase at various temperatures. This calculation assumes that the reaction between two reactants E and S (or in this case an enzyme and substrate) proceeds through a transition state $ES^\ddagger$ to product D according to the following reaction:



The rate constant, k , for the reaction can be described using the standard entropy and enthalpy of the products and reactants by the Eyring equation:

$$k = \frac{k_b T}{h} e^{\left(\frac{\Delta S^\ddagger}{R}\right)} e^{\left(\frac{-\Delta H^\ddagger}{RT}\right)}$$

where k_b is Boltzmann's Constant, T is the temperature in Kelvin, h is Planck's constant, R is the gas constant, and $\Delta S^\ddagger$ and $\Delta H^\ddagger$ are the standard entropy and the energy of activation of the transition state, respectively. Dividing through by temperature and applying a logarithmic transformation gives:

$$\ln\left(\frac{k}{T}\right) = \ln\left(\frac{k_b}{h}\right) + \left(\frac{\Delta S^\ddagger}{R}\right) + \left(\frac{-\Delta H^\ddagger}{R}\right)\left(\frac{1}{T}\right)$$

which can be further simplified to the following equation

$$\ln\left(\frac{k}{T}\right) = \left(\frac{1}{T}\right)c_1\Delta H^\ddagger + (c_2\Delta S^\ddagger + c_3)$$

where c_1 , c_2 , and c_3 are combinations of constants. It is therefore obvious that plotting $\ln(k/t)$ against $(1/T)$ gives a slope containing $\Delta H^\ddagger$ and an intercept with $\Delta S^\ddagger$. The equation for i_{cat} can be rearranged to the following equation:

$$k_{cat} = \frac{i_{cat}}{AFn\Gamma}$$

Substituting k_{cat} for k in the Eyring equation and simple rearrangement gives the following equation:

$$i_{cat} = \frac{AFn\Gamma k_b T}{h} e^{\left(\frac{\Delta S^\ddagger}{R}\right)} e^{\left(\frac{-\Delta H^\ddagger}{RT}\right)}$$

Which, following the same manipulations from above, can be transformed to the following equation:

$$\ln\left(\frac{i}{T}\right) = \left(\frac{1}{T}\right) c_4 \Delta H^\ddagger + (c_2 \Delta S^\ddagger + c_5)$$

where c_4 and c_5 are consolidated constants. Thus, graphing $\ln(i/t)$ against $(1/t)$ gives a slope containing $\Delta H^\ddagger$ and intercept containing $\Delta S^\ddagger$ (though $\Delta S^\ddagger$ cannot be solved for by PFE alone because Γ is unknown which is a term in c_5).

The catalytic current at five temperatures (10°C, 15°C, 20°C, 25°C, 30°C) was measured with a slow (5 mV/sec) cyclic voltammogram returning to initial conditions every two scans to account for film loss.

2.10.3.4 Michaelis constant

The Michaelis constant describes the concentration of substrate required to achieve a rate which is $1/2v_{max}$. When plotted in the traditional Michaelis-Menten plot, it becomes clear that there are three distinct regions of activity response. At concentrations of substrate significantly higher (III) and lower (I) than the $K_M^{H_2}$, the plot is best described as pseudo-linear (Figure 2.6). At concentrations near the $K_M^{H_2}$ the plot is curved, and it is data from this section that is necessary to calculate $K_M^{H_2}$. For different hydrogenases, the limits demarcating the sections of the Michaelis-Menten plot may vary widely with $K_M^{H_2}$.

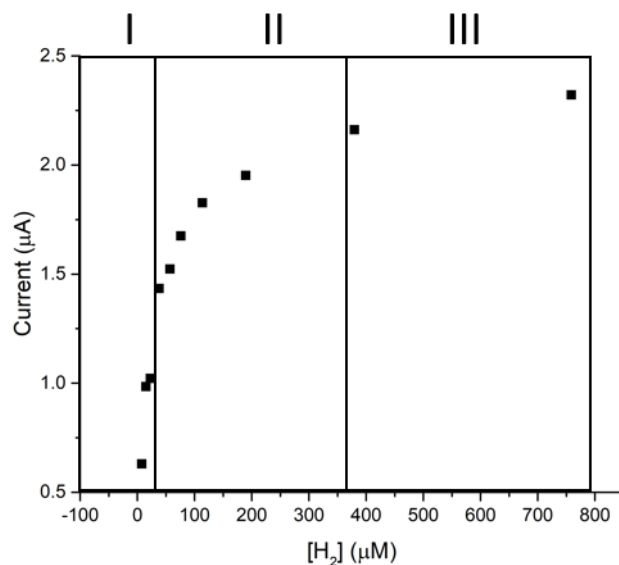
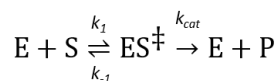


Figure 2.6: An example of a Michaelis-Menten plot of a [NiFe]-hydrogenase with a $K_M^{H_2}$ of approximately 25 μM .

In order to calculate K_M , the equation describing the reaction proceeding through a transition state is useful.



It is then assumed that k_1 is rapid compared to k_{-1} and that the enzyme is in steady-state, thus giving the equations:

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1} + k_{cat})[ES] = 0$$

Which can be rearranged to

$$[ES] = \frac{k_1[E][S]}{(k_{-1} + k_{cat})}$$

or

$$K_M = \frac{[E][S]}{[ES]}$$

where

$$K_M = \frac{(k_{-1} + k_{cat})}{(k_1)}$$

If the equation for the K_M is substituted with an equation reasoning that the active enzyme is the difference between the total enzyme pool and enzyme bound to substrate.

$$[E] = [E_{total}] - [ES]$$

$$K_M = \frac{[E_{total}][S]}{[ES]} - [S]$$

The equation for the rate of reaction (v) is given by the following equation:

$$v = k_{cat}[ES]$$

Max rate is the rate at which the full pool of enzyme is available which gives the following equation:

$$v_{max} = k_{cat}[E_{total}]$$

which if substituted in the equation for K_M gives

$$K_M = \frac{v_{max}[S]}{v} - [S]$$

Dividing through by v_{max} and rearranging this equation gives the Hanes method for determination of K_M which was used for this thesis:

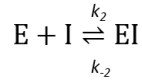
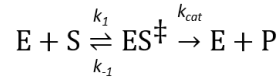
$$\frac{[S]}{v_{max}} = \frac{[S]}{v} + \frac{K_M}{v_{max}}$$

Thus, plotting $[S]/v$ vs. $[S]$ gives a plot with an x-intercept that is $-K_M$.

In order to calculate $K_M^{H_2}$, the catalytic current at ten hydrogen concentrations was measured with a slow (5 mV/sec) cyclic voltammogram returning to initial conditions every three scans to account for film loss.

2.10.3.5 Inhibition constant (k_i)

In this thesis, inhibition of hydrogenase by CO and H₂ is investigated. The former molecule predominantly binds to the enzyme in Ni-SI_a (Figure 1.2), which is the same state to which H₂ binds before heterolytic cleavage. Thus, the binding of CO is considered a competitive inhibitor of hydrogenase during H₂ oxidation and its inhibition can be described by the combination of the following equations:



where E is the enzyme, S is the substrate, I the inhibitor, and P the product. Competitive inhibitors have no effect on the v_{max} of the enzyme, but rather alter the apparent K_m by inhibiting a portion of E_{total} that requires a higher substrate concentration to outcompete the inhibitor. The inhibitor constant K_i is given by (k_{-2}/k_2) . To calculate K_i , one must again make a steady-state assumption for both the reaction of the enzyme with substrate, and inhibitor with enzyme, giving

$$k_1[E][S] = (k_{-1} + k_{cat})[ES]$$

$$k_2[E][I] = (k_{-2})[EI]$$

$$[E_{total}] = [E] + [ES] + [EI]$$

Combining the above equations and inserting them into the equation for v and v_{max} gives the following equation:

$$v = \frac{v_{max}}{1 + \frac{K_M}{[S]} \left(1 + \frac{[I]}{K_i} \right)}$$

For the inhibition of Hyd-2 by CO during H₂ oxidation, the activity depends on the following equation:

$$i = \frac{i_{max}}{1 + \frac{K_M}{[H_2]} \left(1 + \frac{[CO]}{K_i}\right)}$$

A plot of i_{max}/i vs. $[CO]$ gives a straight line of intercept $(K_M/[S])+1$ and gradient $K_M/(K_i[S])$.

To measure K_i , first a film was activated for a long period of time (>1.5h) so that film loss was in the pseudo-linear region of an exponential decay curve. The film was then held at a hydrogen oxidation potential (-0.175 vs. SHE) at 20°C, rotation rate 3000 rpm, and 20% H_2 while the $[CO]$ was increased. The film was returned to initial conditions at the end to account for film loss.

For inhibition by H_2 during proton reduction, the product inhibition observed is considered to be uncompetitive because H_2 binds at a different state (Ni-R) than the state that the proton binds (Ni-SI_a; see Figure 1.2).²¹ Such inhibition changes both the apparent K_M and apparent v_{max} , and K_i is therefore calculated using measurements of the activity at different concentrations of inhibitor. Thus, the current at any given concentration can be described by the following equation:

$$i = \frac{i_{max}}{1 + \frac{[H_2]}{K_i}}$$

Thus, a plot of i_0/i vs. $[CO]$ gives a straight line of gradient $(1/K_i)$. To measure product inhibition, the working electrode was held at an hydrogen oxidation potential (-0.660 vs. SHE) at 20°C, rotation rate 3000 rpm, and initially at 0% H_2 while the $[H_2]$ was increased returning to initial conditions (0% H_2) every three H_2 concentrations.

2.10.3.6 E_{SWITCH}

After full anaerobic inactivation (at 0.25 V vs. SHE) at pH 8 under 100% Ar (1000 scc/min) at 30°C, the electrochemical cell solution was changed for pH 8 buffer and allowed to

equilibrate at the correct temperature before ensuring that activity was still negligible. The gas flow was then switched to 100% H₂ and a backwards scan completed (0.1 mV/sec). By definition, E_{SWITCH} is the minimum of the first derivative of the backwards reactivation scan.

2.10.3.7 Oxygen injection

Prolonged O₂ exposure for Hyd-2 is not feasible, and all oxygen exposure involved brief exposure to an O₂ saturated buffer. Mixed hydrogenase buffer (MHB) was purged with 100% O₂ for at least 10 min before being injected into the electrochemical cell under 100% Ar (1000 scc/min) rotating at 3000 rpm running a cyclic voltammogram (1 mV/sec). Typically, the electrochemical cell contained 5 mL of MHB and the final concentration of O₂ injected was calculated based on the percent volume of O₂ saturated buffer that was added.

2.10.4 Covalent modification of electrodes

Covalent attachment of enzyme to the electrode was carried out following the previously outlined protocol.²² Briefly, a dispersion of multiwalled nanotubes (1 mg/mL) in dimethyl formamide (DMF) is ultrasonicated for 15 min immediately prior to being placed on a PGE electrode and allowed to dry. The MWNT-coated PGE electrode was then coated with a solution of 1-pyrenebutyric acid (10 mM in DMF) for 1 h in the dark. The electrode was rinsed and then activated for 1 h using a solution of freshly prepared 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Flurochem, 0.4 M) and N-hydroxy sulfosuccinimide (NHSS, Sigma-Aldrich, 0.1 M). The electrode was rinsed and covered in Hyd-2 for 1 h. This technique relies on the π - π stacking between the pyrene and carbon

nanotubes. The carboxylic functionality is activated by the EDC/NHSS mixture to covalently bind to the hydrogenase via surface lysines.²²

2.10.5 General electrochemical methods

All electrochemical experiments were performed in mixed hydrogenase buffer pH 3- pH 9 (MHB, 100 mM NaCl, 15 mM MES, 15 mM HEPES, 15 mM TAPS, 15 mM CHES, 15 mM CH₃COONa). This investigation relied on a saturated calomel electrode (SCE) that is based on the redox reaction of elemental mercury to mercury chloride and was calibrated annually against a Pt wire saturate quinhydrone solution at pH 4.0 and pH 7.0. Volts versus SCE is converted to V vs SHE with the following equation:

$$V_{\text{SHE}} = V_{\text{SCE}} + 0.2412 - 6.61 * 10^{-4} * (T - 298) - 1.75 * 10^{-6} * (T - 298)^2 - 9 * 10^{-10} * (T - 298)^3$$

where T is the temperature in Kelvin.²³ All electrochemistry was performed using an Autolab PGSTAT128N with NOVA software to control the potentiostat and record the results. PGE electrodes have an average surface area of 0.03 cm².

2.11 In Vivo experiments with *Shigella sonnei*

2.11.1 Hydrogen production assay

Strains of *Shigella sonnei* were grown anaerobically (approximately 2.5% H₂, 12.5% CO₂, 85% N) in LB supplemented with fumarate for 20 hours until stationary phase was reached. Cells were harvested and resuspended in 5 mL of PBS buffer with 170 mM glycerol in 20 mL vials sealed with #25 suba seals. Cell solutions were then purged with nitrogen for 20 min at a rate of 75 scc/min and incubated shaking at 37°C. Samples (15

μL) of the headspace were taken periodically which were then assayed by GC (Agilent 7890B, ShinCarbonST 100/120 Column, N carrier gas, 32°C). After each injection, the suba seal was reinforced with parafilm.

2.11.2 Hydrogen uptake assay

Strains of *Shigella sonnei* were inoculated to the same OD ($OD_{600}=0.01$) in M9 minimal medium (5 mL) supplemented with 0.2% casamino acids, 0.5% fumarate, and purged for 10 min with 10% H₂ (Ar carrier gas, 100 scc/min total flow rate) before incubation shaking at 37°C. Samples (15 μL) of the headspace were taken periodically which were then assayed with the GC (Agilent 7890B, ShinCarbonST 100/120 Column, N₂ carrier gas, 32°C). After each injection, the suba seal was reinforced with parafilm. For H₂ uptake experiments before and after O₂ exposure, there was a 10x gas displacement in the vial with air followed by 1h or 4h of incubation in an aerobic environment followed by a full purge with Ar.

2.11.3 Growth with minimal medium glycerol and H₂

Strains of *Shigella sonnei* were inoculated to the same OD ($OD_{600} = 0.01$) in M9 minimal medium (150 mL) supplemented with 0.2% casamino acids 0.5% fumarate with 100% H₂ constantly purging the headspace. The cells were grown in a flask joined with a crystal cuvette so that measurements were taken by simply moving the cuvette. Every 4h the gas flow was removed briefly for O.D. measurements.

2.12 Ag nanocluster experiments

All Ag nanocluster (AgNC) experiments were performed by Dr. Liyun Zhang, University of Oxford, and the AgNCs-PMAA/TiO₂ nanoparticles were prepared as described previously.²⁴ Briefly, a freshly prepared aqueous solution of silver nitrate (0.1 M, 10 mL, Sigma Aldrich, 99+%) was mixed with an equal volume of polymethylacrylic acids (PMAA, 5 mg/mL, pH 3.0 adjusted with nitric acid). After mixing, the solution was purged with N₂ for 15 min followed by exposure to sunlight. Upon solar irradiation the solution gradually changed from colourless to magenta. The solution was then centrifuged (2105 RCF, 15 min), the supernatant was decanted, and the pellet of aggregated magenta pellets were resuspended in water. The AgNCs were centrifuged and the nanoclusters were resuspended in EtOH. TiO₂ nanoparticles (100 mg) were then suspended into 20 mL of the purified AgNCs (5 mg/mL) in EtOH and vigorously stirred in a vessel protected from the light by aluminium foil overnight. The resulting suspension of AgNCs-PMAA/TiO₂ was centrifuged, washed in water, then acetone, and dried. The AgNCs-PMAA/TiO₂ particles (5 mg) were dispersed in 5 mL aqueous solution of 0.1 M triethanolamine (TEOA), 0.1 M NaCl, pH 7.0 by sonicating for 15 min, all sealed in a Pyrex pressure vessel (total volume = 22.5 mL), then the solution was purged with nitrogen for 15 min. Subsequently, Hyd-2 (25 µL of 10 µM solution) was added and the suspension stirred gently for 3 minutes to allow binding by AgNCs-PMAA. For photocatalysis measurements, the stirred suspension was irradiated with visible light using a 300 W Arc Lamp, (Newport 67005) fitted with a 420 nm filter and held 5 cm from the vessel. Temperature was controlled by immersing the vessel in a water bath. Production of H₂ was monitored at regular intervals by removing small volumes (20 µL) of headspace gas for gas chromatography (GC) analysis.

An Agilent 7890A series gas chromatograph (GC) with electronic pneumatic control optimized for trace gas analysis was used to monitor the production of H₂ at regular intervals. Headspace gas samples of the reaction mixture were injected into the GC operating in pulsed splitless mode using a gas-tight Hamilton syringe. A 1.5 mm diameter split/splitless liner, a Restek ShinCarbon ST micropacked column, a micro thermal conductivity detector and high-purity N₂ carrier gas with a constant pressure of 25 psi were used.

2.13 Water

All buffers were prepared in ultrapure water (minimum resistivity 18.2 MΩ cm, Millipore). Media was prepared with deionized (DI) water.

2.14 Strains used

Table 2.5

Strain Name	Strain Description	Source/Reference
FTH013	Native Hyd-2 strain. MC4100-derived strain carrying an engineered HybO protein bearing a His ⁶ affinity tag at its extreme C terminus used to produce and isolate native Hyd-2	25
FTH2018	ΔTM Hyd-2 strain. FTH013 derivative without the transmembrane domain coding region from <i>hybO</i>	25
SEB001	R479K Strain. FTH013 derivative with a codon change in <i>hybC</i> using pMAK_hybC_R479K	This Work
SEB002	D103A Strain. FTH013 derivative with a codon change in <i>hybC</i> using pMAK_hybC_D103A	This Work
HJ001	MC4100-derivative, $\Delta hycE$, $\Delta hycE \Delta hybO \Delta hybA$, $\Delta iscR$	26
HJ001-hyp	HJ001 with synthetic operon encoding <i>Ralstonia hypA1B1F1CXDEX</i> integrated into the chromosome in place of <i>tatD</i>	26,27
HJ001-hyp_R479K	R479K Strain. HJ001-Hyp derivative with a codon change in <i>hybC</i> using pMAK_hybC_R479K	This Work
HJ001-hyp_D103A	D103A Strain. HJ001-Hyp derivative with a codon change in <i>hybC</i> using pMAK_hybC_D103A	This Work
HJ001-hyp_E14Q	E14Q Strain. HJ001-Hyp derivative with a codon change in <i>hybC</i> using pMAK_hybC_E14Q	This Work
HJ001-hyp-3S	HJ001-Hyp derivative with the three surface cysteine residues replaced by serine residues (C197S, C432S, C433S)	This Work
GlpG	<i>Shigella sonnei</i> $\Delta spa33 \Delta hyaB \Delta hycE$	Guangyu Liu
$\Delta GlpG$	<i>Shigella sonnei</i> $\Delta spa33 \Delta hyaB \Delta hycE \Delta glpG$	Guangyu Liu
Δhyb	<i>Shigella sonnei</i> $\Delta spa33 \Delta hyaB \Delta hybC \Delta hycE$	Guangyu Liu
G296F	<i>Shigella sonnei</i> $\Delta spa33$ point mutation	Guangyu Liu

2.15 Plasmids used

Table 2.6

Plasmid Name	Plasmid Description	Source
pJL1	pMAK 705 derivative with <i>hybC</i> flanked by 500 base pairs of <i>hybB</i> and <i>hybD</i> with a V63C codon change. Note that <i>hybC</i> and the flanks of <i>hybC</i> and <i>hybD</i> are inserted in reverse to the direction of replication by the plasmid.	pMAK, Bonnie Murphy unpublished
pMAK_hybC_WT	pJL1 derivative with <i>hybC</i> restored to wild type <i>hybC</i>	This Work
pMAK_hybC_R479K	pMAK_hybC_WT derivative with R479K codon change	This Work
pMAK_hybC_D103A	pMAK_hybC_WT derivative with D103A codon change	This Work
pQE-80L	Plasmid with T5 promoter inducible with 1 mM of IPTG	Qiagen
pO ^c	pQE-80 derivative with <i>hybO</i> without the TAT signal or the transmembrane domain (ΔTAT , ΔTM) and C-terminal His ⁶ tag inserted downstream of the promoter	This Work
pO ^c CD	A pO ^c derivative with <i>hybCD</i> inserted downstream of <i>hybO</i>	This Work
pMAK_hybC_E14Q	pMAK_hybC_WT derivative with E14Q codon change	This Work
pO ^c Y'222C	As pO ^c with the Y222C mutation in <i>hybO</i>	This Work
pO ^c D81N	A pO ^c derivative with D81N codon change	This Work
pO ^c G'24C/G'125C	A pO ^c derivative with G24C and G125C codon changes	This Work

2.16 Primers used

Table 2.7

Primer Name	Primer Function	Primer Sequence
pMAK_overlap_F	Primer pair for pMAK-HybC forwards mutagenic primers	5'-CGGTCCAATGATCGAAGTTAGGCTGGTAAGA GCCG-3'
pMAK_overlap_R	Primer pair for pMAK-HybC reverse mutagenic primers	5'-CGGCTCTTACCAGCCTAACTTCGATCATTGGAC CG-3'
hybC_C63V_F	<i>hybC</i> C63V (mutate back to WT) mutagenesis forwards primer	5'-GGATGATTGTGCAACGTATCTGTGGC <u>G</u> TATGT ACTAC CACTCACGCG-3'
hybC_C63V_R	<i>hybC</i> C63V (mutate back to WT) mutagenesis reverse primer	5'-CGCGTGAGTGGTAGTACAT <u>A</u> CGCCACAGATAC GT TGCACAATCATCC-3'
hybC_R479K_F	<i>hybC</i> R479K mutagenesis forwards primer	5'-GTGTTGGCTTCCTCGAAGCGCCG <u>A</u> AAGGTATG CTCTC TCACTGGATGGTTATTAAAG-3'
hybC_R479K_R	<i>hybC</i> R479K mutagenesis reverse primer	5'-CTTTAATAACCATCCAGTGAGAGAGCATACCTT <u>I</u> CG GCGCTTCGAGGAAGCCAACAC-3'
hybC_D103A_F	<i>hybC</i> D103A mutagenesis forwards primer	5'-GTAACATCATTCTGGCTGCGCACACCACGCAT <u>G</u> CGC ATATTGTTCA ~~TT~~ TCTATCAGC-3'
hybC_D103A_R	<i>hybC</i> D103A mutagenesis reverse primer	5'-GCTGATAGAAATGAACAATATG <u>C</u> GCATGCGTG GTGTG CGCAGCCAGAATGATGTTAC-3'
hybC_E14Q_F	<i>hybC</i> E14Q mutagenesis forwards primer	5'-GCCAGAGAATTACTATTGATCCGGTAACCCGT ATT <u>C</u> AGGGGCATTTACGCATCGATTGCG-3'
hybC_E14Q_R	<i>hybC</i> E14Q mutagenesis reverse primer	5'-CGCAATCGATGCGTAAATGCCCCT <u>G</u> AATACGG GTTA CCGGATCAATAGTAATTCTCTGGC-3'
hybO_F	Amplify ΔTAT ΔTM <i>hybO</i> forwards primer	5'-GAATTCGAGCTCGCGGCCGCACTAGTCACAGA GGAACAG GTATGGAGATGGCCGAATCGGTTACTAACC-3'

	with pQE-80L overlap	
hybO_R	Amplify ΔTAT ΔTM hybO reverse primer with pQE-80L overlap	5'- GTCGACGCGGCCGCCCTAGGTTATTAGTGATG GT GATGGTGATGGCCGCCCTCTTTAGCGTTAACA TCC-3'
OpenpQE80_F	Amplify linear pQE-80L construct forwards primer	5'- CCTAGGGCGGCCGCGTCGACCTGCAGCCAAG CTTA ATTAGCTGAGC-3'
OpenpQE80_R	Amplify linear pQE-80L construct reverse primer	5'- ACCTGTTCTCTGTGACTAGTGCGGCCGCGAG CTCGA ATTCTGTGTGAAATTGTTATCCGCTCACAATTG -3'
pQE80_GibOverla p_F	Primer pair for pQE-80 HybO reverse mutagenic primers	5'- CCTGACGTCTAAGAAACCATTATTATCATGAC- 3'
pQE80_GibOverla p_R	Primer pair for pQE-80 HybO forwards mutagenic primers	5'- GTCATGATAATAATGGTTTCTTAGACGTCAGG- 3'
hybO_G24C_F	<i>hybO</i> G'24C mutagenesis forwards primer	5'- GCGCAGGAGTGCACCTGTTGTACGGAATCTC- 3'
hybO_G24C_R	<i>hybO</i> G'24C mutagenesis reverse primer	5'-GAGATTCCGTACAACAGGTGCACTCCTGCG- 3'
hybO_G125C_F	<i>hybO</i> G'125C mutagenesis forwards primer	5'-TCTGCGTGGGGCTGTGTTGCCGCAG-3'
hybO_G125C_R	<i>hybO</i> G'125C mutagenesis reverse primer	5'-CTGCGGCAACACAGCCCCACGCAGA-3'
hybO_D81N_F	<i>hybO</i> D'81N mutagenesis forwards primer	5'- GCAGTATGTGTTAGTGGTGAACGGTTCCATCC- 3'
hybO_D81N_R	<i>hybO</i> D'81N mutagenesis reverse primer	5'- GGATGGAACCGTTCACCACTAACACATACTGC- 3'

HybO_Y222C_F	Y'222C mutagenesis forwards primer	5'- CCACCTCGGCTGTAAAGG-3'
HybO_Y222C_R	Y'222C mutagenesis reverse primer	5'-TAAGTTTCTGGCCCTTTACAGCCGAGGTG GCACAGGCACCAGCCTTCG-3'
HybC_C433S_C43 2S_F	C433S_C432S mutagenesis forwards primer	5'- CCACGCTGGGCCGTATTATTGGTCGTACCGTT CACAGT AGTGAATTGCAGGAT-3'
HybC_C433S_C43 2S_R	C433S_C432S mutagenesis reverse primer	5'-GTGAACGGTACGACCAATAATACGGCCCAG CGTGGA-3'
HybC_C197S_F	C197S mutagenesis forwards primer	5'- TAGCGCACTACCTGCAAGCGTTGGAGAGCCAG CGT GACGC-3'
HybC_C197S_R	C197S mutagenesis reverse primer	5'- GCGTCACGCTGGCTCTCCAACGCTTGCAGGT AGTGCGCTA-3'
hybCD_F	Amplify <i>hybCD</i> forwards primer with pQE-80 HybO overlap	5'- CACAGAGGAACAGGTATGAGCCAGAGAATTA CTAT TGATCCGGTAACC-3'
hybCD_R	Amplify <i>hybCD</i> reverse primer with pQE-80 HybO overlap	5'- TTATTAGTCATGAATCGCCTCCCGTGGGATGG CTT CCACGCC-3'
pQEHybO_Open_ F	Amplify linear pQE-80 HybO construct forwards primer	5'- GCCATCCCACGGGAGGCGATTCATGACTAATA AG CGGCCGCGTCGACCTGCAGCCAAGCTTAATTA G-3'
pQEHybO_Open_ R	Amplify linear pQE-80 HybO construct reverse primer	5'- GGTTACCGGATCAATAGTAATTCTCTGGCTCAT ACCT GTTCTCTGTGAGGTTATTAGTGATGGTGATG GTG ATGGCCGCCCTCTTTAGCGTTAACATC-3'

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Chapter 3

A novel overproduction system for Hyd-

2

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3.1 Introduction

3.1.1 Expression of genes as proteins

Proteins are the functional units in biology that are responsible for most processes necessary for life. Hyd-2 (see Section 1.5), like all bacterial proteins, is encoded by deoxyribonucleic acid (DNA), either on the chromosome or in a plasmid (see later), which is then transcribed by cellular machinery into messenger ribonucleic acid (mRNA).¹ Transcription of the gene of interest is initiated by a promoter sequence, and the messenger RNA is then translated into transfer RNA (tRNA) at which point the mRNA is read in three-base segments, known as codons, that code for specific amino acids. As only a subset of the total proteins encoded in the chromosome is needed at any certain time and growth condition, the cell also has mechanisms for downregulating or upregulating the expression of certain genes by altering access to the promoter that signals to start DNA transcription.¹

Translation begins at the ribosomal-binding site (RBS), and the amount of protein produced depends greatly on the characteristics of the RBS. After a protein's primary structure (amino acid sequence) is assembled, it relies on chaperone and maturase proteins for proof-reading, folding, and transportation to its final location, where, depending on the protein in question, it may be associated with the cell membrane or other protein subunits. Within these final steps, chaperone proteins can detect poorly formed protein and direct them to proteases for destruction if important, functional parts of the protein, such as redox centres or other cofactors, are missing.^{1,2}

Besides genetic factors, protein production is also dependent on a variety of growth conditions, as these conditions regulate the genes expressed and level of

expression. Fortunately, *E. coli* is one of the most researched organisms and existing literature maps out ideal general growth conditions and guidelines for systems that overexpress proteins within *E. coli*.^{3,4} This is in part due to the fact that *E. coli* is a resilient and metabolically flexible organism capable of growing in different growth conditions. *E. coli* is capable of using oxygen, nitrate, fumarate, trimethylamine-N-oxide (TMAO), dimethyl sulfoxide (DMSO), and nitrite as terminal electron acceptors.⁵

3.1.2 Hyd-2 yield

The first isolation and thorough characterization of Hyd-2 occurred in 2010,⁶ and to easily isolate the protein, it was engineered with a histidine affinity tag on the C-terminus of *hybO* (in this thesis, affinity tagged Hyd-2 expressed from the chromosome is referred to as Hyd-2-N, i.e. natively expressed), confirming previous experiments demonstrating that Hyd-2 is most highly expressed during the exponential phase.^{7,8} Purification of Hyd-2 also revealed that the protein yield is significantly lower (0.015 mg of native Hyd-2 per gram of cells) than that of its homologue, Hyd-1 (0.15 mg of Hyd-1 isolated for each gram of cells).⁶

The reason that Hyd-2-N suffers from such low levels of protein production is not entirely clear. Both the *hyb* and *hya* operons are regulated by other factors including the presence of O₂ and exogenous nitrate.⁹ Downregulation of genes that encode Fe-S cluster-containing proteins like *hybO* in the presence of O₂ is performed by IscR (iron sulphur cluster regulator)—an Fe-S cluster-containing protein that binds to a specific sequence at the promoter of *hybO*.^{10,11} Moreover, because Hyd-2 is expressed during mid-to-late exponential phase, and the media in which *E. coli* is grown for Hyd-2

expression begins fully aerobic, the window between anaerobicity and exponential phase for *E. coli* may limit expression of the *hyb* operon.

Hyd-2-N is a highly complex multi-subunit protein. It is primarily processed by the maturase proteins from the *hyb* operon HybD, HybE, HybF, and HybG. HybD is responsible for cleaving the C-terminal peptide; this cleavage helps lock in place the [NiFe]-cofactor.¹² HybE is a chaperone protein that directs the interaction between HybO and HybC before translocation to the membrane.^{13,14} HybF and HybG assist in Ni and Fe insertion, respectively.^{15,16} The processing of Hyd-2 N is also regulated by proteins from both the *hyp* operon, responsible for general hydrogenase maturation, and the Tat system, which is responsible for the proof-reading and subsequent translocation of proteins containing a Tat-cleavable peptide to the cytoplasmic membrane.¹⁷ Bottlenecks in either of these systems could decrease the level of Hyd-2-N that can be properly matured—a prerequisite for translocation with the Tat system.¹³ HybO is the only subunit of the Hyd-2 catalytic core (HybOC) with a Tat signal, and HybC must therefore be targeted for translocation by the Tat system by ‘piggybacking’ on the Tat signal from HybO.^{18,19} Evidence also suggests that the presence of HybO regulates the level of HybC synthesis. Analysis of proteins present in the cytoplasm, membrane, and periplasm consistently identify excess HybC in the cytoplasm.⁵

The low yield of Hyd-2 N has made a full characterization of Hyd-2 difficult, and original experiments were focused on protein film electrochemistry with limited electron paramagnetic resonance (EPR) and infrared (IR) spectroscopy.^{6,20} Thus, increasing the expression of Hyd-2 was necessary for a more robust characterization, especially structural characterization and analyses of site-directed variants.

3.1.3 Overexpression systems for hydrogenase

In order to boost production of important proteins, significant effort has been devoted to genetically develop overexpression systems, categorized into two different methods. Firstly, the genes of interest can be kept in situ and the chromosome manipulated to increase expression. This approach is ideal for complex proteins that require significant post-translational modification because these proteins typically benefit from co-located translation and transcription of chaperone proteins that are encoded on the same operon.^{21–23} For example, the yield of the [NiFe]-hydrogenase Hyd-5 from *Salmonella enterica* was significantly increased by deletion of the transmembrane (TM) domain of the small subunit and by exchanging the operon promoter for a strong, constitutive T5 promoter.²⁴

The second approach to increasing protein expression is to insert the genes of interest into a plasmid that has a strong promoter and then express the plasmid in a strain that has ideally been optimized for expression of the plasmid. This procedure is ideal for genes that will undergo a wide array of mutations: plasmid mutations are typically simpler and quicker than chromosomal mutations. Moreover, plasmids can be expressed in different strains and species, which would allow further optimization of gene expression.

Attempts to express [NiFe]-hydrogenases from a plasmid have been met with limited success, likely due to the complicated maturation necessary for fully functional protein (see Section 1.3.1).²⁵ Due to the importance of [NiFe]-hydrogenases, work has been done to increase the production of hydrogenase maturation and chaperone proteins to sustain hydrogenase overproduction systems. One promising line of research has shown that the hydrogenase assembly genes from *R. eutropha* can be utilised by *E.*

coli for the production of *R. eutropha* soluble hydrogenase, and importantly, also assist in the maturation of hydrogenases native to *E. coli*.²⁶

3.1.4 Aims of this chapter

This chapter details attempts to increase the yield of Hyd-2. First, growth conditions were optimized in an attempt to increase Hyd-2-N yields. Resulting growth curves and yields were measured. Additionally, an overexpression system to overproduce Hyd-2 (termed Hyd-2-NOP) was created. A thorough enzymatic characterization of Hyd-2-NOP was completed showing that Hyd-2-NOP and Hyd-2-N are functionally identical.

3.2 Results

3.2.1 Hyd-2 and identification of contaminants

Recent attempts to express Hyd-2-N have shown similar yields as reported by Lukey et al.⁶ No error was reported for previous expression levels, but recent experiments have shown large variability in Hyd-2-N yields, perhaps because the yield is affected by a variety of factors including anaerobicity and growth rate.

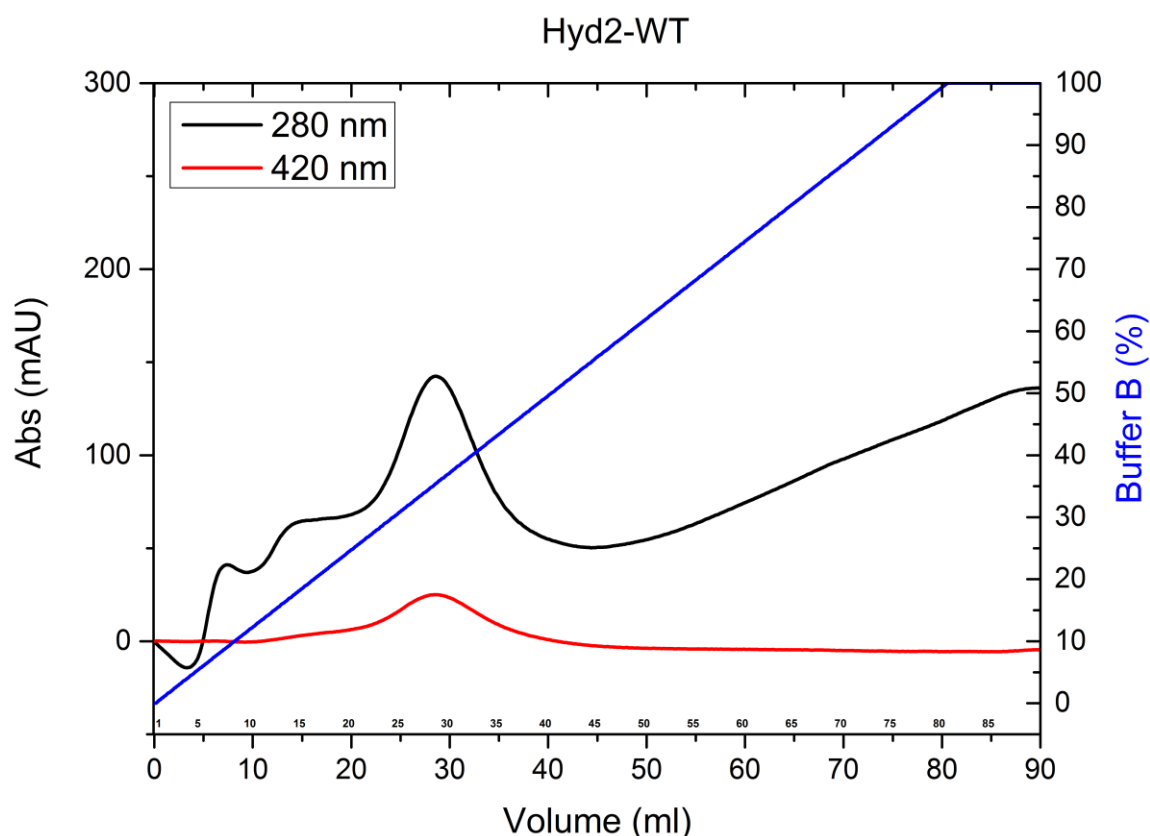


Figure 3.1: Elution profile of *E. coli* Hyd-2-N from a Ni^{2+} -loaded HisTrap column. Hyd-2 from the solubilized membrane (0.002% Triton X-100) of *E. coli* (60L) grown under quasi-anaerobic conditions loaded onto the column and eluted with Buffer B (50 mM Tris, 350 mM NaCl, 0.002% v/v Triton X-100, 1mM DTT, 500 mM imidazole). Note that the imidazole contains impurities that also absorb at 280 nm.

The elution profile of the protein from a Ni^{2+} -loaded HisTrap column remained similar to that reported by Lukey et al, and the presence of protein in the eluent was monitored by UV/visible light absorption at 280 nm and 420 nm (Figure 3.1). These wavelengths measure the concentration of protein (more precisely, the aromatic rings in the amino acids of the protein) and iron content of the eluent, respectively. For [NiFe]-hydrogenase, absorbance at the two wavelengths are usually at a ratio of approximately 5:1. Because the catalytic core of Hyd-2 is co-purified with variable amounts of HybA (a circa 30 kDa protein with 4 [4Fe-4S] clusters, see Figure 1.3), the absorbance at both wavelengths of the chromatogram overestimates the amount of catalytic core.

Moreover, the absorbance at 420 nm depends greatly on the oxidation state of the Fe-S clusters; thus, the elution profile can only give an approximation of the concentration of the catalytic core of Hyd-2.



Figure 3.2: SDS-PAGE denaturing gel of Hyd-2-N fractions eluted from Ni-column in Buffer B (50 mM Tris, 350 mM NaCl, 0.002% v/v Triton X-100, 1mM DTT, 500 mM imidazole) (Figure 3.1). A sample (10 μ L) of fractions from the peak in the chromatogram are mixed with SDS-PAGE loading dye, boiled, and loaded. SDS-PAGE gel run at 200 V for approx. 55 min. HybC (61 kDa) and HybO (37 kDa) are present along with variable amounts of HybA (32 kDa). Additional low molecular weight contaminants (31 kDa, 27 kDa, and 15 kDa) are also present. Relative size of proteins determined by comparison with Broad Range Prestained Protein Marker (NEB, P7708S).

SDS-PAGE denaturing gels reveal the content of the eluent following Ni-affinity chromatography: Hyd-2-N consistently co-purifies with high concentrations of various low-molecular weight contaminants (Figure 3.2). Although previous work has identified three subunits of Hyd-2 in the SDS-PAGE, no work has been done attempting to identify the contaminant bands. Given that the contaminants remain in the sample even after the protein undergoes extended concentration in a concentrator with a 50 kDa cut-off

(Figure 3.3), the small contaminating proteins must interact with different proteins (possibly including Hyd-2) as part of a larger protein complex.

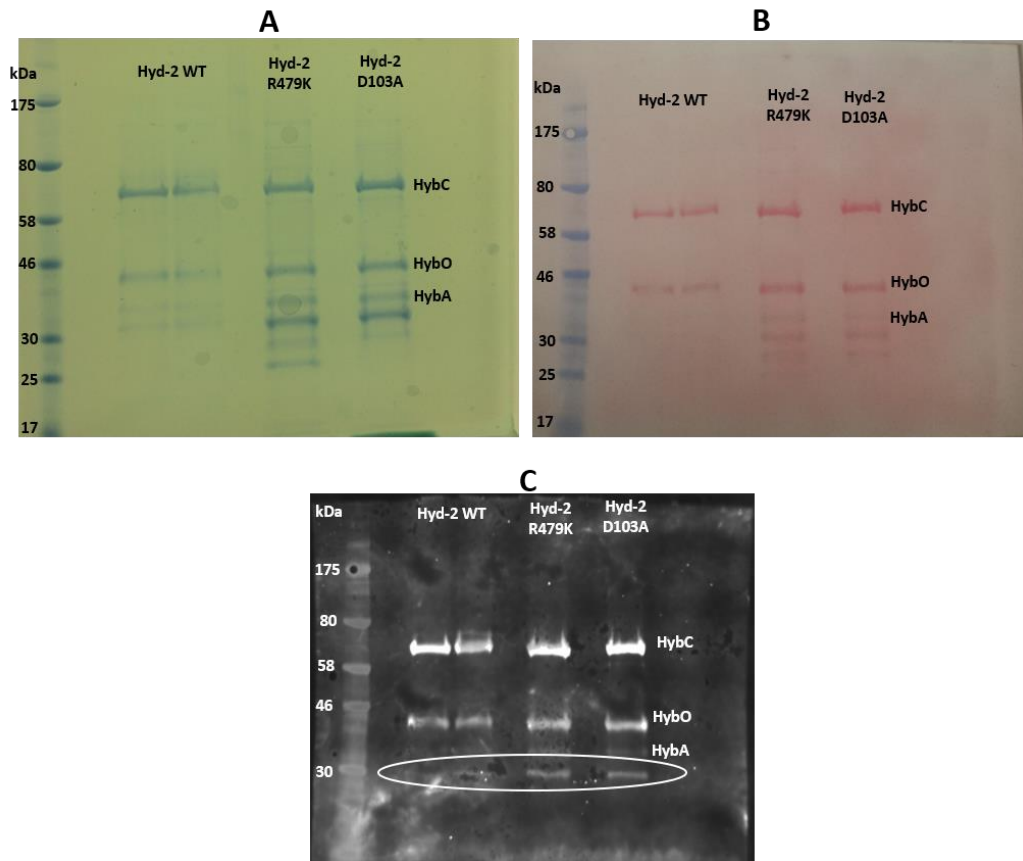


Figure 3.3: SDS-PAGE of Hyd-2 in buffer containing 0.002% v/v Triton X-100 (**A**) and western blot (**B** Ponceau stain, **C** fluorescence) of Hyd-2-N and variants (Chapter 6) following Ni-affinity purification and subsequent concentration (50,000 MWCO). Western blot was performed with primary antibody = anti-Hyd-2-N antibody (Davids Biotechnologie GmbH, raised in rabbit against the products of a similar purification procedure to that shown in Figures 3.1 and 3.2) and secondary antibody = goat anti-rabbit IgG (H&L) DyLight 650. Hyd-2 subunits are labeled corresponding to HybC, HybO and HybA as subsequently confirmed by mass spectrometry analysis (see later). An additional band circled at mass ~30 kDa, could correspond to an additional form of HybA.

Mass spectrometry (MS, Advanced Proteomics Facility, University of Oxford) and Western blot analysis were performed to verify the identity of the protein bands seen in the SDS-PAGE gel (Figure 3.3). First, these techniques suggested that any high molecular bands above 80 kDa were indeed contaminating proteins rather than the dimerization of Hyd-2-N. Moreover, the results positively confirmed the bands that are labelled in Figure 3.3. Finally, these results showed that most of the low molecular bands were unlikely to

be degraded or cleaved Hyd-2-N subunits, but rather were contaminants of unknown identification.

However, a single band of mass approximately 30 kDa, previously assigned as a contaminant, warranted renewed analysis. In the Western blot this band appeared to have significant interactions with anti-Hyd-2-N, and MS results allowed for the possibility that this band at least partially consists of HybA of lower molecular weight (data not shown). This suggested that HybA could exist in various states during Hyd-2 N purification which prompted further explorations of the role that HybA cleavage could play in regulating Hyd-2 activity (see Chapter 5). Further exploration of contaminant identity was unnecessary as increasing yields of Hyd-2 (*vide infra*) made the ratio of contaminants negligible.

3.2.2 Varying conditions for Hyd-2 yield

The first attempt to increase expression of Hyd-2-N was to supplement the growth media of FTH013 with TMAO (trimethylamine N-oxide) rather than sodium fumarate (Section 3.1.1). While the strain reached a higher O.D. (Figure 3.4), the result was a statistically insignificant increase in the amount of Hyd-2 isolated. Across three trial growths with TMAO, the average Hyd-2 yield was within one standard deviation of each other (data not shown). Supplementing the media with TMAO did not change the large variations in the yield between different preparations of the enzyme (data not shown). Increasing the concentration of supplemented sodium fumarate and TMAO also failed to increase the Hyd-2 yield (data not shown).

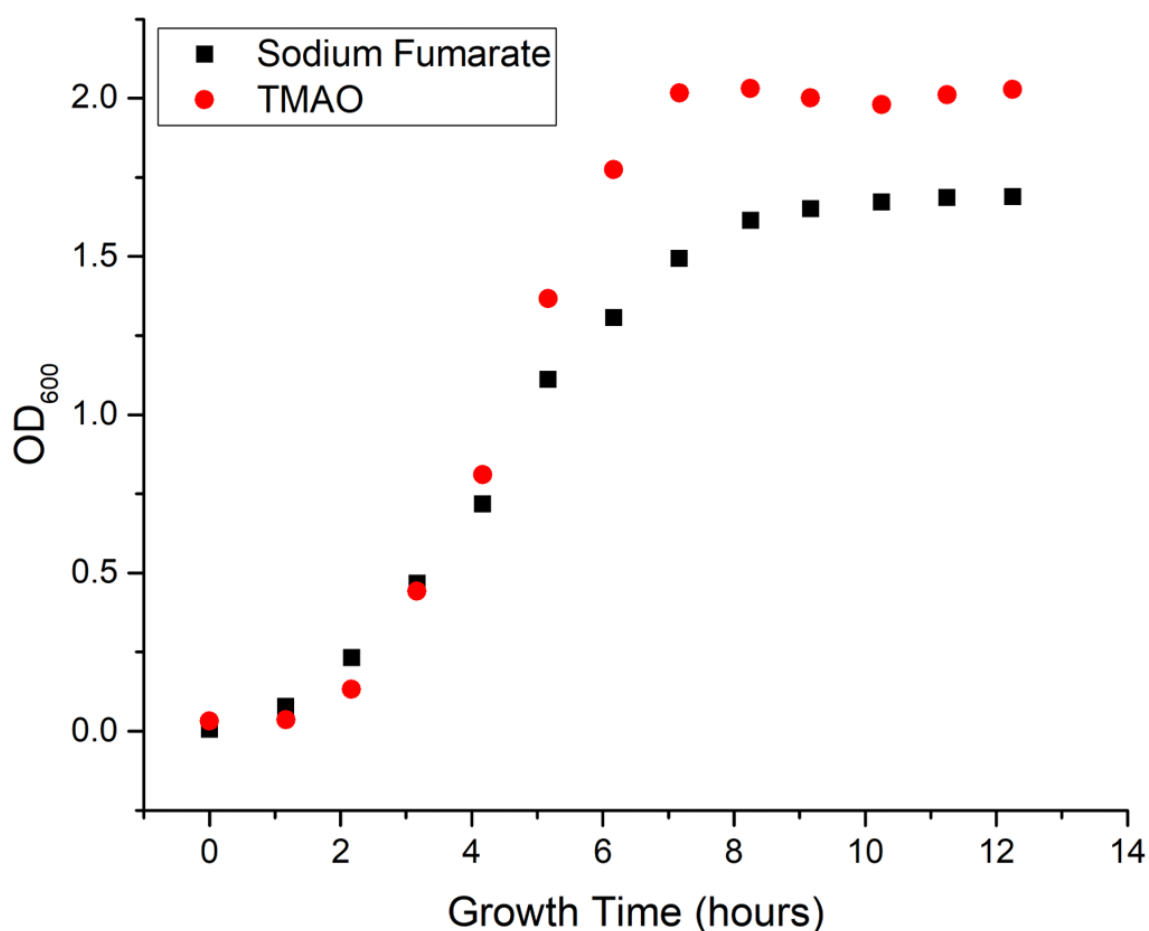


Figure 3.4: Growth curve of FTH013 supplemented with TMAO (red) and sodium fumarate (black). Both growth conditions were also supplemented with glycerol.

Another approach to increase Hyd-2 production was an attempt to modulate the temperature in order to maintain *E. coli* in the exponential growth phase for a long period of time (18+ hours) to extend the phase during which *E. coli* maximally expresses Hyd-2 (Section 3.1.2). This approach proved detrimental to the protein and the cells resulting in a lower pellet weight as well as significantly impaired hydrogenase production (data not shown).

3.2.3 Genetic approach to increased yield

While overproduction of the HybO and HybC peptides is straightforward, the more challenging part of overexpression of Hyd-2 subunits is maintaining balance between all the enzyme maturation processes including cofactor biosynthesis and insertion. Previous work suggested that cellular levels of the small subunit (HybO) may be a limiting factor in Hyd-2 assembly since excess large subunit (HybC) was often observed in the cell cytoplasm in anaerobically-grown cells.⁵ Overexpression of the gene encoding the small subunit alone therefore could be a useful strategy to increase the yield of Hyd-2.

In order to confirm that simply overexpressing HybO would increase the yield of Hyd-2, a plasmid was designed to encode an affinity-tagged and soluble small subunit. Previous work²⁵ had shown the importance of designing HybO with a C-terminal His-tag, as opposed to an N-terminal His-tag that would result in poorly folded protein, and initial efforts therefore focused on HybO with a C-terminal His-tag. Moreover, to prevent any downregulation from bottlenecks in the Tat system, the Tat signal was deleted from *hybO* which also ensures that the HybOC complex would not be translocated from the cytoplasm to the cytoplasmic membrane. Deletion of the TM domain was also included to prevent the formation of inclusion bodies within the cell. Thus, $\Delta Tat \Delta TM$ *hybO* was amplified with a C-terminal His-tag from the chromosome of strain FTH013 and inserted downstream of the T5 promoter in a pQE-80L plasmid (see Tables 2.5, 2.6, and 2.7 for table of strains and plasmids). The resulting plasmid is termed pO^c (Figure 3.5).

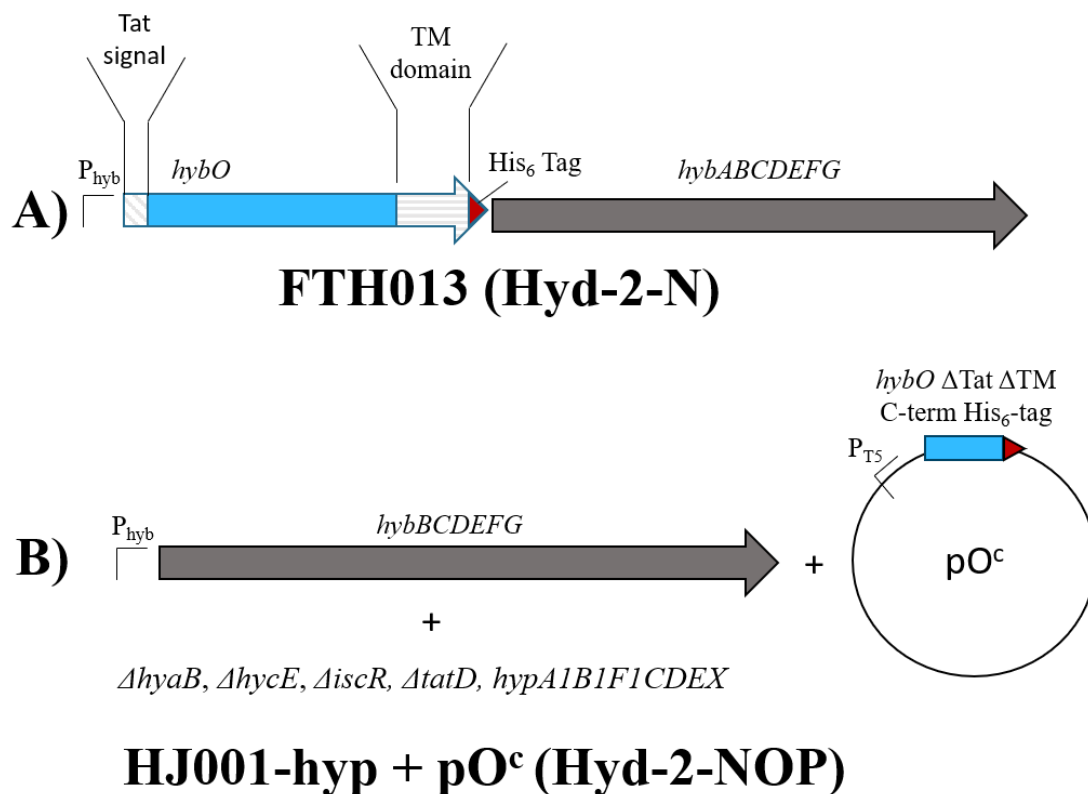


Figure 3.5: A representation of the *hyb* operon for Hyd-2-N used for original Hyd-2-N production and characterization **(A)**,⁶ and a representation of the genotype of strain HJ001-hyp used with plasmid *pO^c* to produce Hyd-2-NOP **(B)**. Figure adapted from reference.²⁷

The *E. coli* mutant expression strain HJ001-hyp lacked the genes for the Hyd-1 and Hyd-3 large subunits and also carried in-frame, non-polar deletions of *hybO* and *hybA* (see Table 2.5).²⁶ Although still encoding the Hyd-2 large subunit *hybC*, this strain had no hydrogenase activity without a plasmid containing *hybO* (*vide infra*). Additionally, the strain contained a deletion of *iscR* and the non-essential *tatD* had been replaced with a synthetic version of the *Ralstonia eutropha* *hypA1B1F1C1D1E1X* operon that have been shown to be active in maturing the membrane-bound [NiFe]-hydrogenases of *E. coli* (Section 3.1.3).²⁶

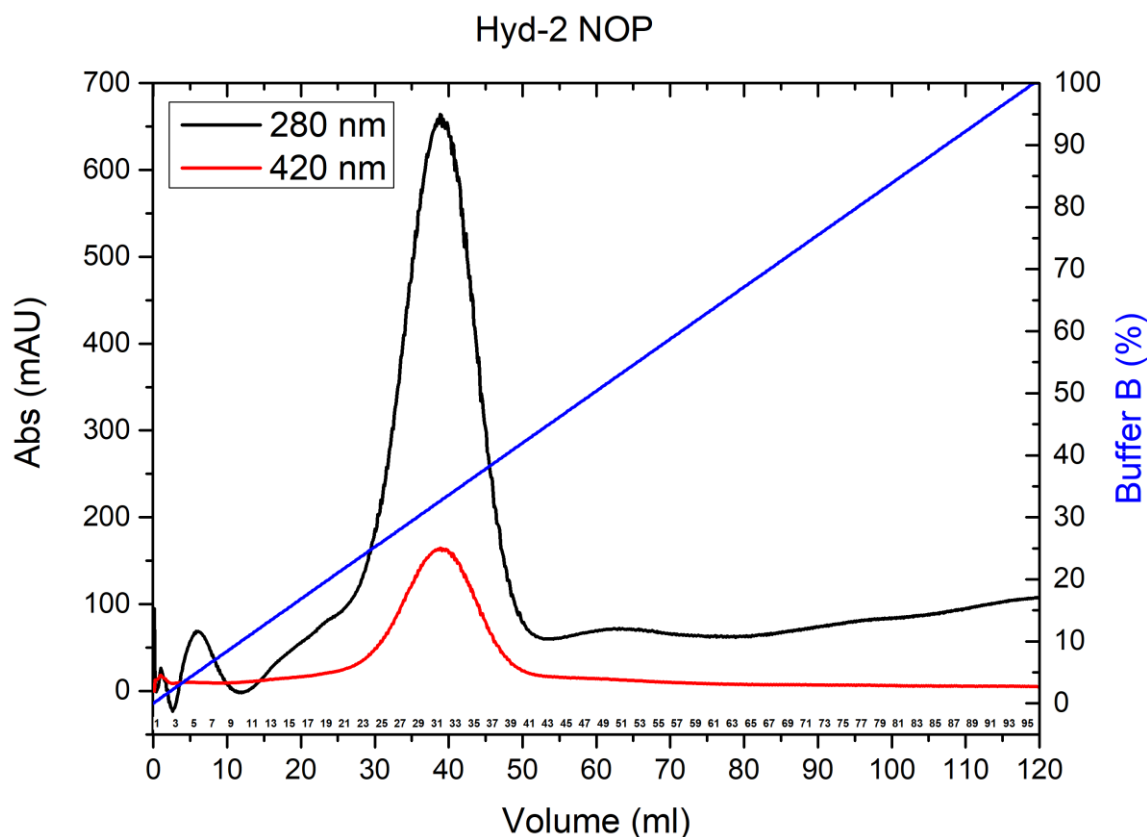


Figure 3.6: Elution profile of Hyd-2-NOP from a Ni^{2+} -loaded HisTrap column. Hyd-2-NOP from the cytoplasm of *E. coli* (120L) grown under quasi-anaerobic conditions loaded onto the column and eluted with Buffer B (50 mM Tris, 350 mM NaCl, 0.002% v/v Triton X-100, 1mM DTT, 500 mM imidazole). Note that the imidazole contains impurities that also absorb at 280 nm.

This expression system showed significantly increased yield for Hyd-2-NOP compared to the natively expressed Hyd-2-N. Purification by a Ni^{2+} -loaded HisTrap column resulted in a similar chromatographic profile with a single peak that later analysis with SDS-PAGE and PFE confirmed is Hyd-2 (Figure 3.6). SDS-PAGE analysis of the protein revealed that Hyd-2-NOP purifies as 2 peptides of masses corresponding to processed HybC and $\Delta\text{Tat } \Delta\text{TM HybO}$ only, i.e. with no HybA, as expected (Figure 3.7). However, significant impurities remained. Thus, the protein required further purification when preparing the protein for crystallization: because the impurities appear at sizes that are

significantly different than HybOC, Hyd-2 was further purified by size exclusion chromatography (SEC).

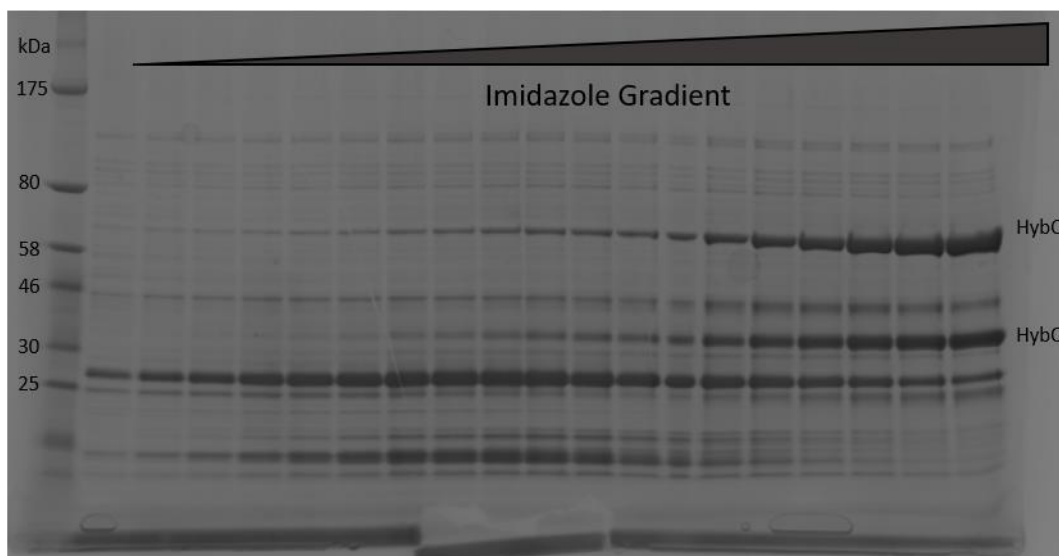


Figure 3.7: SDS-PAGE gel of Hyd-2-NOP fractions eluted from Ni-column in Buffer B (50 mM Tris, 350 mM NaCl, 0.002% v/v Triton X-100, 1mM DTT, 500 mM imidazole) (Figure 3.6). A sample (10 μ L) of fractions from the peak in the chromatogram are mixed with SDS-PAGE loading dye, boiled, and loaded. SDS-PAGE gel run at 200 V for approx. 55 min. HybC (61 kDa) and HybO (33 kDa), and additional low molecular weight contaminants (27 kDa and 15 kDa) are also present. Relative size of proteins determined by comparison with Broad Range Prestained Protein Marker (NEB, P7708S).

The chromatogram from the SEC showed that there are significant impurities both larger and smaller than HybOC (Figure 3.8). There is also a ‘shoulder’ on the Hyd-2 peak (at approx. 27 mL in the chromatogram) in some SEC runs. This could indicate that Hyd-2 exists in different oligomeric states that result in different elution times during SEC purification. For Hyd-1, the shoulder of the peak represents the difference in elution time dependent on the quaternary conformation [i.e. whether the protein is in a dimer of heterodimers ($\alpha\beta$)₂ or simply a heterodimer ($\alpha\beta$)].²⁸ The purest fractions from SEC yielded Hyd-2-NOP that successfully crystallized (Chapter 4), and this purification protocol was therefore used for all future Hyd-2 variants.

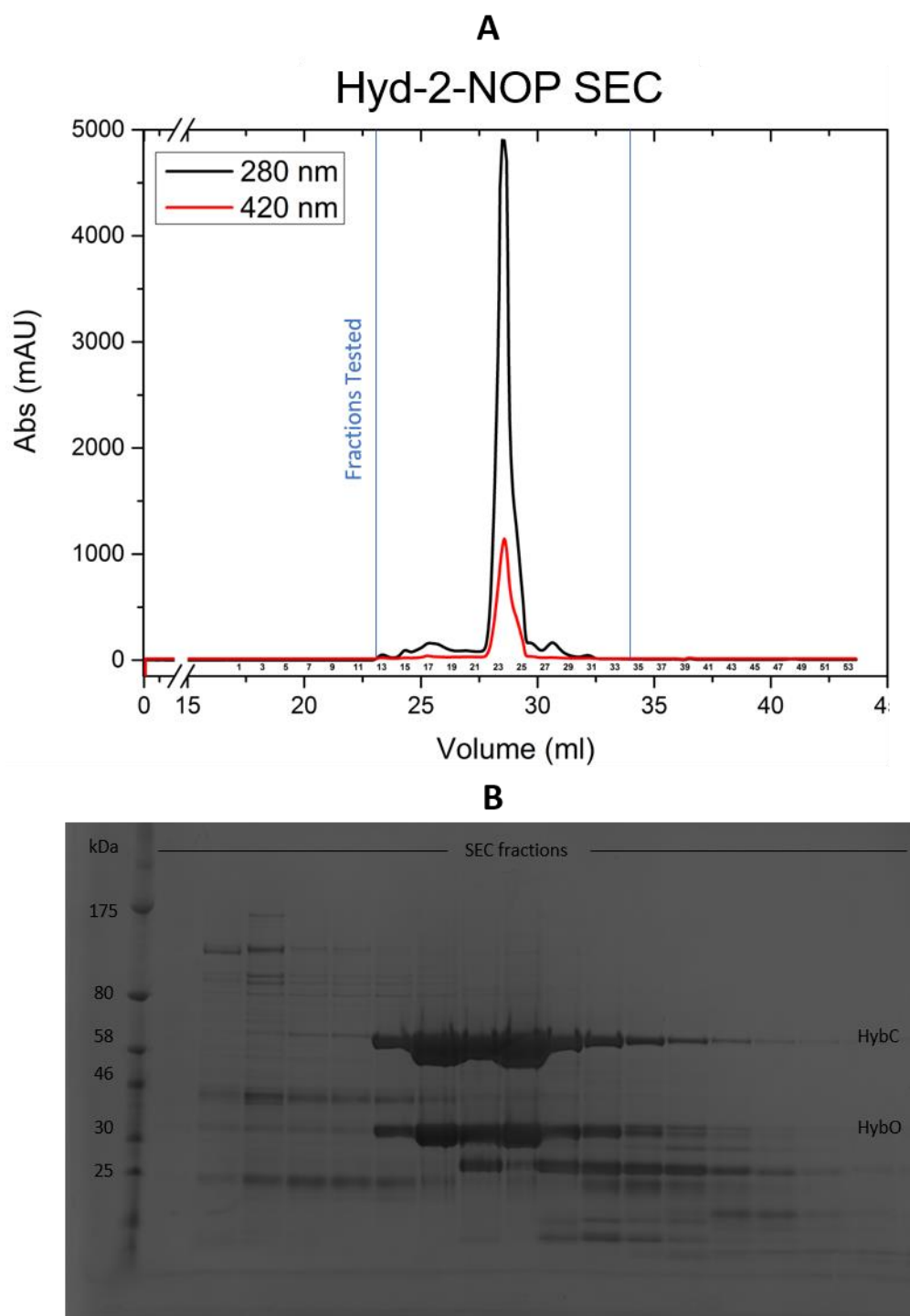


Figure 3.8: Purification of Hyd-2-NOP by SEC in buffer with 0.02% w/v DDM. **(A)** Elution profile of Hyd-2-NOP from SEC column as a secondary purification technique. **(B)** A sample (10 μ L) of fractions from the peak in the chromatogram are mixed with SDS-PAGE loading dye, boiled, and loaded. SDS-PAGE gel run at 200 V for approx. 55 min. HybC (61 kDa) and HybO (33 kDa). SEC purification succeeded in separating the catalytic core from low molecular weight contaminants (27 kDa and 15 kDa) that are also present in other fractions. Relative size of proteins determined by comparison with Broad Range Prestained Protein Marker (NEB, P7708S).

While the absorbencies of the SEC chromatogram (Figure 3.8) indicates that the Hyd-2-NOP yield is roughly an order of magnitude larger than for Hyd-2-N, this is only an approximation. In order to have a more quantitative analysis of the yield of active Hyd-2, the plasmid was sent for comparative analysis following expression in strains HJ001 ($\Delta hyaB$, $\Delta hycE$, $\Delta hybOA$, $\Delta iscR$) and HJ001-Hyp (see Table 2.5), carried out by Prof. Frank Sargent (University of Dundee). As a negative expression control, collaborators at the University of Dundee also created a plasmid encoding $\Delta Tat \Delta TM hybO$ with an N-terminal His-tag (termed pO^N).

The two strains were transformed with either empty vector (pQE-80L), pO^N , or pO^C and grown anaerobically in the presence of IPTG (1 mM) before a soluble protein extract was prepared. A control strain of FTD674, which contains a $\Delta Tat \Delta TM hybO$ gene on the chromosome, (Table 2.5)¹⁷ was cultured under identical conditions. The relative amounts of active Hyd-2 were then assessed by rocket immunoelectrophoresis (Figure 3.9).⁸ Rocket immunoelectrophoresis^{7,8} is a technique that can assay the activity of Hyd-2 (precipitated with anti-Hyd-2) within an agarose gel by incubating the gel in a H_2 environment with a redox dye. The larger the coloured rocket-shaped arc, the greater the quantity of active Hyd-2. This technique is ideal in that it distinguishes active Hyd-2 from poorly folded, inactive protein, as Hyd-2 that has not undergone proper folding will not oxidize the H_2 and reduce the dye.

Under the specified growth conditions, expression of pO^C produced stable HybO that was able to interact with the HybC and generate active Hyd-2 enzyme (Figure 3.9). Moreover, the assays confirmed that the relative amount of enzyme produced by the plasmid-based system greatly exceeded that normally found at native levels (Figure 3.9). The controls also demonstrated that the expression level is higher compared to that for

soluble Hyd-2 encoded by the chromosome. Additionally, the trial confirms that an N-terminal His-tag prevents proper expression of Hyd-2.

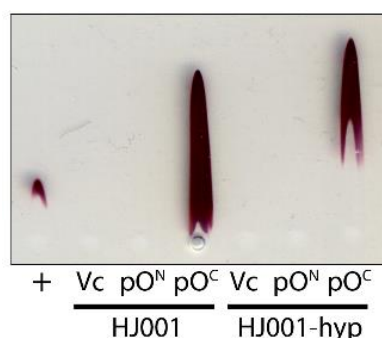


Figure 3.9: Comparison of yield for Hyd-2 overexpression systems in strains HJ001 and HJ001-hyp containing either no plasmid (vector control abbreviated V_c), Δ Tat Δ TM hybO with an N-terminal His-tag (pO^N), or Δ Tat Δ TM hybO with a C-terminal His-tag (termed pO^C). A positive control of natively expressed Δ Tat Δ TM Hyd-2 (+) from strain FTD674 is included for comparison. Rocket immunoelectrophoresis performed with cell extracts washed with 50 mM Tris-HCl in 1% (w/v) agarose containing 5 μ l of anti-Hyd-2 serum buffered with 20 mM barbitone. HCl at pH 8.3. Following electrophoresis, activity staining with 10 mM TrisHCl pH 7.5 containing 0.5 mM benzyl viologen and 1 mM tetrazolium red was carried out in a 100% H₂ atmosphere. Assay performed by Alexander Finney and Prof. Frank Sargent (University of Dundee). Figure adapted from reference.²⁷

3.2.4 Purification and catalytic activity of recombinant Hyd-2

Based on the high yield of protein purified from the cytoplasm of HJ001-Hyp with pO^C, this combination of strain and plasmid was used for the overproduction of Hyd-2 for the remainder of this thesis.

The T5 promoter was induced upon the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) and the timing for the addition of IPTG to the growth medium affected the level of expression. The conditions of growth including induction timing, concentration of IPTG, and concentration of ampicillin were all optimized. The best condition (1 mM IPTG, 100 μ g/mL ampicillin, induced at an OD₆₀₀ of 0.25) were used in all further protein growth and purification (Figure 3.10).

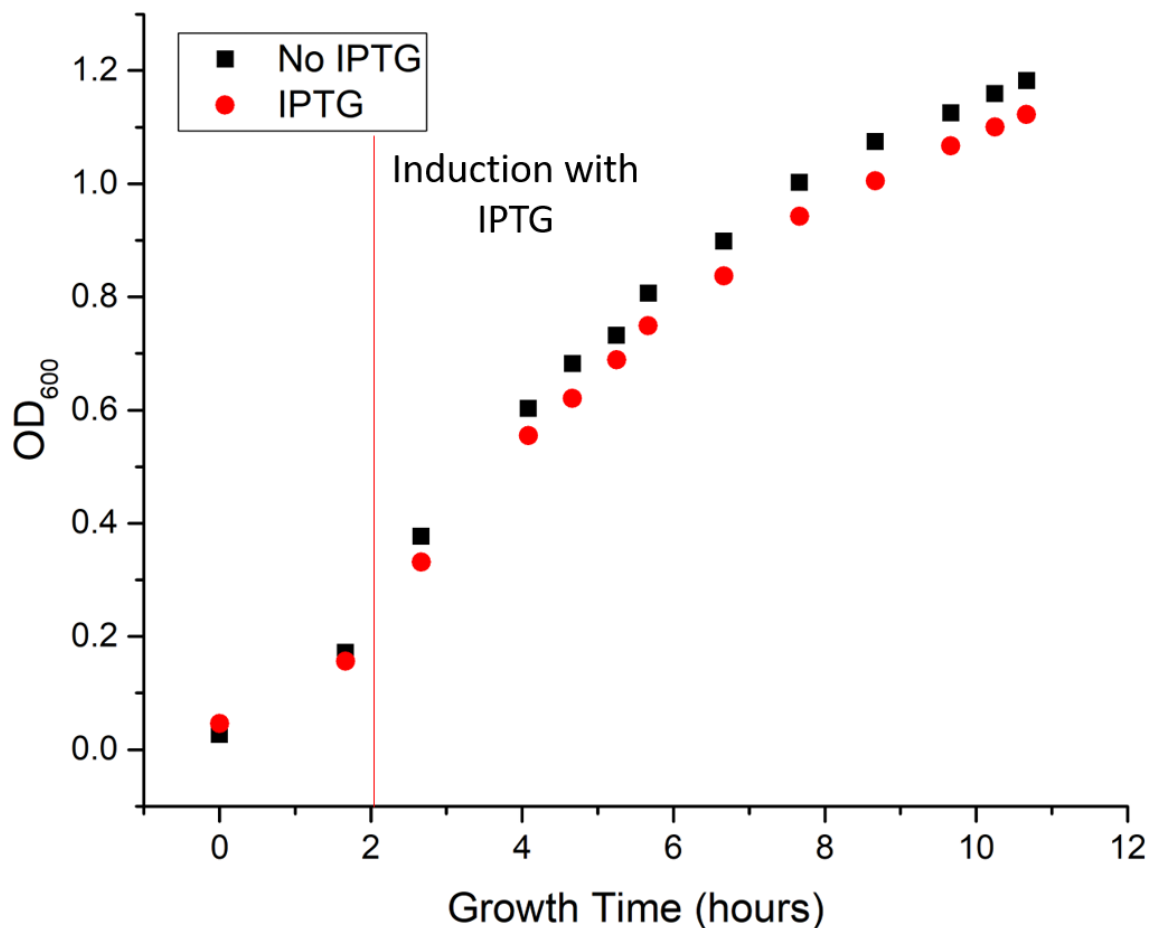


Figure 3.10: Growth curve of HJ001-hyp with pO^C with (red) and without (black) induction by IPTG.

3.2.5 Further attempts to increase yield of Hyd-2

Further attempts to increase expression of Hyd-2 were unsuccessful: first, the large subunit gene (*hybC*) as well as the gene for the protease that cleaves and processes it (*hybD*) were amplified from the chromosome and added to pO^C (termed pO^C CD) in an attempt to express the full catalytic core from the plasmid (Figure 3.11). Because *hybC* is followed by *hybD* on the chromosome, both genes were amplified and added as a single cassette downstream of *hybO*. This plasmid was expressed in HJ001-hyp following the same growth and purification protocols established when purifying Hyd-2-NOP.

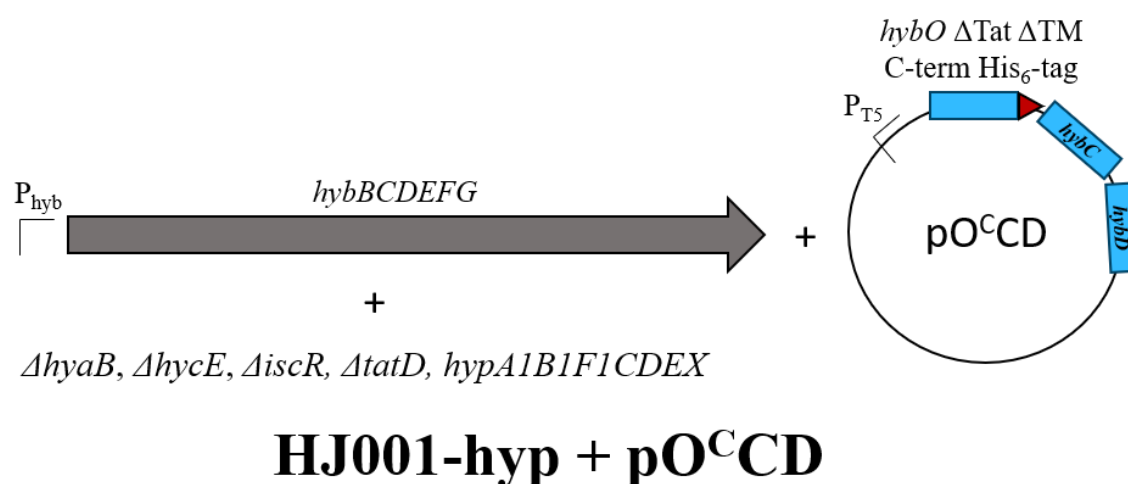


Figure 3.11: A representation of the genotype of strain HJ001-hyp used with plasmid pO^CCD. In an attempt to further increase the yield of Hyd-2, *hybC* and *hybD* were expressed from pQE-80 plasmid in addition to being expressed from the chromosome.

This approach led to a significant decrease in yield when compared to only overexpressing *hybO* from plasmid pO^C in strain HJ001-hyp (data not shown). The decrease in Hyd-2 yield occurred in spite of the fact that in the test expression *hybC* and *hybD* were expressed both from a plasmid and from the chromosome (Figure 3.11). Second, attempts to extend the exponential phase by dropping the temperature of the growth medium during preparation of Hyd-2-NOP from 37 °C to 24°C upon induction of plasmid resulted in a lower cell pellet weight as well as decreased the yield (data not shown).

3.2.6 Electrochemical comparison of Hyd-2-N and Hyd-2-NOP

While the protein appeared intact on SDS-PAGE, the growth conditions, presence of foreign maturation proteins from *R. eutropha* *hypA1B1F1C1D1E1X*, and absence of HybA could have changed the enzymatic parameters of Hyd-2 NOP, so a full enzymatic characterization was done for Hyd-2-NOP to ensure that the protein was functionally identical to Hyd-2-N.

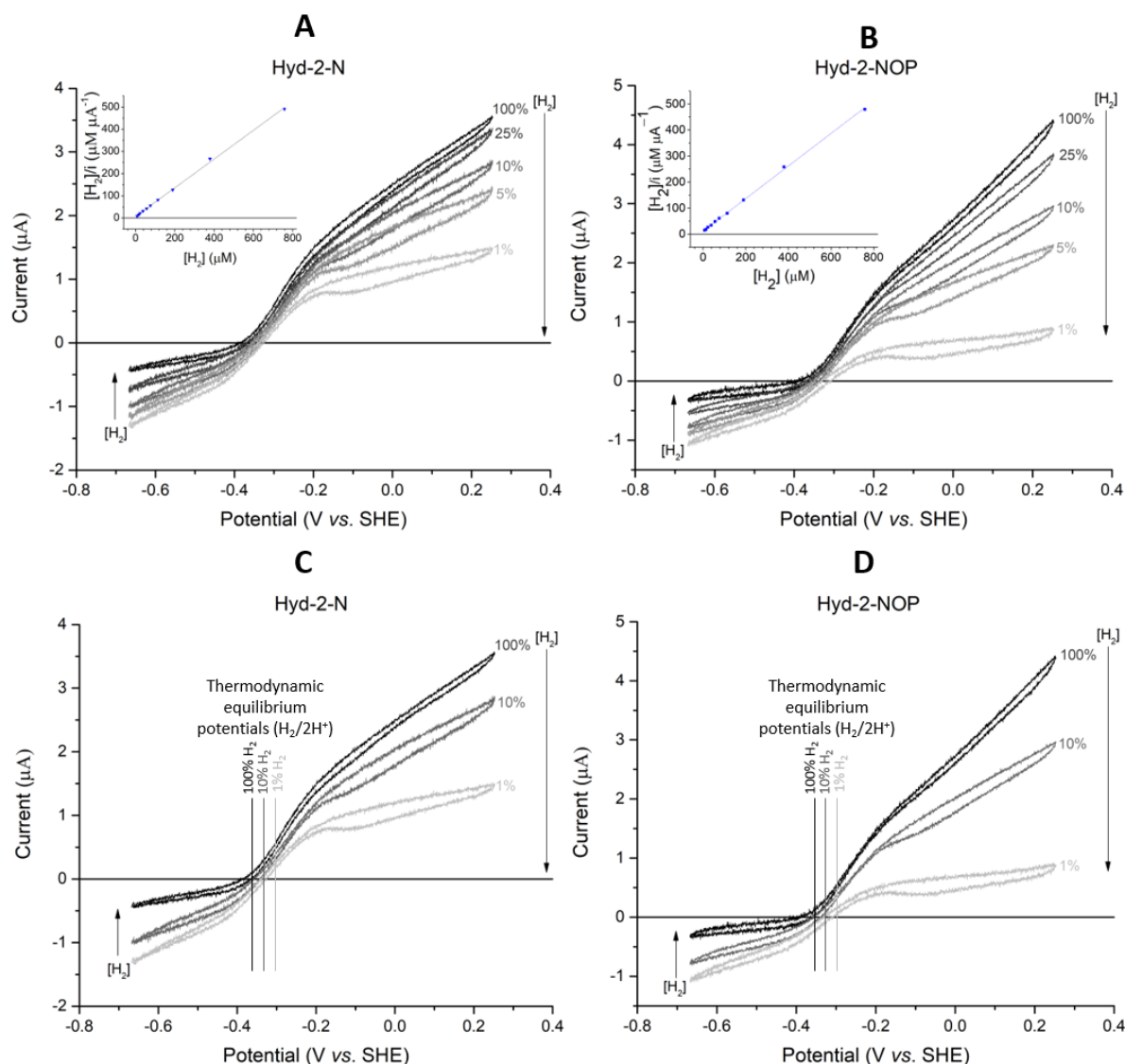


Figure 3.12: A comparison of the electrochemical profiles of Hyd-2-N and Hyd-2-NOP. Measurements of K_m for Hyd-2-N (panels A and C) and Hyd-2-NOP (panels B and D) are shown. Increasing activity corresponds with increasing hydrogen concentration (panels A and B) and the zero-current potential corresponds well to the values predicted by the Nernst equation (panels C and D). K_m values were calculated with a Hanes-Wolfe plot at -0.175 V vs. SHE (panels A and B inset). Figure adapted from reference.²⁷

The previously published electrochemical profile of Hyd-2-N showed it to be a reversible hydrogenase, i.e. capable of both H₂ oxidation and H⁺ reduction, the latter being greatly product inhibited.^{6,29} The electrochemical profile of recombinant, overproduced Hyd-2-NOP is also consistent with these characteristics in that the adsorbed enzyme requires minimal overpotential for H₂ oxidation or H⁺ reduction, i.e. Hyd-2-NOP produces a cyclic voltammogram that sharply cuts through the zero-current

axis, and the H^+ reduction current drastically decreases with increasing partial pressures of hydrogen (see Figure 3.12, Figure 3.13, and Figure 3.14).

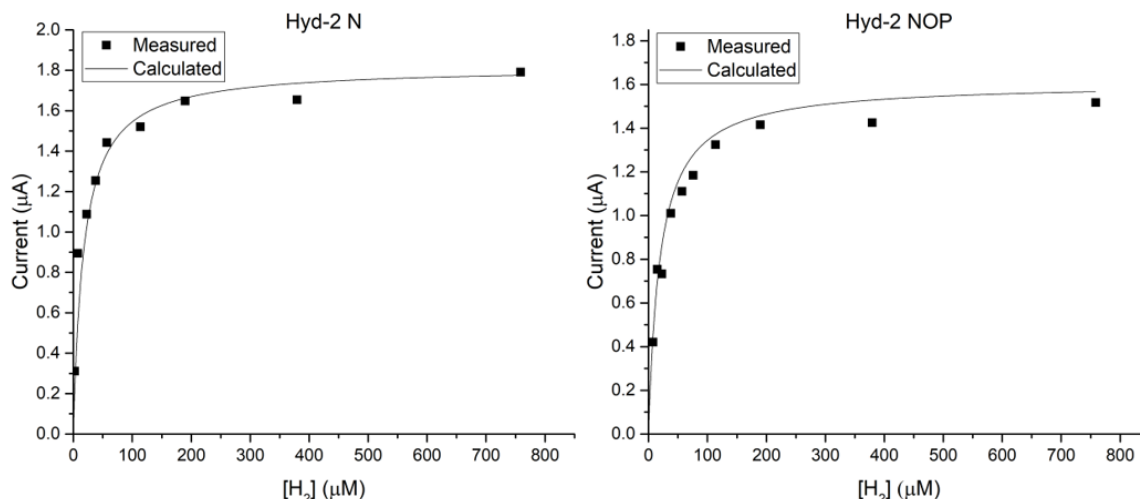


Figure 3.13: Example of the Michaelis-Menten plots of the calculated K_M and v_{max} for Hyd-2 N (left) and Hyd-2 NOP (right) compared with the measured activity. Other conditions for measurement of the activity: pH 6.0, 30°C, 5 mV/sec, 100% H_2 , and $\omega=3000$ RPM.

Several key enzymatic parameters are used to describe the characteristics of enzymes in general and [NiFe]-hydrogenases in particular. One of them is the Michaelis Constant, $K_M^{H_2}$, which describes the concentration of H_2 required to reach half the enzyme's max turnover rate. Because $K_M^{H_2}$ is a combination of three enzymatic parameters (see Section 2.10.3.4 for a detailed description of $K_M^{H_2}$), it is an important measurement to confirm that the activity of Hyd-2-NOP is similar to Hyd-2-N. One of the enzymatic parameters that makes up $K_M^{H_2}$ is k_{cat} , which is potential dependent. Thus, $K_M^{H_2}$ is reported with the potential at which it was measured. For Hyd-2-NOP the measured $K_M^{H_2}$ at 0.175 V is 23 ± 7 μM whereas the measured $K_M^{H_2}$ at 0.175 V for Hyd-2-N is 21 ± 4 μM . Both of these figures are in good agreement with previously reported figures.⁶ Another important enzymatic parameter is the inhibition constant, $K_i^{H_2}$, that describes the concentration of H_2 required to reach half of total inhibition (see Section

2.10.3.5). Like $K_M^{H_2}$, $K_i^{H_2}$ is potential dependent. At -0.670 the $K_i^{H_2}$ for Hyd-2-NOP is $240 \pm 11 \mu\text{M}$ and the $K_i^{H_2}$ for Hyd-2-N is $219 \pm 14 \mu\text{M}$. Both figures are in good agreement with data previously reported for Hyd-2, and the calculated Michaelis-Menten curves for both enzymes agree well with the measured data (Figure 3.13).⁶

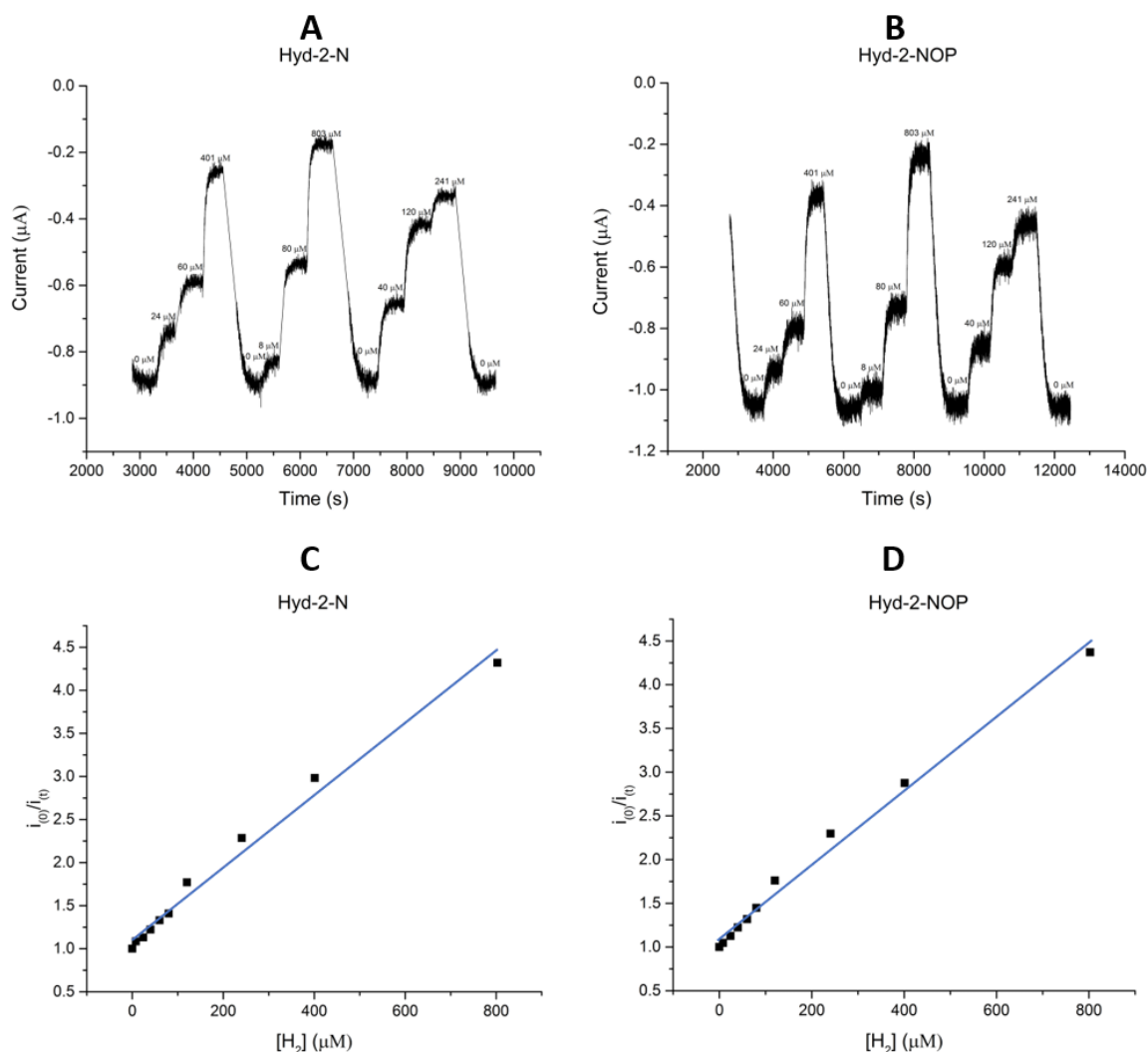


Figure 3.14: Comparison of H_2 production activity with varying concentration of H_2 at -0.175 V vs. SHE between Hyd-2-N (A) and Hyd-2-NOP (B). The effect of product inhibition on current is nearly identical for both enzymes. The inhibition constant ($K_i^{H_2}$) is calculated as the inverse slope when plotting the ratio of the uninhibited current ($i_{(0)}$) to inhibited current ($i_{(i)}$) against the concentration of H_2 (panels C and D). Figure adapted from reference.²⁷

The electrochemical profiles of Hyd-2-N and Hyd-2-NOP are similar despite the fact that the Hyd-2-N HybOC catalytic core is co-purified in detergent with variable

amounts of HybA⁶ whereas *hybA* is deleted from the chromosome of strain HJ001-hyp, used to produce Hyd-2-NOP. The similarity in the general characterization of Hyd-2 shows that HybA has a negligible effect on the electrochemistry of Hyd-2.

However, there are nuanced differences between the two electrochemical profiles. Firstly, Hyd-2-NOP experiences significantly quicker ‘film loss’, which is understood as an irreversible decrease in current over time, related to enzyme desorption, unfolding, or reorientation at the electrode. This decrease in the stability of the film could be explained by the loss of the transmembrane domain on HybO and the complete lack of HybA (which also has a transmembrane helix). The presence of these two transmembrane helices in Hyd-2-N may provide greater film stability due to the increased hydrophobic residues available to interact with the pyrolytic graphite. Secondly, films of Hyd-2-NOP are less likely to show the effects of a dispersion of interfacial electron transfer (IET) due to a greater homogeneity of the sample population in Hyd-2-NOP (Figure 3.15).³⁰ Dispersion can be seen as an increase in current with potential that corresponds to increasing quantities of fully electroactive enzyme. As the potential increases, enzymes that are in a non-ideal orientation have sufficient driving force to drive fast IET. This effect is particularly pronounced on larger films (>10 μA) that have not undergone a prolonged activation procedure.

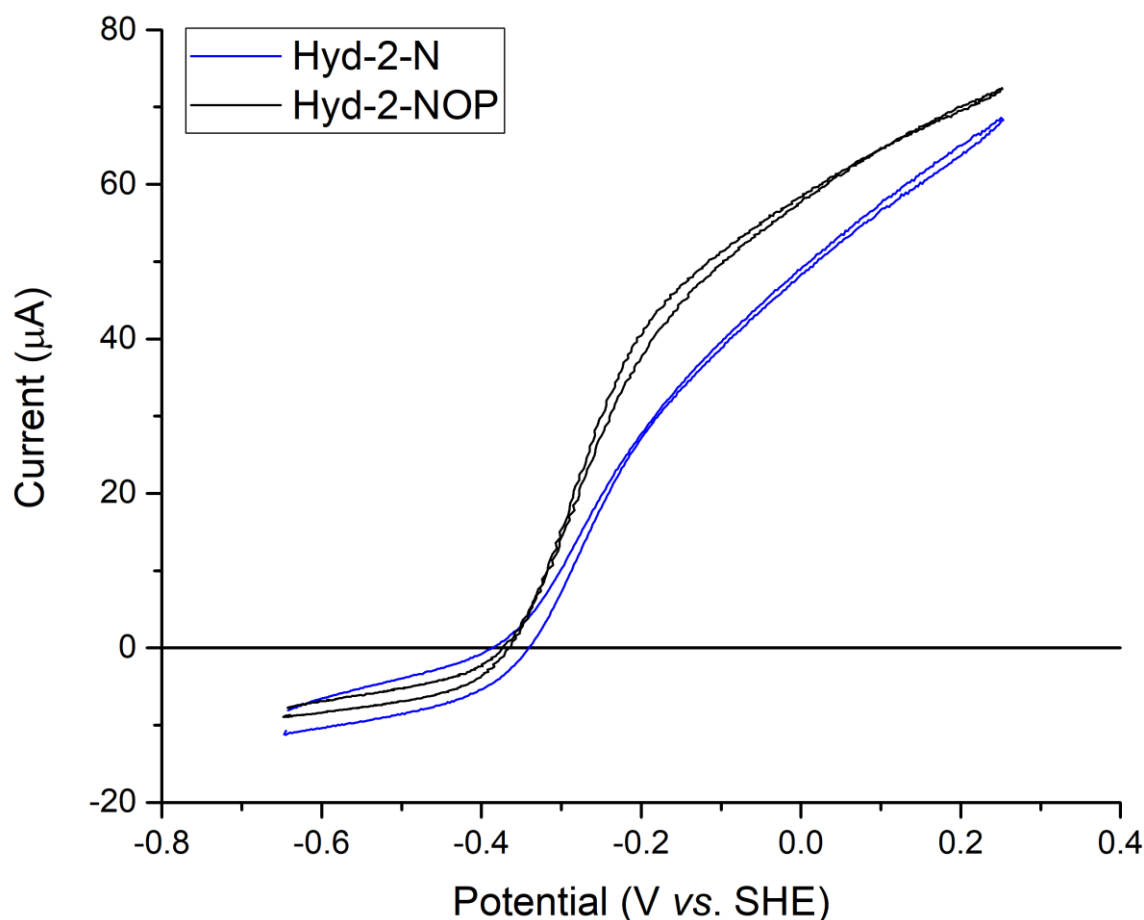


Figure 3.15: Comparison of early CVs for Hyd-2-N (black) and Hyd-2-NOP (red). Both scans taken on films before activation at pH 6.0, 30°C, 30 mV/sec, 100% H₂, and 1000 RPM.

Another important enzymatic parameter, though more qualitative in nature than most other enzymatic parameters, is E_{SWITCH} . On a fully anaerobically inactivated enzyme film, E_{SWITCH} is measured as the minimum of the first derivative (i.e. inflection point on the normal scan) of the backwards reactivation scan (see Section 2.10.3.6). For Hyd-2-N and Hyd-2-NOP the measured E_{SWITCH} among three trials for both enzymes is within one standard deviation from each other making them statistically equivalent (Figure 3.16).

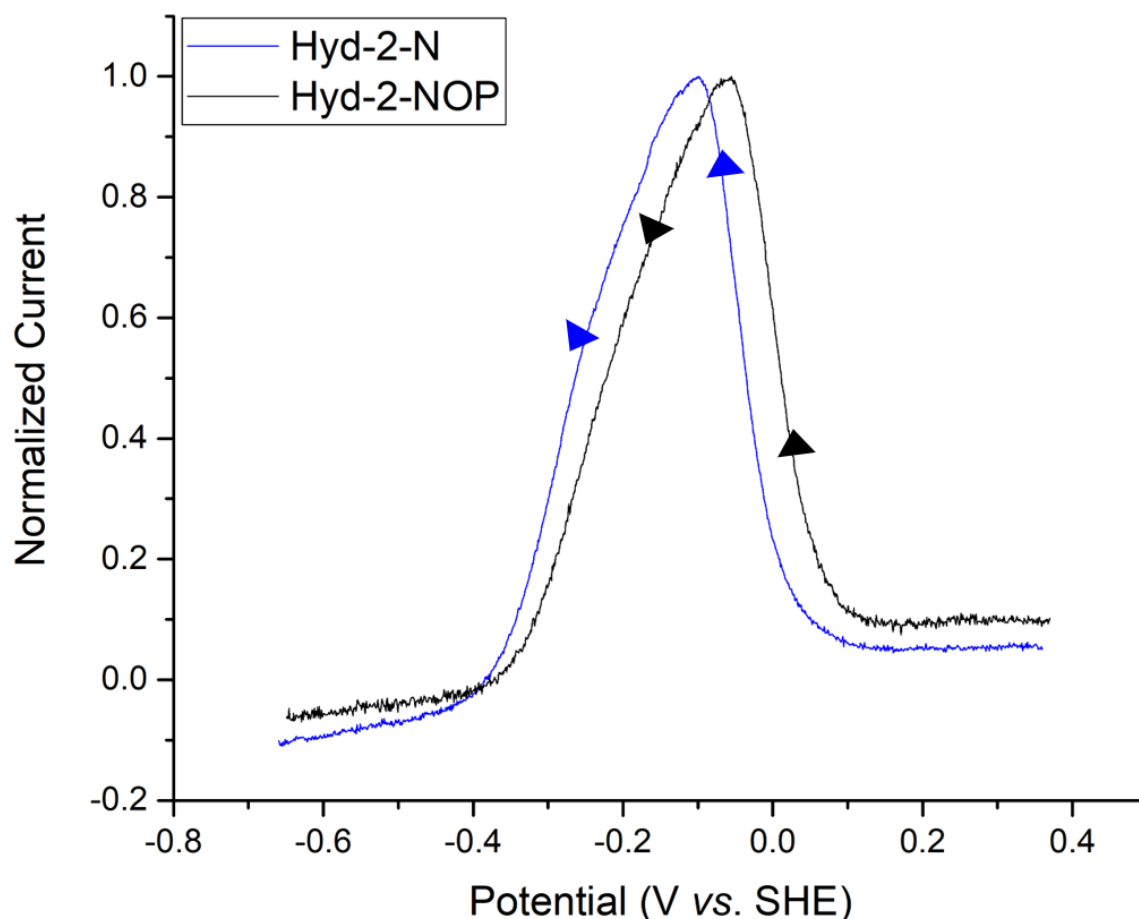


Figure 3.16: Overlay of E_{SWITCH} scans (0.1 mV/sec, 30°C, 1000 rpm) following anaerobic inactivation (pH 8.0, 100% Ar) at 0.35 V. The driving force required to reactivate Ni-B gives insight into the stability of the resting inactive state. Data have been normalized to max current for each experiment for easier comparison. Data for E_{SWITCH} has been smoothed with the Savitzky-Golay method, 41 points per window. The variance between Hyd-2-N and Hyd-2-NOP is within error of each other. Adapted from reference.²⁷

Activation enthalpy ($\Delta H^\ddagger$) for H_2 oxidation is related to the energy required to form the transition state in the rate-limiting step of steady-state turnover. Any change in the rate-limiting step can cause large changes in the $\Delta H^\ddagger$ which makes it an important measurement to confirm the fidelity of the active site. Again, in Hyd-2-N and Hyd-2-NOP the measured $\Delta H^\ddagger$ are nearly identical at all potentials. Previously, $\Delta H^\ddagger$ has been reported at 0.200 V, and at that potential Hyd-2-NOP has a $\Delta H^\ddagger$ of $47 \pm 2 \text{ kJ mol}^{-1}$ and Hyd-2-N has a $\Delta H^\ddagger$ of $43 \pm 3 \text{ kJ mol}^{-1}$. The agreement between these figures, as well as previously reported data,^{25,31} suggests the active site of Hyd-2-NOP is well-formed and Hyd-2-NOP follows the same catalytic cycle as Hyd-2-N (Figure 3.17).

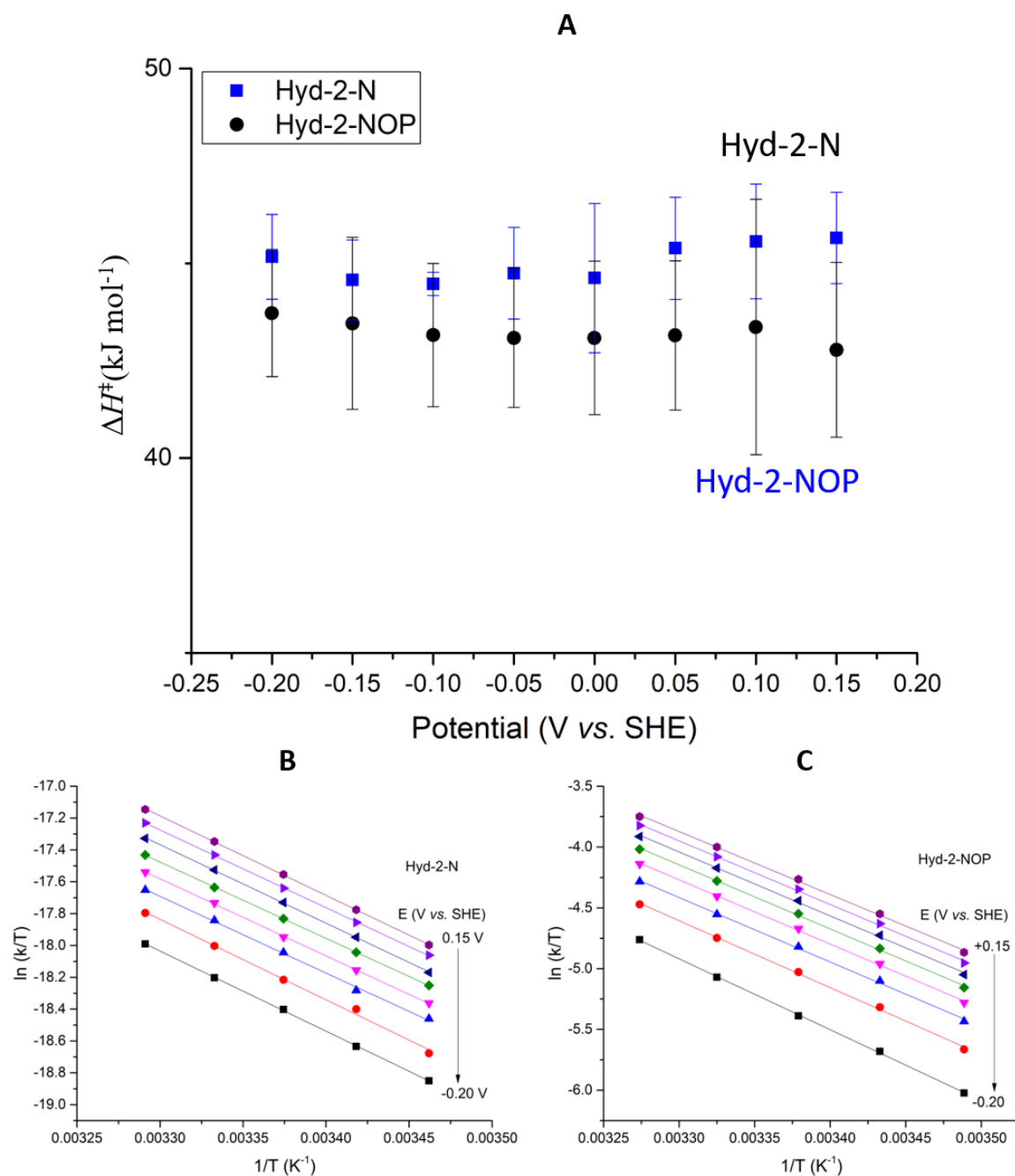


Figure 3.17: Hydrogen oxidation activity (at -0.200 V vs. SHE) at varying temperatures (in the range 10°C to 30°C) is analysed with Eyring plots (panels B and C) to reveal similar activation enthalpies $\Delta H^\ddagger$ for Hyd-2-N and Hyd-2-NOP (A). Figure adapted from reference.²⁷

3.2.7 EPR comparison of Hyd-2-N and Hyd-2-NOP

EPR is a powerful tool to interrogate the spin states of metals buried deep inside protein. EPR is typically more sensitive to small changes in the spin environment around the active site than PFE. This technique therefore allows greater confirmation that the

NiFe active site cofactor has been properly assembled and inserted into active Hyd-2. Fortunately, the main paramagnetic states of Hyd-2 have been previously mapped.^{6,32} There are two inactive paramagnetic states of Hyd-2: Ni-A ($g_x=2.31$, $g_y=2.24$, and $g_z=2.01$) and Ni-B ($g_x=2.31$, $g_y=2.16$, and $g_z=2.01$).^{6,32} These two states appear at oxidizing potentials, and upon exposure to O_2 that typically occurs during purification. For Hyd-2 there are three active paramagnetic states that are commonly seen: Ni-C ($g_x=2.194$, $g_y=2.137$, and $g_z=2.011$), Ni-L ($g_x=2.297$, $g_y=2.119$, and $g_z=2.048$), and Ni-X ($g_x=2.242$, $g_y=2.095$, and $g_z=2.053$).^{6,32} Two of these signals (Ni-C and Ni-L) have a known corresponding state in the catalytic cycle, but the physical meaning of the third state (Ni-X) is still unknown (Figure 3.18).

While Ni-L has been shown to be an intermediate in Hyd-1,³³ it is still unknown whether the same is true for Hyd-2. However, Ni-L is commonly found in Hyd-2 EPR samples prepared in ambient light. Ni-L was first discovered (and consequently named) by exposing Ni-C to light at cryogenic temperatures. The light has sufficient energy to break the bonds coordinating the hydride to the [NiFe]-cofactor. Upon returning Hyd-2 to the dark, Ni-L returns to Ni-C, and previous enquiries have focused on measuring the rate of relaxation from Ni-L to Ni-C to help better understand the thermodynamics of the transition.³² Interestingly, Ni-X is the only reduced species that is not affected by the light, but the physical meaning of the species remains elusive.

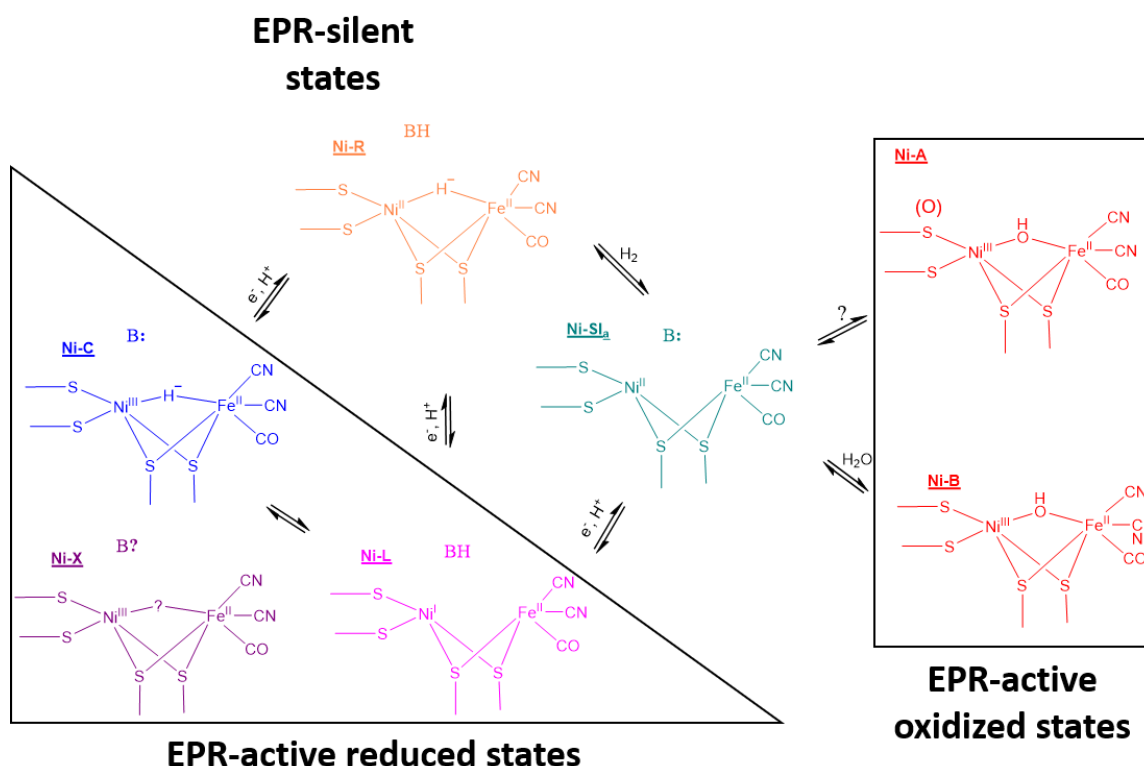


Figure 3.18: Diagram of the mechanism of Hyd-2 including the reduced state seen in EPR of unknown significance. EPR species are outlined whereas Ni-SI and Ni-R are both EPR silent species. The mechanistic relevance of Ni-L is debated, but it is included here as an intermediate as EPR samples of Hyd-2 purified in ambient light typically contain significant quantities of Ni-L.

The Fe-S clusters also give a strong signal at low temperatures (15 K), yet at higher temperatures (70 K) the Fe-S clusters relax too quickly to be measured, which facilitates the collection of clean EPR spectra for the active site without interference by Fe-S clusters. Under oxidizing conditions and at low temperature, the medial cluster gives a strong radical signal from the $[3\text{Fe-4S}]^+$ ($g_x=2.03$, $g_y=2.02$, and $g_z=2.01$).^{6,32}

For Hyd-2-NOP, potentials were set by titrating the sample with $\text{Na}_2\text{S}_2\text{O}_4$ or $\text{K}_3[\text{Fe}(\text{CN})_6]$ (see Section 2.6.2 for complete description of sample prep). The EPR spectra at two different potentials and temperatures show EPR signals that are nearly identical to those published for Hyd-2-N (Figure 3.19).³²

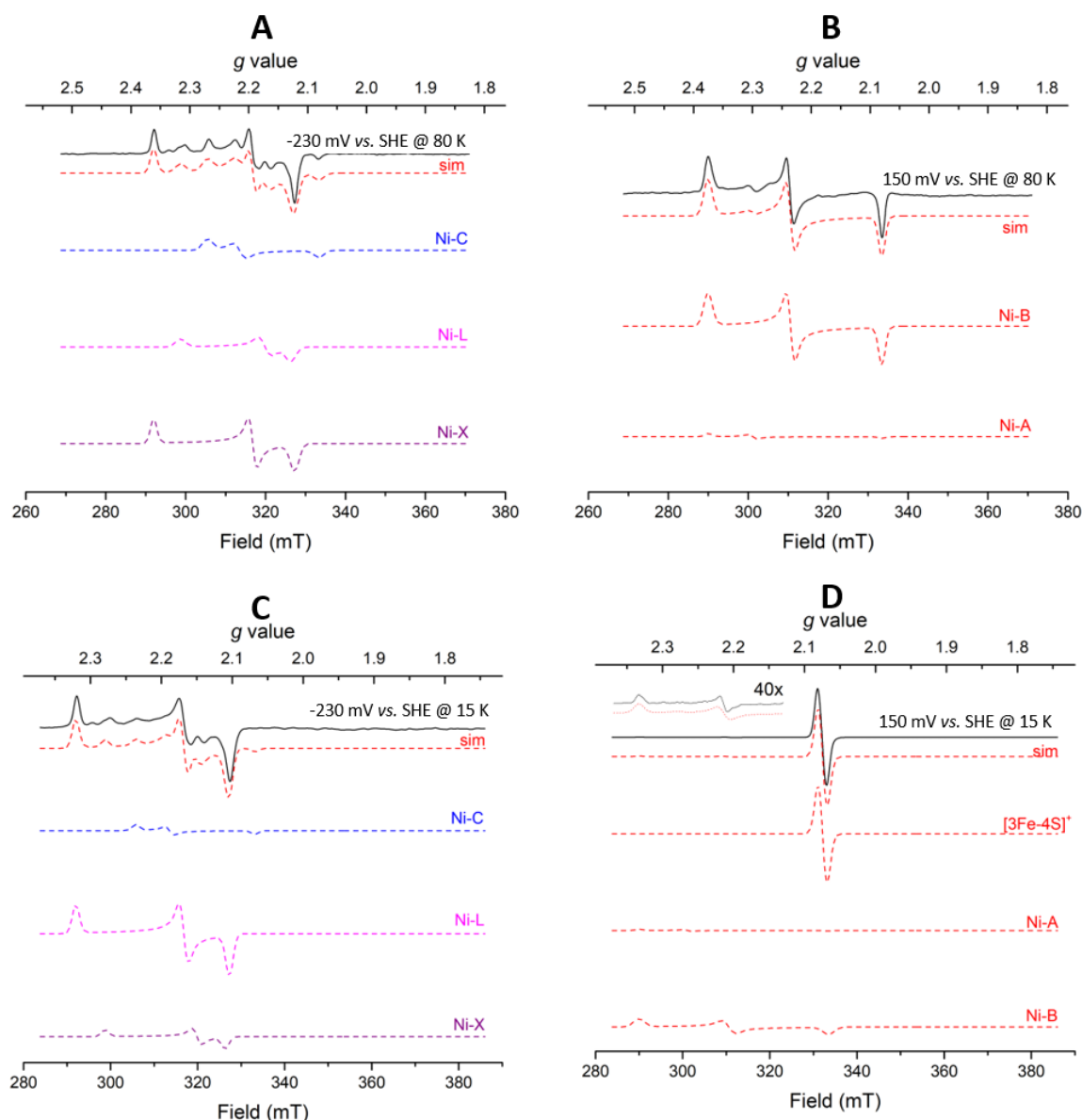


Figure 3.19: X-band continuous wave electron paramagnetic resonance (EPR) measurements of Hyd-2-NOP at 80K at -230 mV (A) and 150 mV (B) and 15K at -230 mV (C) and 150 mV (D). Microwave power 2.00 mW; modulation amplitude 1.0 mT. Protein concentration was $\sim 25 \mu\text{M}$. Figure adapted from reference.²⁷

The spectra are in good agreement with those previously reported. As expected, the inactive states, Ni-A ($g_x=2.309$, $g_y=2.225$, $g_z=2.009$) and Ni-B ($g_x=2.309$, $g_y=2.157$, $g_z=2.009$), appear as the sole signals at high temperature (80 K) under oxidizing conditions. At low temperatures under those same conditions, the spectrum is dominated by the $[3\text{Fe-4S}]^+$ signal ($g_x=2.02$, $g_y=2.017$, $g_z=2.015$). The clearest reduced signal comes from

high temperature (80 K) revealing the three reduced species: Ni-X ($g_x=2.242$, $g_y=2.095$, $g_z=2.053$); Ni-C ($g_x=2.193$, $g_y=2.137$, $g_z=2.01$); and Ni-L ($g_x = 2.293$, $g_y = 2.115$, $g_z = 2.047$). These data also further confirm that Ni-X is a signal associated with the active site, rather than an impurity or a signal from a subunit that copurified with Hyd-2-N.

The simulated signals were generated by Hyperfine Spectrum (WR Hagen Visual Software) and the sum of the individual signals also agree well with the original complete spectrum.

3.2.8 Turnover rate comparison of Hyd-2-N and Hyd-2-NOP

Because PFE of hydrogenases on PGE cannot be used to calculate rate (see Section 2.10.2), determining the turnover rate of the hydrogenase relies on conventional spectroelectrochemical assays using coloured redox dyes. For Hyd-2 the rate of H_2 oxidation is determined with benzyl viologen ($E = -358$ mV) whereas the rate of H_2 production is determined with fully reduced methyl viologen ($E = -446$) (see Section 2.6.3). Turnover frequencies obtained by conventional assays are within error for the two enzymes. Table 3.1 summarizes the enzymatic parameters of Hyd-2 N and Hyd-2-NOP showing that the two enzymes are nearly identical.⁶

3.3 Discussion

By expressing *hybO* on a plasmid, the level of hydrogenase production has increased by nearly an order of magnitude. Moreover, this yield may be close to the highest possible level of Hyd-2 production without further manipulation of the maturase protein since further genetic engineering by expressing the large subunit from a plasmid only decreases the Hyd-2 yield. This decrease in protein synthesis is likely because HybC

requires significant amounts of unique processing including the addition of carbonyls, nitriles, and nickel. This complex synthesis may drive a requirement for *hybC* to be transcribed and translated from the chromosome where it is in close proximity to the translation of its assembly proteins.^{22,23} The fact that Hyd-2 expression decreases when *hybC* is expressed from both a plasmid and the chromosome would support this reasoning. For example, immature HybC expressed from the plasmid could dimerize with mature HybO to decrease the pool of active Hyd-2 even though *hybC* expression from the chromosome remains unchanged.

Table 3.1: Summary of key enzymatic parameters for Hyd-2-N and Hyd-2-NOP. Figure adapted from reference.²⁷

	Hyd-2-N	Hyd-2-NOP
solution assay oxidation turnover frequency w/ benzyl viologen (s⁻¹)^a	204± 16	218± 17
solution assay H₂ production turnover frequency w/ methyl viologen (s⁻¹)^a	10± 3	7± 1
$K_M^{H_2}$ at -0.175 V (μM)^b	21± 4	23± 7
$\Delta H^\ddagger$ at -0.200 V (kJ mol⁻¹)	43 ± 3	47± 2
E_{switch} (mV)^c	-51± 10	-37± 27
$K_i^{H_2}$ at -0.670 V (μM)^d	219± 14	240 ± 11

All measurements done at pH 6.0 ^a Measured at room temperature ^b measured at 30°C ^c $K_i^{H_2}$ measured at 20°C.

The increased yield of Hyd-2-NOP is important for structural characterization of the hydrogenase, yet the utility of the insight gained from structural characterization depends upon the similarity between the overexpressed hydrogenase and the native enzyme. The nearly identical enzymatic characteristics of Hyd-2-NOP and Hyd-2-N

provides evidence that the overexpression system increases the yield without significantly altering the enzyme (Table 3.1).

However, there were minor changes in the shape of the CV of Hyd-2-NOP in that it cut more sharply through the zero-current axis (Figure 3.15). These minor changes observed in the behaviour of the Hyd-2-NOP on an electrode can be explained by a more homogenous enzyme sample that is missing the transmembrane domain as well as its ferredoxin partner HybA. From a Hyd-2 enzyme pool comprised of the catalytic core with variable presence of HybA, Hyd-2-N films are likely to be heterogeneous in their phenotype. Additionally, the presence of at least one TM domain from HybO (which must always be present), possibly a second TM domain from HybA (which may or may not be present for each catalytic core), and the ability for each TM domain to orientate at the electrode surface in a random fashion all contribute to an increased level of dispersion of IET in Hyd-2-N. By contrast, Hyd-2-NOP lacks the level of ambiguity of protein adsorption, and thus it only experiences dispersion from the random orientations of the adsorption of the catalytic core. Therefore, the effects of dispersion are seen to a much lesser degree. The increased effects of dispersion in Hyd-2-N may mask some features near the thermodynamic equilibrium of $H_2/2H^+$, which become clearer with Hyd-2-NOP. The effect of missing HybA, though, may not be limited to simply having a more homogenous sample. Because the potential of the entry/exit point for electrons controls the bias of the [NiFe]-hydrogenase,³⁴ the presence of HybA could slightly change the bias as well as contribute to slightly different IET. Nevertheless, the similarity between the enzymatic profiles of Hyd-2-N and Hyd-2-NOP confirm that the enzyme is active and intact.

3.4 Conclusion

The overexpression system developed here establishes that HybO availability is an important limiting factor for native Hyd-2 synthesis and maturation. Successful overexpression of Hyd-2 allows for in-depth investigation of Hyd-2-NOP and possible products of site-directed mutagenesis.

Beyond Hyd-2, successful overproduction of a single [NiFe]-hydrogenase subunit provides a new strategy for increasing yields of hydrogenase or other proteins processed by the Tat system, with obvious advantages for their biophysical and biochemical analysis. This new approach also provides easier access for mutations and other molecular biology techniques of subunits expressed on the plasmid.

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Chapter 4

Determination of a draft quaternary structure of Hyd-2

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4.1 Introduction

4.1.1 Single crystal X-ray diffraction

X-ray diffraction of single crystals is a powerful tool to interrogate the molecular structure of proteins. Molecules ranging from 10s of atoms to proteins up to 100s of kDa in size can crystallize. If the molecules in these crystals are properly ordered, they will orderly diffract X-rays in such a manner that reveals electron density. Solving the structure of small molecules with tens of atoms has been done for over 100 years, yet solving the structure of relatively complex proteins with a size above 10,000s atoms is more difficult and by comparison is a relatively recent development. The detail of the electron density maps is a function of their resolution.

Typically, resolutions better than 3.0 Å are required for solving the structure of a protein in detail. A resolution of at least 2.0 Å is required for accurately solving the position of all the sidechains in the protein. Because X-ray scattering depends on the number of electrons in an atom, larger atoms are more visible whereas smaller atoms, particularly when connected to large ones, require better resolution to distinguish. For example, in a structure with a resolution better than 1.0 Å, it is possible to begin to see the electron density of individual hydrogen atoms.

In addition to the difficulty in interpreting the electron density maps for large proteins, these proteins are less likely to crystallize. Proteins are large and flexible which makes crystallization less entropically favourable. Scientists still cannot predict ideal crystallization conditions for a given molecule. Thus, crystallization usually depends on trying a plethora of different crystallization conditions (>500 on first attempt) and refining the crystallization parameters that yield initial successes. This 'shotgun

approach' is one of the main reasons why proteins must have a high yield to be structurally characterized, yet once ideal conditions for crystallization are refined, the amount of protein required for structural characterization is minimal (possibly less than 100 μg). The likelihood of protein crystallizing is based on a variety of factors such as buffer, age of protein, history of protein, and concentration of protein particulate. These factors make it difficult to predict the ability of each batch of protein produced to crystallize.

Notwithstanding the difficulties in solving the structure of a protein from X-ray diffraction of a single crystal, attaining the structure of a protein is an important tool when characterizing an unknown protein. Solving the structure is particularly important if the protein has been subjected to site directed mutagenesis, as point mutations have the potential to destabilize the protein, create poorly matured protein, or interrupt interactions with chaperone proteins that ensure the protein fully folds. Thus, enzymatic changes like attenuated activity observed after the mutation cannot be confidently assigned to a specific mechanistic role of the mutated amino acid without ensuring that the mutation did not have a large effect on the structure.

4.1.2 Hydrogenase structures

Although the first crystallization of [NiFe]-hydrogenase occurred in 1987,¹ the first crystal structure of [NiFe]-hydrogenase was not published until 1995.² The publication reported the structure of the standard hydrogenase from *D. gigas* at a resolution of 2.85 Å and was soon followed by the structures of other [NiFe] and [NiFeSe] hydrogenases.^{3,4} Overall, the structures of [NiFe]-hydrogenases are similar across both O₂-sensitive and O₂-tolerant hydrogenases.

Typically, [NiFe]-hydrogenases are crystallized as heterodimers of the large (α) and small (β) subunits (Section 1.3.1). Membrane-bound hydrogenases, like Hyd-1 from *E. coli*, are typically crystallized as tetramers $(\alpha\beta)_2$ with the notable exception of the membrane-bound hydrogenase from *R. eutropha*, which has been crystallized as a dimer $(\alpha\beta)$,⁵ though there is evidence to suggest that it exists as a triheterodimer $(\alpha\beta)_3$ *in vivo*.⁶ Most reported hydrogenase crystal structures contain Ni-A and Ni-B states, since these oxidized states dominate proteins purified in an aerobic environment. In an attempt to gain insight into the catalytic cycle, recent efforts have focused on crystallizing the protein in reduced states either by exposing the protein to hydrogenase's natural substrate, H_2 , or by treating the crystallized protein with strong reducing agents.^{7–9} While the latter approach is likely to produce a heterogeneous mixture of states, particularly as states like Ni-B need both electrical reduction and a chemical step to enter the catalytic cycle, the former approach can be difficult for hydrogenases like Hyd-2 that are less stable over time and the resulting degradation impairs crystallization.

Nonetheless, a recent 0.89 Å reduced structure of the [NiFe]-hydrogenase from *D. vulgaris* Miyazaki F was stunning in its ability to resolve a hydride at the active site confirming the bridging ligand of Ni-R.⁹ As the identity of the catalytic states are of the utmost interest, it is important to continue to solve the structure of hydrogenases in their active states to help parse the catalytic mechanism.

4.1.3 Function predicted by structure: O_2 -tolerance

Part of the importance of solving the structure of a protein is that it gives key insight into the function and mechanism of enzymatic characteristics. For example, because the first structures of [NiFe]-hydrogenases were of standard, O_2 -sensitive [NiFe]-

hydrogenases,^{2,3} it was assumed that the small subunits and Fe-S clusters of the O₂-tolerant hydrogenases were structurally similar (see Section 1.3.1 for general introduction to the Fe-S clusters of [NiFe]-hydrogenase). However, spectroscopic data mounted that there could be an additional redox transition possible in the proximal cluster of O₂-tolerant hydrogenases that could be related to a structural shift of the proximal cluster.^{10–12} The possibility of a proximal cluster in O₂-tolerant [NiFe]-hydrogenases with unique properties was highlighted by a study showing that there are two additional cysteines that surround the structure of the proximal cluster in O₂-tolerant hydrogenases that are replaced by glycine residues in standard O₂-sensitive [NiFe]-hydrogenase. The importance of these two cysteines (originally referred to as the supernumerary cysteines) was probed by studies in which Lenz *et al.* mutated the two additional cysteines that coordinate the proximal cluster in O₂-tolerant [NiFe]-hydrogenases, C19 and C120 in *E. coli* Hyd-2 numbering (C19 and C117 in *R. eutropha* numbering), to glycine in the MBH of the *Knallgas*-bacterium *R. eutropha*. The MBH from *R. eutropha* is required for growth in the standard conditions for *Knallgas*-bacteria: a mixture of O₂ and H₂. Studies of the single and double variant proteins showed that the cysteine to glycine mutations resulted in significantly increased O₂ sensitivity. Interestingly, the *R. eutropha* strain with the C120G mutation could no longer grow on H₂ in the presence of O₂ whereas the double mutant (C19G/C120G) was still capable of severely limited growth.¹³ A separate study on the mutations of the homologous residues in Hyd-1 from *E. coli* showed that the purified cysteine to glycine variants were significantly more sensitive to exposure to air, though the resulting Hyd-1 C19G variant was less O₂-tolerant than the C19G/C120G double variant.¹⁴

The structures of the O₂-tolerant [NiFe]-hydrogenases from *Hydrogenovibrio marinus* and *Ralstonia eutropha* confirmed that O₂-tolerant hydrogenases indeed contain a unique proximal cluster.^{5,15} The structures revealed that rather than the standard cubane [4Fe-4S] cluster present in standard [NiFe]-hydrogenases, the O₂-tolerant [NiFe]-hydrogenase contained a [4Fe-3S]6cys proximal cluster.^{5,15} Rather than coordination to 4 cysteines like the [4Fe-4S] cluster from standard [NiFe]-hydrogenases, the [4Fe-3S]6cys proximal cluster is coordinated to 6 cysteines including the two cysteines previously regarded as supernumerary. One prominent change in the coordination between the two Fe-S clusters is that the [4Fe-3S]6cys lacks a sulphide found in [4Fe-4S] clusters, and that sulphide is replaced by the cysteine-S thiolate of C19 (termed S2 in Figure 4.1).

Structures solved of the [NiFe]-hydrogenase from *H. marinus* and *R. eutropha* in the chemically oxidized and H₂-reduced states also revealed that upon reduction the [4Fe-3S]6cys cluster is capable of undergoing a structural rearrangement.^{5,15} As the Fe1 atom coordinated by the sulphur atoms of C19, C20, and S2 is oxidized from Fe(II) to Fe(III), it breaks away from S2 to coordinate the backbone N of C20 (Figure 4.1).^{5,15} In Hyd-1 from *E. coli*, there is also evidence a glutamate and the Fe1 atom (E76 in Hyd-1 numbering, D81 in Hyd-2 numbering) display multiple conformations, one of which places one of the Oδ atoms within bonding distance (2.05 Å) of a population of the Fe1.⁷ The homologous change is not seen in the MBH from *R. eutropha*,⁵ but in Hyd-1 the E76 has been theorized to deprotonate amide of C20. For a population of the E76 to deprotonate the amide of C20 and then coordinate the Fe1, the proton from the amide C20 would have to be transferred away from the E76 and a proton pathway has been proposed.⁷

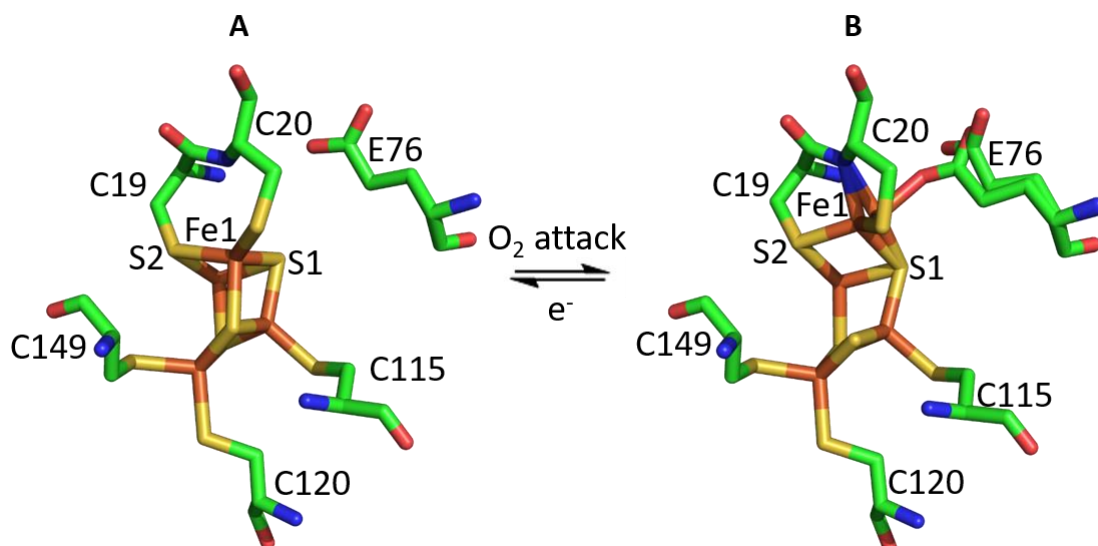


Figure 4.1: Schematic of the proximal cluster rearrangement in O_2 -tolerant *E. coli* Hyd-1. **(A)** Conformation of the H_2 -reduced proximal $[4Fe-3S]6cys$ cluster of Hyd-1 (Cys17 omitted for clarity, PDB 3UQY). **(B)** Conformation of the superoxidized proximal $[4Fe-3S]6cys$ (Cys17 omitted for clarity, PDB 3USC) cluster in Hyd-1. The superoxidized clusters show an alternate conformation in which Fe_1 is displaced towards the backbone N atom of C20 causing elongation and eventual rupturing of the Fe_1-S_2 bond. The Fe atom and E76 residue exist in two conformations: (1) the Fe coordinates the amide backbone of C20, S1, and S2 and (2) the Fe coordinates the amide backbone of C20, S1, and the $O\delta$ of E76.

Theoretical studies combined with the previous spectroscopic evidence confirmed the correlation between the structural rearrangement and the ability for the proximal cluster to undergo two redox transitions.^{7,16} The redox transition of $4+/3+$ occurs without a structural rearrangement, whereas the $5+$ oxidation state is termed the superoxidized state and requires the structural rearrangement described above.

Moreover, solving the structure of the Hyd-1 C19G variant showed that the mutation had caused the insertion of a unique $[4Fe-4S]$ instead of the $[4Fe-3S]6cys$ cluster found in Hyd-1.¹⁷ The $[4Fe-4S]$ cluster found in C19G also differs from the standard cubane $[4Fe-4S]$ cluster found in Hyd-2 as it is coordinated by 5 cysteines.¹⁷ This $[4Fe-4S]$ cluster is not able to form the superoxidized state and is therefore incapable of the rapid sequential donation of two electrons to the active site that occurs in the native enzyme. The lack of the $[4Fe-3S]6cys$ cluster conferred the Hyd-1 C19G variant with a greater

sensitivity to O₂ than standard [NiFe]-hydrogenase like Hyd-2.¹⁸ These efforts have shown success at making the proximal cluster of O₂-tolerant [NiFe]-hydrogenases more like the proximal cluster found in standard [NiFe]-hydrogenases. Unfortunately, attempts to make the inverse mutations (G24C/G125C) in Hyd-2 numbering have been unsuccessful,¹⁹ and structural characterization of Hyd-2 could help elucidate the exact coordination around the proximal Fe-S cluster.

Another postulated defence against inactivation by O₂ attack is dimerization ($\alpha\beta$)₂. After oxidative inactivation, the hydrogenase must regain electron supply to convert the hydrogenase from an oxidized inactive state into one that is catalytically active. Solving the structure of Hyd-1 revealed that the distance between the two distal clusters of separate ($\alpha\beta$) heterodimers is 12.4 Å, which could allow for rapid electron transfer between the two dimers.^{7,17,18} This mechanism suggests the potential for 'rescue' between the two active sites in the case that one is active and the other is inactive.⁷ Further, purifications of Hyd-1 were able to separate the heterodimer ($\alpha\beta$) and the tetramer ($\alpha\beta$)₂, and when tested showed evidence that the tetramer ($\alpha\beta$)₂ was better capable of withstanding oxygen attack than the heterodimer alone.²⁰ This data confirms that the structure of a hydrogenase is important in determining key enzymatic characteristics.

4.1.4 Aims of this chapter

This chapter details the structure of the catalytic core of Hyd-2 with a focus on the coordination of the proximal cluster. The implications of the findings on the enzymatic characteristics of Hyd-2 are discussed as well as the implications on a model of the entire Hyd-2 enzyme.

4.2 Results

4.2.1 Crystallization conditions for Hyd-2

Initially, Hyd-2 NOP was set up for crystallization in a variety of buffers and pH values at two different concentrations (5 mg/mL and 8 mg/mL). These first plates were seeded by an automated system (Mosquito robot, TTP labtech, UK), which facilitated the screening of nearly 1000 different crystallization conditions. Within 24 hours, crystals of Hyd-2 NOP in 5 different conditions grew, yet the crystals that best diffracted came from protein at 5 mg/mL grown in a mixture of Bis-tris (100 mM, pH 5.5), MgCl_2 (200 mM) and PEG 3350 (25% w/v). Subsequent crystallization trials came from slight alterations of those crystallization conditions and usually involved altering the pH (+/- 0.2 pH units) and PEG 3350 concentration (19%-22% w/v). These conditions consistently grew Hyd-2 crystals within 24 hours for most preparations of Hyd-2 NOP.

4.2.2 Structure of Hyd-2

Hyd-2 NOP was first crystallized in the as-isolated condition and the protein came from aerobic purification by HisTrap column and SEC followed by crystallization in semi-aerobic conditions (Section 3.2.3). After setting up the crystal screening trays for the as-isolated protein, they were taken into anaerobic glove boxes and allowed to briefly equilibrate in anaerobic conditions before being sealed, and the plates were placed in temperature regulated, aerobic conditions. These semi-anaerobic conditions of crystallization did not change the states of the as-isolated enzyme but helped prevent total degradation of the protein during crystallization.

Crystal trays in subsequent experiments were set up by hand and grown in an anaerobic glove box to prevent degradation as well as to isolate them in reduced states

(Section 2.8.1). Some crystals were chemically reduced by being soaked in a cryoprotection solution with 5 mM DTT to reduce them before being flash frozen. In other crystallization trials Hyd-2 NOP was treated with 12 h of 100% H₂ prior to crystallization. Each protein treatment slightly changes the optimal crystallization conditions, and so for each protein treatment, 24 crystallization conditions within the above parameters were set up to find the ideal condition for that particular protein. The growth of reduced crystals required seeding of the crystallization drops using the streak seeding method with aerobic crystals as the source of the seeds, but because X-ray diffraction presents an average of the molecular positions, the effect of the seeding on the final structure is negligible. The x-ray data collection and refinement statistics for the structures are in Table 4.1 (see Section 2.8 on how the structures were solved by Dr. Steve Carr).

Table 4.1: X-ray data collection and refinement statistics for Hyd-2-NOP. Dr. Steve Carr solved all structures.

Sample	As-Isolated/aerobic	Chemically Reduced	Hydrogen Reduced
Data Collection			
PDB ID	6EHQ	6EHS	6EN9
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions (Å)	a=99.06, b=100.09, c=168.14	a=99.89, b=100.07, c=168.80	a=99.60, b=100.54, c=168.58
Resolution (Å)	85.4 – 2.2 (2.24-2.10)	65.2- 1.5 (1.53-1.50)	86.35-1.5 (1.53-1.50)
Total reflections	549,679 (27,487)	2,394,760 (111,154)	1,380,818 (74,652)
Unique reflections	84,222 (4,242)	269,411 (12,174)	255,707 (13,573)

Completeness (%)	98.7 (95.5)	100 (99.9)	99.2 (98.2)
Multiplicity	6.5 (6.5)	8.9 (8.4)	5.4 (5.5)
$\langle I/\sigma \rangle$	6.4 (1.7)	11.2 (1.4)	10.9 (1.6)
R_{merge} (%)	19.0 (98)	10.8 (145)	7.3 (85.8)
R_{pim} (%)	12.0 (62.9)	5.6 (79.5)	3.9 (43.9)
$CC_{1/2}$	0.978 (0.681)	0.998 (0.554)	0.998 (0.714)
Refinement			
$R_{\text{work}}/R_{\text{free}}$ (%)	16.3/18.9	15.6/18.4	15.2/17.5
No. of atoms	13,242	13,570	13,903
Macromolecule	12,718	12,723	12,723
Solvent	524	847	1,180
Average B-factors			
Macromolecule	30.6	19.85	19.28
Solvent	29.2	24.26	25.7
RMSD bond lengths (Å)	0.0128	0.0167	0.017
RMSD angles (°)	1.53	1.72	1.78
Ramachandran plot (%) favoured / outliers	96.3 / 0	96.3 / 2	96.3 / 0
Clashscore	0.44	1.57	1.42

Values in parenthesis refer to data in the highest resolution shell. See Section 2.8 for more information about R_{merge} , R_{meas} , R_{pim} , R_{work} , R_{free} , and $CC_{1/2}$.

4.2.3 Overall structural arrangement

The structure of Hyd-2-NOP was solved to a maximum resolution of 1.5 Å (reduced) or 2.2 Å (as-isolated) (Table 4.1). As previously predicted by native PAGE and AUC measurements,²¹ the catalytic core of Hyd-2 is a dimer of heterodimers $(\alpha\beta)_2$ similar to that observed for almost all membrane-bound [NiFe]-hydrogenases (Figure 4.2).

Moreover, the extensive interface between the two dimers would suggest that this arrangement is not a crystallographic artefact but instead is its native arrangement.

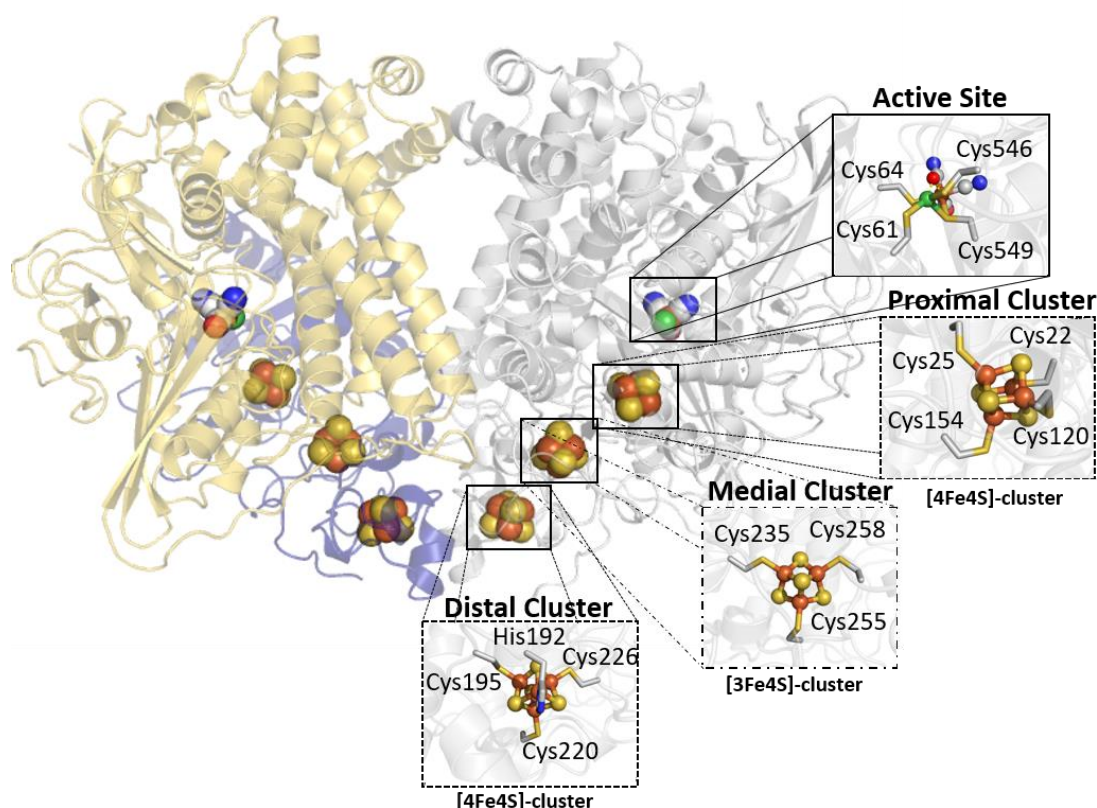


Figure 4.2: Structure of the catalytic core of Hyd-2-NOP. In one dimer the small subunit (HybO) is coloured blue and the large subunit (HybC) gold, in the second both subunits are grey to accentuate the position of the metals centres, (displayed as spheres with Fe-S clusters coloured bronze/yellow and the [NiFe] centre bronze/green). Panels show the coordination of each metal centre. Figure adapted from reference.²²

The interface between the dimer of heterodimers ($\alpha\beta$)₂ is large, burying over 2678 Å² of the solvent accessible surface. The dimer interface is stabilized by 17 salt bridges and 16 hydrogen bonds. Further analysis by size exclusion chromatography-multi angle light scattering (SEC-MALS) shows the prevalence of the ($\alpha\beta$)₂ conformation of Hyd-2-NOP in solution. Even in a buffer that contains detergent, which stabilizes hydrophobic patches like those found in between the dimer interface, the clear majority of the enzyme still exists in the ($\alpha\beta$)₂ conformation (Figure 4.3).

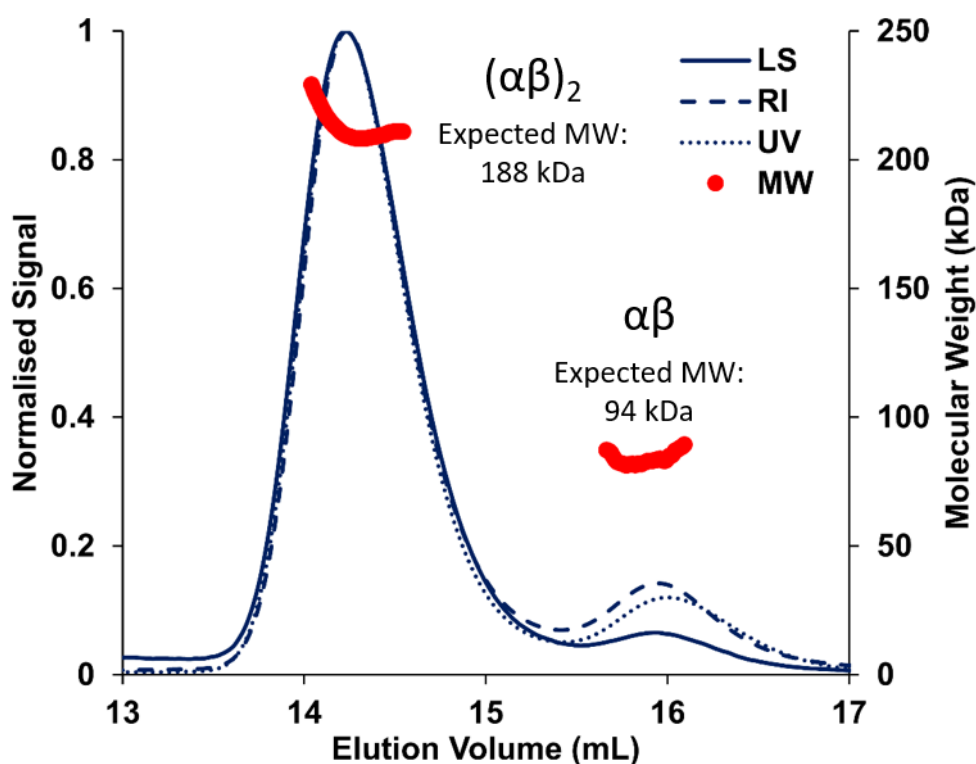


Figure 4.3: SEC-MALS of Hyd-2-NOP in buffer containing detergent (Tris-HCl 20 mM, NaCl 150 mM, DTT 1 mM, DDM 0.02%, pH 7.2). LS-laser scattering, RI-refractive index, MW-molecular weight. Data collected by Dr. Gemma Harris. Figure adapted from reference.²²

Analysis of HybOC²³ by the DALI server (http://ekhidna.biocenter.helsinki.fi/dali_server/) comparing Hyd-2 and other proteins shows that the hydrogenase exhibits an oxidoreductase fold that is very similar to [NiFe]-hydrogenases from a range of organisms. Both copies of HybC show electron density for residues 2-552 of 552. Although *hybC* encodes 567 amino acids, residues 553-567 are cleaved in post-translational modification after proper assembly.²⁴ The C-terminal carboxylate group on His552 is clearly visible in the electron density signifying that HybC of Hyd-2 NOP is fully processed.

Both copies of HybO in the asymmetric unit show electron density for residues 10-276 of 299 (294-299 are His-tag). Further testing determined that the His-tag on HybO was still present in the crystals of Hyd-2 NOP (Figure 4.4). This result confirms that

residues 277-299 were still present in the crystal, suggesting that residues 277-299 are flexible and disordered in the crystal lattice.

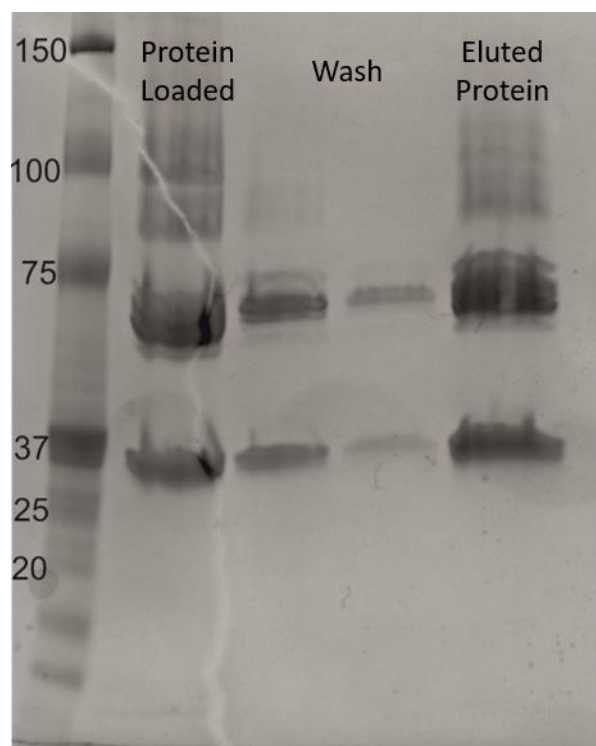


Figure 4.4: Approximately 20 crystals of Hyd-2-NOP were looped and washed in crystallization buffer before being dissolved in water. Dissolved crystals were mixed with 20 μ L Ni sepharose beads pre-equilibrated with 20 mM tris pH 7.2, 350 mM NaCl, 60 mM Imidazole. The beads were washed twice with the same buffer before protein was eluted with 20 mM tris pH 7.2, 350 mM NaCl, 500 mM imidazole. Fractions from each step were analysed by SDS-PAGE. SDS-PAGE gel performed by Dr. Steve Carr (Research Complex at Harwell). Figure adapted from reference.²²

4.2.4 Large subunit

The Ni atom of the of [NiFe] cofactor contained in each HybC is terminally coordinated by two cysteine thiolate ligands (Cys61 and Cys546) and coordinated to the Fe by two cysteine thiolate ligands (Cys64 and Cys549). The Fe atom is further coordinated by small inorganic ligands (2 CN^- and CO) (Table 4.2, Figure 4.2). The active site is structurally identical to that of Hyd-1, and to almost all [NiFe]-hydrogenases, with similarity extending into the ‘canopy’ residues (Asp103, Arg479, and Asp544) that form

part of the secondary coordination sphere. For the as-isolated enzyme, there is additional electron density around the S-atom of Cys546 which indicates partial oxidative damage.

Table 4.2: Table of distances of active site atoms for Hyd-2 NOP in both subunits (termed L and M).

Hyd-2 NOP As Isolated										
	Residue	D103		D544			Ni	Fe	CN	OH
	Atom	Od1	Od2	Od1	Od2	-CO				
R479	N η 1 (L)	3.35	3.06	4.52	4.04	5.18	4.58	4.40	3.18	3.26
	N η 1 (M)	3.38	3.09	4.49	4.05	5.16	4.55	4.34	3.21	2.99
	Average	3.37	3.08	4.51	4.05	5.17	4.57	4.37	3.20	3.13
	N η 2 (L)	2.72	3.86	2.79	3.14	4.58	5.66	6.00	5.42	4.71
	N η 2 (M)	2.70	3.83	2.79	3.16	4.56	5.66	6.24	5.41	4.38
	Average	2.71	3.85	2.79	3.15	4.57	5.66	6.12	5.42	4.55
	Ne (L)	4.61	5.20	2.93	3.14	2.87	5.03	5.01	4.37	3.70
	Ne (M)	4.63	5.19	2.93	3.14	2.86	5.06	5.00	4.34	3.39
	Average	4.62	5.20	2.93	3.14	2.87	5.05	5.01	4.36	3.55
		D103	Od1 (L)	4.72	4.06	6.97	7.32	7.65	6.00	6.40
			Od1 (M)	4.72	4.13	6.97	7.28	7.60	6.09	6.15
			Average	4.72	4.10	6.97	7.30	7.63	6.05	6.28
			Od2 (L)	6.40	5.62	7.97	6.41	6.48	4.90	5.59
			Od2 (M)	6.35	5.60	7.95	6.42	6.46	4.98	5.47
			Average	6.38	5.61	7.96	6.42	6.47	4.94	5.53
					D544	Od1 (L)	7.53	7.93	7.01	6.47
						Od1 (M)	7.54	7.92	7.02	6.13
						Average	7.54	7.93	7.02	6.30
						Od2 (L)	8.03	7.73	6.01	6.66
						Od2 (M)	8.05	7.73	6.05	6.36
						Average	8.04	7.73	6.03	6.51
						-CO (L)	6.91	6.85	6.57	5.77
						-CO (M)	6.93	6.88	6.56	5.48
						Average	6.92	6.87	6.57	5.63
	S-Cys 61	S-Cys 546	S-Cys 64	S-Cys 549						
	2.22	2.17	2.53	2.5		Ni (L)		2.95	5.16	1.72
	2.21	2.19	2.52	2.47		Ni (M)		2.85	5.06	1.90
	2.22	2.18	2.53	2.49		Average		2.90	5.11	1.81
			2.34	2.35		Fe (L)			3.02	1.99
			2.30	2.32		Fe (M)			2.95	2.18
			2.32	2.34		Average			2.99	2.09
									CN (L)	3.62
									CN (M)	3.53
									Average	3.58

At each active site there was obvious electron density between the Ni and the Fe, which is typical as the bridging position is occupied in almost every state of catalysis. The

as-isolated enzyme was purified and crystallized aerobically, and thus the enzyme was likely in a mixture of Ni-A and Ni-B states. The size of the electron density in the bridging position and its position relative to the Ni and Fe atoms were consistent with, and assigned as, a hydroxide. The bridging hydroxide was also visible in the chemically reduced protein, which would be expected as full removal of the bridging hydroxide requires both reduction potential and protons.

The H₂-reduced structure shows that the [NiFe] cofactor experiences a slight contraction upon reduction. The contraction is almost entirely due to the movement of the Ni atom, which, in turn, pulls in the terminal ligating thiolates (Cys61 and Cys546) to decrease the distance between the Ni and Fe from 2.94 Å in the as-isolated structure to 2.66 Å in the reduced structure (Figure 4.5). This contraction coincides with a shift that shortens the distance between the active site and Glu14, which has been shown to be essential for proton transfer away from the active site.²⁵

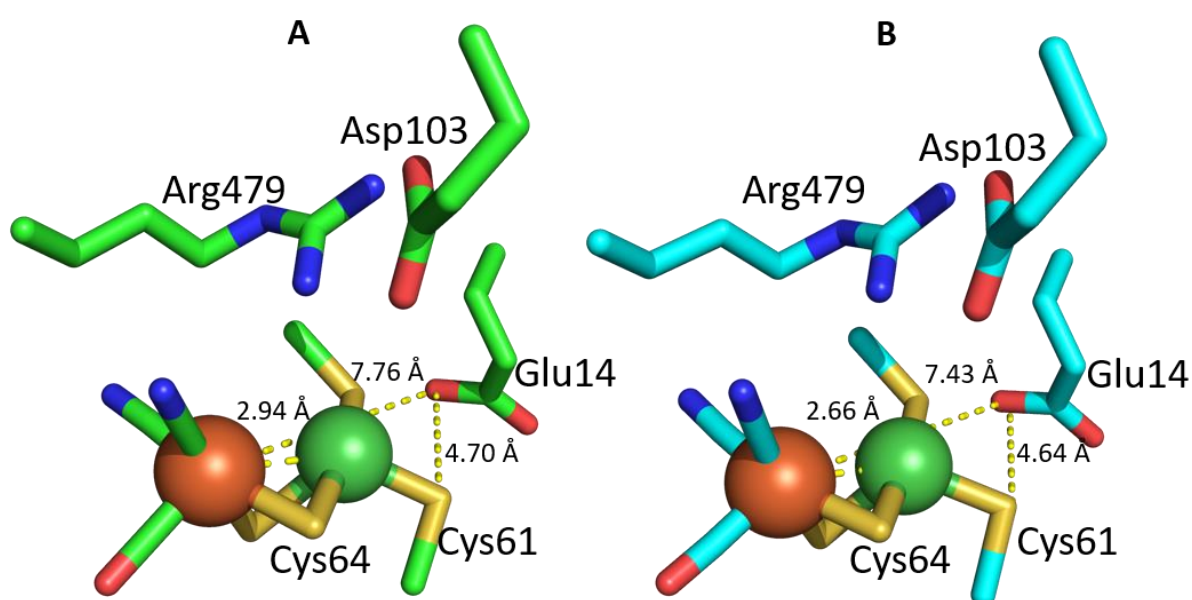


Figure 4.5: Comparison of (A) as-isolated and (B) H₂ reduced active sites including Glu14 which has been shown to be essential for proton transfer in [NiFe]-hydrogenase.²⁵ The contraction of the active site also causes the Glu14 to draw closer to the active site. Bridging electron density has been omitted for clarity.

Moreover, for the reduced structure, the electron density observed in one of the active sites is consistent with a hydride that overlays well with the bridging hydride in the Ni-R form of *D. vulgaris* Miyazaki F (PDB 4U9H).⁹ This electron density is centred between the two metals of the [NiFe] cofactor—1.8 Å away from the Fe and 1.7 Å away from the Ni (Figure 4.6). Additionally, there is residual electron density in the other active site of the reduced structure, yet the centre of the electron density is farther away from the Fe atom (2.4 Å) and closer to the Ni atom (1.6 Å) than would be expected for Ni-R. Like all membrane-bound [NiFe]-hydrogenases, each HybC contains a structural magnesium ion coordinated by His552 (end of C-terminal), Glu42, and the main chain carbonyl of Ala498. For the chemically reduced structure, there was evidence of oxidative damage on Cys546. However, the hydrogenase reduced by H₂ showed no evidence of oxidative damage on any cysteine sulphurs at the active site.

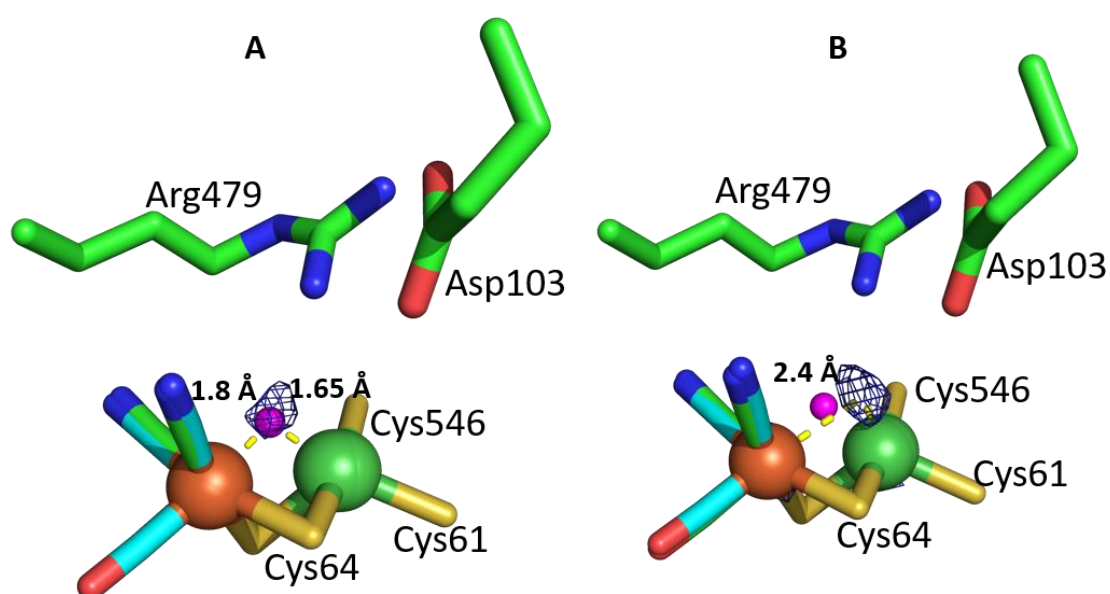


Figure 4.6: Comparison of the active sites in Hyd-2 after hydrogen reduction. Panel A shows the superposition of the Ni-R active site from *D. vulgaris* Miyazaki F (cyan) and Hyd-2 (green). The bridging hydride is centred between the two metals and within a peak of Fo-Fc difference density, suggesting the active site of Hyd-2 is also in the Ni-R state. Panel B shows that the active site of the second HybOC dimer from the hydrogen reduced structure of Hyd-2 also contains Fo-Fc density between the metals, but it is shifted towards the Ni atom such that its centroid is 2.45 Å from the Fe and 1.6 Å from the Ni.

4.2.5 Small subunit

Each Fe-S cluster of Hyd-2 is located at a maximum of 10.7 Å from its electron transfer partners, which allows rapid electron transfer between the active site and the surface of the protein. Moreover, the distal clusters of the two $\alpha\beta$ dimers are within 12.3 Å of each other suggesting that electron transfer could occur between the two dimers. The coordination of the proximal Fe-S cluster is highlighted in Figure 4.7. As expected, the proximal cluster of Hyd-2 is a standard cubane, though additional features were visible in the 2Fo-Fc map around the proximal cluster of the as-isolated enzyme.

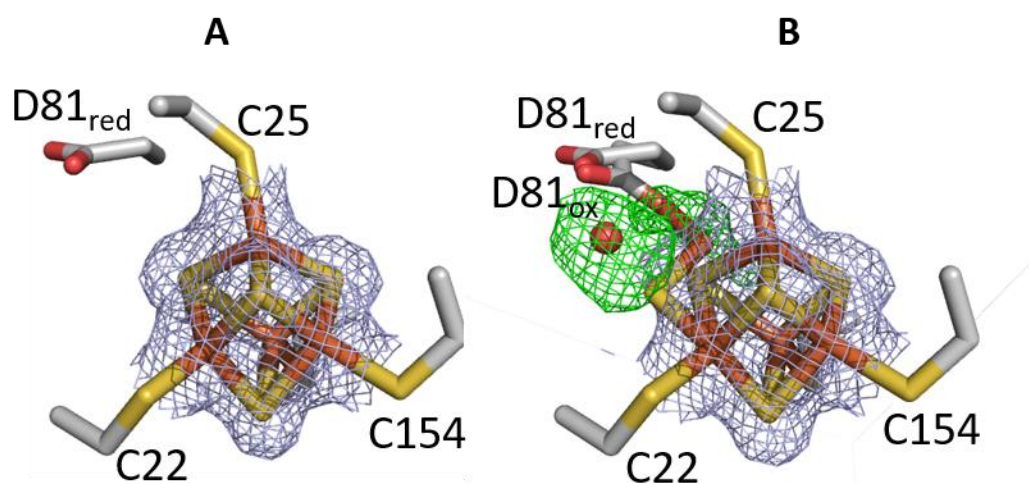


Figure 4.7: Structure of the proximal [4Fe-4S] cluster of HybO in the (A) reduced and (B) oxidative damage conformations with blue density showing modelled density and the green density showing electron density that is present yet not explicitly modelled in the initial protein model. After oxidative damage, the cluster adopts an alternative conformation in which the Fe typically coordinated by Cys25 moves away from the cluster. Rotation of nearby Asp 81 allows the carboxylate group to coordinate the shifted Fe, and an additional water molecule (red sphere) is also observed next to the damaged cluster.

The shift in electron density around the proximal cluster was due to the movement of an Fe atom in the proximal cluster which was modelled as oxidative breakdown of the cluster similar to that observed in the [NiFe]-hydrogenases from *A. vinosum* and *D. desulfuricans*.^{26,27} In this breakdown, the Fe moves away from its coordination with Cys25, and a nearby aspartate residue (Asp81) rotates to coordinate

the Fe in its new position. The relative intensities of the peaks in the anomalous difference map for the proximal cluster shows that approximately 50% of the as-isolated protein in the crystal is in the alternate configuration.

4.3 Discussion

4.3.1 Possible role of D81

The alternative proximal cluster configuration has been observed previously,²¹ and sequence alignment of group 1 hydrogenases reveals an aspartate, glutamate or asparagine residue is conservatively substituted in position 81 (Figure 4.8). This proximal cluster shift has also been observed in addition to the more complex shift in O₂-tolerant hydrogenases (see Section 4.1.3).⁷ The repositioning of the acidic side chain could represent a rudimentary form of oxygen tolerance since the aspartate prevents the loss of Fe from the vicinity of the proximal cluster upon oxidative damage, and addition of reducing equivalents allows reconstitution of the [4Fe-4S] cluster. However, preliminary characterization of a variant designed to test the importance of D81 in Hyd-2 (termed Hyd-2 D'81N-OP, apostrophe indicates Hyd-2 variants with mutations in the small subunit) displayed a similar ability to reactivate from O₂ attack as the native enzyme (Appendix A).

Standard membrane bound [NiFe]-hydrogenases	
	81
<i>E. coli</i> Hyd2 (1c)	NALEKYKGQYVLVVDGSIPLKDN---GIYCMVAG-----EPIVDHIRKAAEGAAA
<i>A. vinosum</i> (1e)	QAMDENRGQYLVIVDGSIPGPDAN--PGFSTVAG-----HSNYSILMETVEHAAA
<i>D. fructosovorans</i> (1b)	QALEGKDG-YYLVEGGGLPTIDG---QWGMVAG-----HPIETTKKAAAKAKG
<i>D. gigas</i> (1b)	EAIKG--D-FVCVIEGGIPMGDG---GYWGVGR-----RNMYDICA EVAPKAKA
<i>D. vulgaris</i> MF (1a)	QAVNSPHG-FIAVVEGGIPTAAN---GIYGVAN-----HTMLDICSRLPKAQA
<i>R. eutropha</i> Actinobacterial-type (1h)	KAARGEIDNFEVLVLEGSIPNERINGEGYWAAMGT---DPQTHQPITIPWLDRLAPKALA
O ₂ tolerant membrane-bound [NiFe]-hydrogenase	
<i>E. coli</i> Hyd-1 (1d)	DIITQYNGKYILAVEGNPPLGEQ---GMFCISSG-----RPFIEKLKRAAGASA
<i>S. typhimurium</i> Hyd5 (1d)	DVMREYKGN YIVAVEGNAPLNED---GMFCILAG-----EPFLEKLKRV SADAKA
<i>H. marinus</i> (1d)	EIKEKYKGN YILAVEGNPPLNQD---GMSCIIGG-----RPFSEQLKRMADDAKA
<i>R. eutropha</i> MBH (1d)	EIMTKYKGN YILAVEGNPPLNQD---GMSCIIGG-----RPFIEQLKYVAKDAKA
<i>A. aeolicus</i> (1d)	RIIKEYWGN YILAVEGNPPLGED---GMYCIIGG-----RPFVEILKESAEAGAKA
[NiFeSe]-hydrogenase	
<i>D. baculatum</i> NiFeSe (1a)	EIAEKFN GNFFLLVEGA IPTAKE---GRYCIVGETLDAKAHHHEVTMMELIRDLAPKSLA
<i>D. vulgaris</i> Hborough NiFeSe (1a)	KVAEKFKGKFFLVIEG SVPEAD---GKYCIIGE-----ANHHEISMVDALKEFGPNAAA
	. : : * *

Figure 4.8: Sequence alignment (Clustal Omega) of the small subunits of selected [NiFeSe]-hydrogenases, highlighting the conservation of D81 at the equivalent position. The subgroup to which each hydrogenase is categorized is given in brackets. Figure adapted from reference. ²²

4.3.2 Comparison of Hyd-1 and Hyd-2

Hyd-2 has an active site that is very similar to Hyd-1, implying the active site architecture of Hyd-1 is indeed poised to allow both oxidation and production of hydrogen and that the driving force that dictates which reaction occurs comes from elsewhere, for example, the potential of the quinone pool or Fe-S clusters. Moreover, the similarity of the positioning of the active site ‘canopy’ suggests their continued importance in Hyd-2. Perhaps the most prominent change in the canopy between Hyd-1 and Hyd-2 is the positioning of D103/D118. In Hyd-1, D118 is extended towards the pendant arginine drawing the O δ 2 atom closer to the active site and pendant arginine while maintaining O δ 1 in a similar position to D103 (Figure 4.9 and see Chapter 6 for an in-depth discussion of D103).

The cysteine that experiences oxidative damage is also different between Hyd-1 and Hyd-2 (Figure 4.9). It is common for one of the sulphur atoms ligating the Ni to exhibit oxidative damage, and typically that oxidative damage occurs on either the ligating

sulphur atom^{8,26,28} or the terminal sulphur.²⁷ Although there is still dispute about the chemical significance of Ni-A, oxidative damage to a terminal cysteine is likely one of the aspects of oxidative damage that forms the more recalcitrant Ni-A.^{8,29} In Hyd-2, this damage occurs at C546 whereas in Hyd-1 the oxidative damage occurs at C79 (equivalent to C64 in Hyd-2). While this distinction may seem trivial, the sulphur that experiences oxidative damage could reveal subtle nuances in the mechanism and where greater electron density is localized during mechanistic steps. The location of the oxidation may also be relevant given that the sulphur of C546 is theorized to be the base that reacts with the [NiFe] cofactor during H₂ activation.^{9,30}

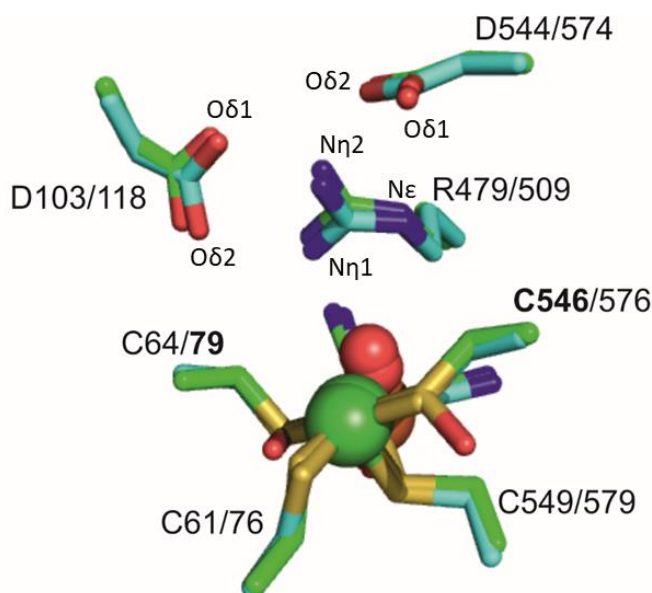


Figure 4.9: Overlay of the active site 'Canopy' region of Hyd-2 (green) and Hyd-1 (cyan). Hyd-2-Numbering of residues is shown first for each residue. Figure adapted from reference.²²

Another large difference between Hyd-1 and Hyd-2 is the presence of the special [4Fe-3S]6cys cluster in Hyd-1 and other similar O₂-tolerant [NiFe]-hydrogenases. The special [4Fe-3S]6cys cluster in O₂-tolerant [NiFe]-hydrogenases is coordinated by six cysteines (Figure 4.1), four of which are present in Hyd-2 (Cys22, Cys25, Cys120 and Cys154) while the two 'supernumerary' cysteines (Cys 19 and Cys120 in Hyd-1) are

replaced by glycine (G24 and G125 in Hyd-2) in their standard hydrogenase [4Fe-4S] counterparts. The C19G and C120G mutations resulted in decreased O₂-tolerance for Hyd-1.¹⁴ However, attempts to increase the O₂-tolerance of standard [NiFe]-hydrogenases by mutating the conserved glycine residues to cysteine residues to incorporate the special [4Fe-3S]6cys proximal cluster into the standard [NiFe]-hydrogenase from *D. fuctosovorans* have been unsuccessful.¹⁹ Moreover, a variant strain of Hyd-2 G'24C/G'125C from mutations in FTH013 (native expression of Hyd-2 from the chromosome) resulted in no purified enzyme.¹⁹ If these mutations had been successful at inserting the [4Fe-3S]6cys cluster into Hyd-2, the result could have been an O₂-tolerant reversible hydrogenase with important implications on the possibility of engineering [NiFe]-hydrogenases. Thus, it is important to understand why the G'24C/G'125C mutations led to no protein expressed. An overlay of the proximal clusters of Hyd-1 and Hyd-2 shows that the backbone of G24 and G125 in Hyd-2 occupy nearly identical positions as C19 and C120 in Hyd-1 (Figure 4.10).

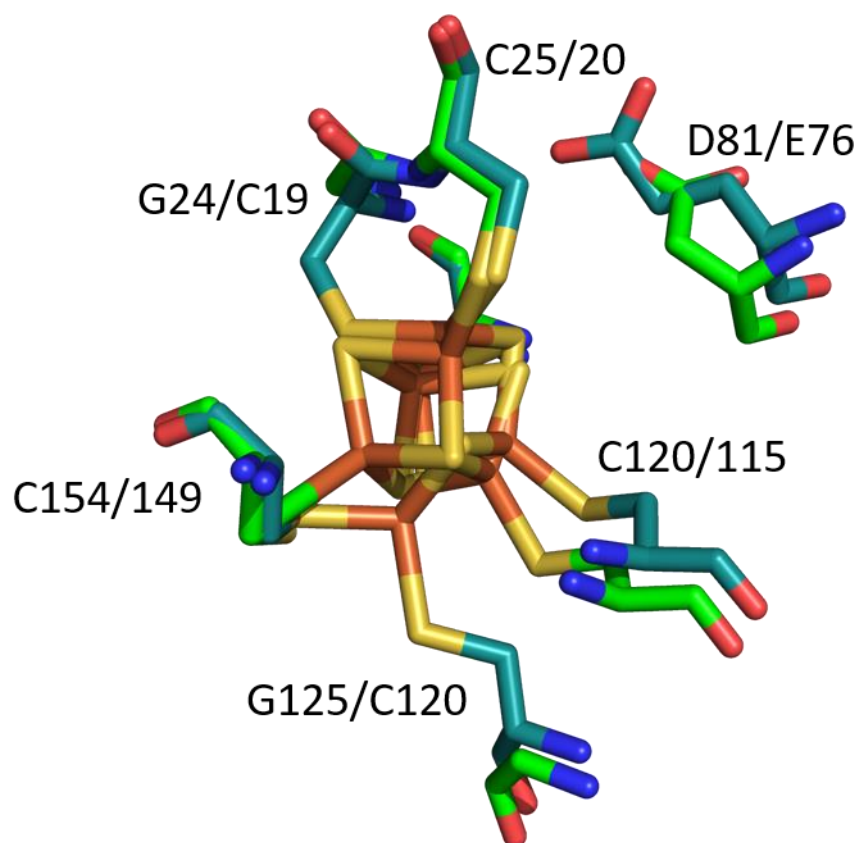


Figure 4.10: Overlay of the reduced proximal cluster from Hyd-1 (cyan) and Hyd-2 (green). The 6th cysteine (Cys17) that coordinates the proximal cluster in Hyd-1 has not been labelled but is shown in the figure. The overlay includes residue that coordinates the proximal clusters as well as D81 that has been shown to coordinate the clusters upon oxidative breakdown. The residues are labelled with Hyd-2 numbering followed by Hyd-1 numbering.

Figure 4.10 also shows that the largest difference in the residues of the regions around the proximal cluster is the positioning of D81 in Hyd-2 (E76 in Hyd-1). However, the lack of expression of the G'24C/G'125C variant protein in *D. fructosovorans*, which conserves a glutamate in the position occupied by E76 in Hyd-1, shows that a glutamate at the position does not necessarily enable production of the glycine to cysteine variant. Overall, the overlay of the structures of Hyd-1 and Hyd-2 suggests that there is no clear structural reason why the G'24C/G'125C variant is not produced, but attempts to produce the variant in the Hyd-2 overproduction system resulted in no protein purified (Appendix A).

The full proteins of Hyd-1 and Hyd-2 are also very similar and when the protein backbones are superimposed upon each other, there are only a few areas of apparent difference between Hyd-1 and Hyd-2. The most obvious difference is that Hyd-1 is slightly larger and therefore contains slightly elongated loops around the periphery of the protein (Figure 4.11). This is typical for homologous proteins that differ in length because changes in the surface loops of the protein are less likely to affect the core function of the protein. Moreover, changes to the periphery of the protein could be driven by the need to interact with other protein subunits specific to each protein.

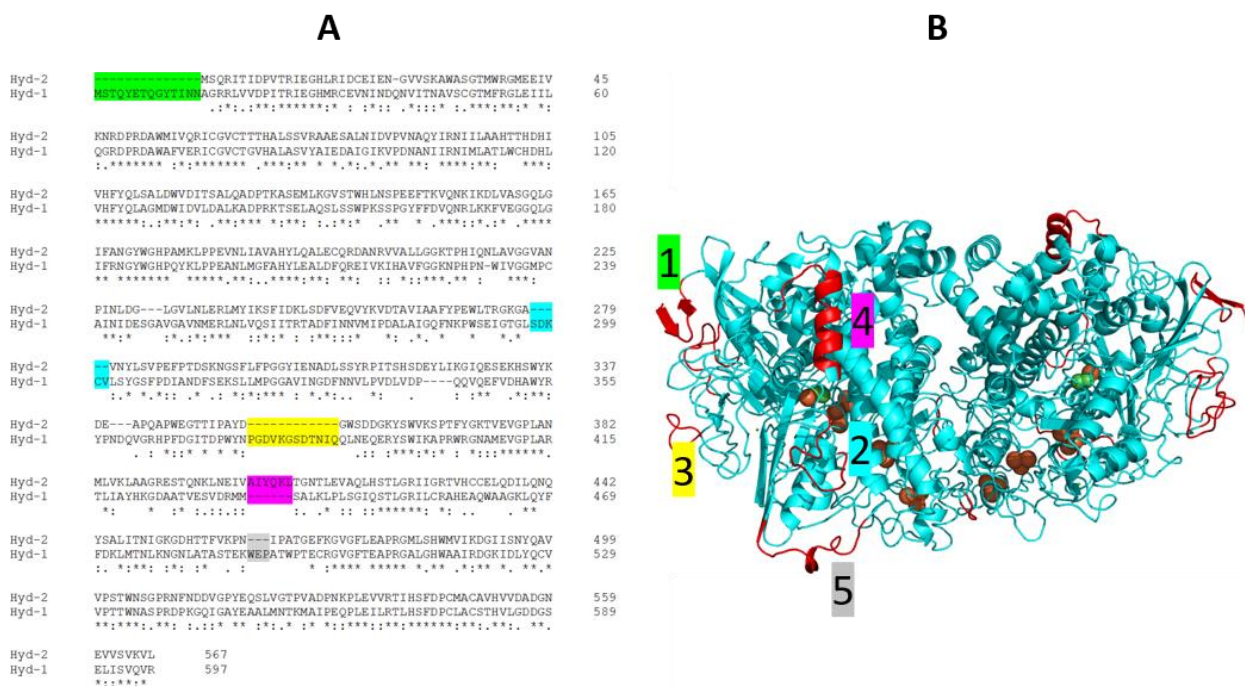


Figure 4.11: Comparison of the structures and sequences of Hyd-1 and Hyd-2. **(A)** Sequence alignment of Hyd-1 and Hyd-2 with the significant external loops that appear in Hyd-1 but not in Hyd-2 are highlighted. The colour corresponds to the loops numbered in the **(B)** superposition (using secondary structure motifs) showing the polypeptide backbones of the two proteins overlay with an r.m.s.d. of 1.2 Å. Differences in the structure and sequences in the large subunit are shown in Hyd-1 with loops coloured red and correspond to the sequence alignment.

4.3.3 Dimeric configuration with in vivo implications

The dimer of heterodimers configuration of the large and small subunits ($\alpha\beta$)₂ seen in Hyd-2 has previously been observed with O₂-tolerant hydrogenases as well as the standard O₂ sensitive hydrogenase from *A. vinosum*.^{20,26} The free energy of dissociation (ΔG^{diss}) of the ($\alpha\beta$) heterodimer in Hyd-2 was calculated using PISA³¹ and found to be approximately 44.0 kcal/mol, whereas the ΔG^{diss} of the ($\alpha\beta:\alpha\beta$) dimer of heterodimers was 23.0 kcal/mol. These calculations represent a lower limit based on the interactions seen in the crystal structure. Given this figure, the equilibrium constant for dimerization of the $\alpha\beta$ heterodimers would be approximately 10¹⁷—a number that would suggest that Hyd-2 always exists in a dimer of heterodimers configuration. Moreover, the SEC-MALS results of Hyd-2-NOP in the presence of detergent that should stabilize the monomeric HybOC heterodimer form provide compelling support for the inherent stability of the ($\alpha\beta$)₂ dimer and suggest that it persists in the in vivo structure.

4.3.4 Possible quaternary configuration

An intact HybOC-AB enzyme has never been isolated, suggesting protein-protein associations beyond the catalytic core are more labile than HybOC interaction. From the calculations above, the primary stabilizing force of Hyd-2 is the interaction between HybO and HybC followed by the dimeric interaction ($\alpha\beta:\alpha\beta$). Although the crystal structure presented here represents only the catalytic core of Hyd-2, it provides insight into how the quaternary structure of the HybOC-AB complex could be assembled in the bacterial membrane. Homology modelling was performed to produce structures for HybA and HybB to help construct a model for the complete HybOC-AB complex.

The models were based on protein templates that share important characteristics with Hyd-2. Most importantly, they were the proteins with the highest degree of sequence similarity with the respective subunits of Hyd-2, and they also come from membrane-anchored, multi protein complexes. Thus, the utility of the template extends beyond the structure of HybA and HybB to the orientation of HybA and HybB relative to the membrane and HybOC heterodimer. The model for HybA was FdnH, which is the ferredoxin subunit of formate dehydrogenase (PDB 1KQF).³² Like HybA, FdnH contains 4 Fe-S clusters and serves to anchor formate dehydrogenase to the membrane with a single C-terminal TM helix. It also shares a similar role as HybA in that it connects the quinone-reactive integral membrane cytochrome with the dehydrogenase catalytic subunit. The 17 amino acids of HybO that are not visible in the crystal structure, which in the native protein connect the TM to HybO, would provide sufficient distance for HybA to be located between HybOC and the cytoplasmic membrane. This arrangement would also allow for the respective TMs of HybO and HybA to interact within the cytoplasmic membrane, which previous biochemical analysis has suggested.³³ Analysis of the structure of HybOC also displays hydrophobic patches close to the distal cluster in both copies of HybO that would imply that such a location would be ideal for protein-protein interactions (Figure 4.12). Interaction via the hydrophobic patches would position an Fe-S cluster of HybA to be in sufficient proximity to the distal cluster of HybO to allow for rapid electron transfer. Such an arrangement is widespread throughout nature. In formate dehydrogenase,³² nitrate reductase,³⁴ and polysulfide reductase,³⁵ the catalytic core and ferredoxin subunits are in a 1:1 ratio, further suggesting that there are likely two HybA subunits in the full HybOC-AB quaternary complex.

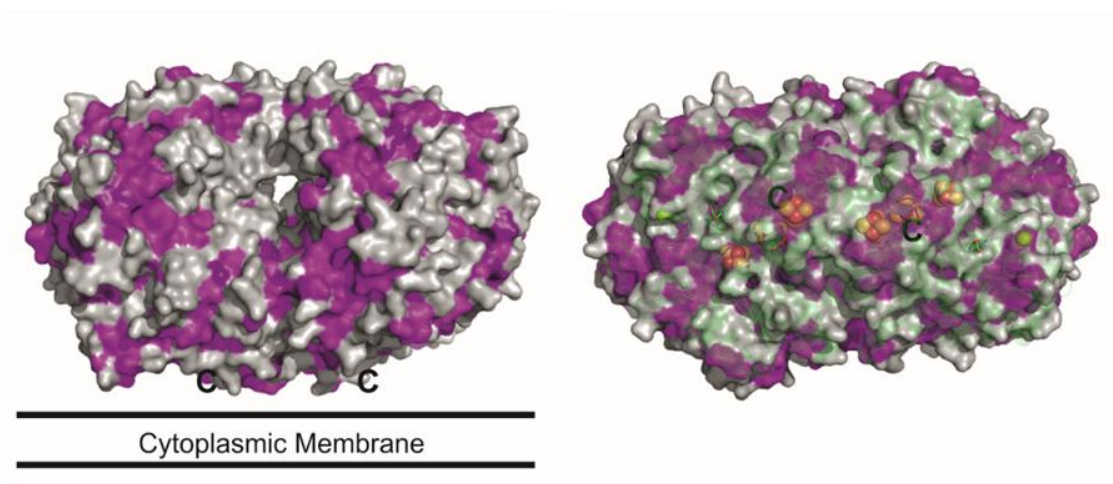


Figure 4.12: Distribution of hydrophobic residues (coloured purple) across the surface of the catalytic core. Obvious symmetrical patches near the C-terminus adjacent to the distal Fe-S clusters (coloured yellow and orange) on the membrane side of HybOC (right) reveal possible locations for interactions with other subunits. Figure adapted from reference.²²

The fourth subunit of Hyd-2, HybB, is an integral membrane protein that is proposed to contain a menaquinone binding domain as well as operate as a true conformational proton pump. It was modelled using the integral membrane component of polysulfide reductase (PDB 2VPZ), PsrC.³⁵ Using PsrC as the template renders a structure for HybB in which 8 of the predicted transmembrane helices are arranged as two four-helix bundles. The polysulfide reductase complex is also dimeric, and that configuration is stabilized mainly by PsrC suggesting that HybB could also play an important role in stabilizing the dimeric configuration of the full HybOC-AB structure. Moreover, the organization of polysulfide reductase is further evidence that the full quaternary structure of HybOC-AB contains two copies of each subunit.

The predicted structure of Hyd-2 can be divided into 3 distinct modules: an outer catalytic module which contains the active site, a central electron relay module, and an integral membrane domain (Figure 4.13). The outer catalytic module is positioned farthest from the membrane and includes the active site. The central electron relay

module comprises HybO and HybA for a total of 7 Fe-S clusters. Aside from anchoring the protein to the membrane, the integral membrane module connects the hydrogen oxidation/production to the redox status of the quinone pool. This arrangement is common in nature from relatively simple, multi-subunit redox proteins like formate dehydrogenase to larger, highly complex redox proteins like Complex I.^{34,36,37}

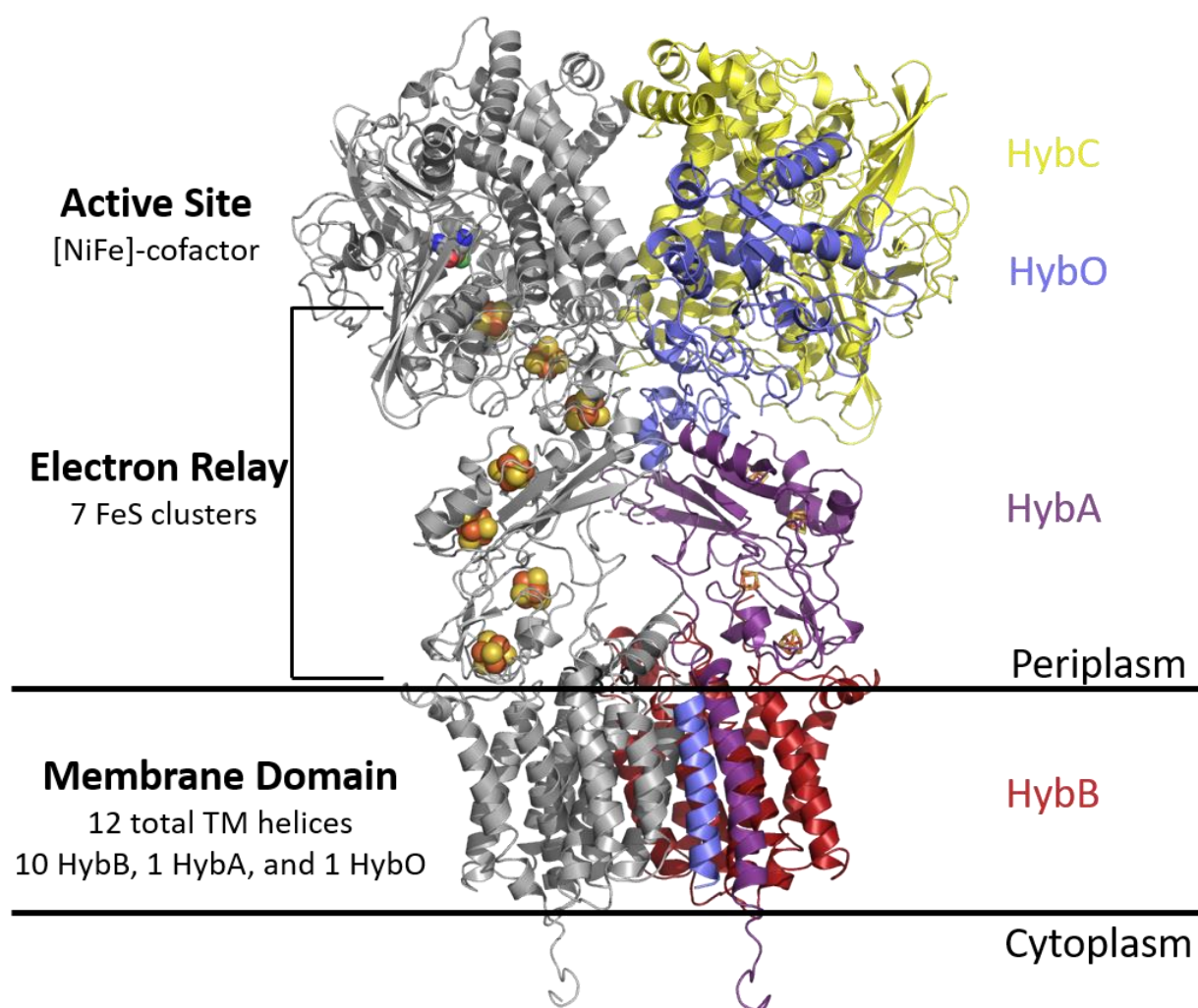


Figure 4.13: Structure of HybOC combined with homology modelling of HybAB to show a possible quaternary configuration of HybOC-AB. Homology modelled by Dr. Steve Carr (Research Complex at Harwell).

4.4 Conclusion

Solving the structure of Hyd-2-NOP proves the utility of the Hyd-2 overproduction system and confirms previously predicted characteristics of Hyd-2 like its dimer configuration and the conserved nature of the active site canopy. Crucially, the development of a straightforward crystallization protocol also allows for future products of site-directed mutagenesis in Hyd-2 to be structurally verified. The first ever structure of HybOC also allows for a more informed model of the quaternary structure of Hyd-2. This quaternary structure is particularly important when trying to understand the role that Hyd-2 orthologues play in pathogens like *S. enterica* or *Shigella sonnei* (Chapter 5).

4.5 References

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Chapter 5

Investigating the cleavage of the Hyd-2
subunit HybA by the rhomboid protease
GlpG

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5.1 Introduction

5.1.1 Intramembrane Proteases

As discussed previously (see Section 1.3.1), Hyd-2 undergoes a range of post-translation modifications before it is fully active including cleavage of a peptide on HybC after full correct insertion of the [NiFe] cofactor.¹ HybO and HybA also have a N-terminal Tat signal that targets the subunits to the twin-arginine translocation system that is cleaved after successful translocation to the periplasmic side of the cytoplasmic membrane.² However, because HybC and HybO are the only subunits that have been consistently purified and characterized, modifications to the catalytic core (HybOC) are the most well-known among the post-translation modifications. However, it is possible that there are additional post-translation modifications to other subunits that have not been detected because HybA and HybB have not been thoroughly characterized.

Recent research done by Guangyu Liu has focused on the importance of a certain class of intramembrane proteases termed rhomboids that could play an important role processing HybA of Hyd-2 from *Shigella sonnei* (a pathovar of *E. coli*). These proteases are of particular interest because their active site is contained within the membrane. Thus, rhomboid proteases can modify a membrane-bound protein from within the membrane.³ This capability makes rhomboid proteases unique as most post-translation modifications studied typically occur before transit to or insertion in the membrane and do not occur within the transmembrane domain. By cleaving the proteins after translocation to the membrane, rhomboid proteases can alter activity of the protein, possibly causing real time effects in metabolic pathways.

Rhomboid proteases are well characterized, yet their role in organisms is still unclear. The structure of a prominent rhomboid protease from *E. coli*, GlpG, was solved in 2006, and this advance enhanced the understanding of the mechanism of rhomboid proteases.⁴ It was shown that rhomboid proteases are made up of at least 6 transmembrane helices containing a catalytic dyad used for cleavage.^{3,4} This catalytic dyad comprises a serine and a histidine located on two different helices. Substrates of rhomboid proteases also typically have a cleavage motif that consists of an amino acid with a small side chain at the P1 position and a large hydrophobic residue at P4 (where P1 is the residue immediately N-terminal to the cleavage site).^{5,6}

However, the full physiological role of rhomboid proteases is still unclear as the natural substrates of most bacterial rhomboids are still unknown.³ In the few known natural substrates of rhomboid proteases, they play an important role in cleaving proteins in a variety of processes ranging from regulating epidermal growth factor receptors in fruit flies^{7,8} to altering proteins key to cell migration and growth in mammals.⁹ The importance of rhomboid proteases has invigorated scientific inquiry into their precise role and possible therapeutic pathways to altering their activity.

Preliminary research into the action of GlpG in *S. shigella*, an orthologue of the GlpG found in *E. coli*, identified the transmembrane domain of HybA as a possible substrate for the rhomboid protease. Although previous work in this thesis (Chapter 4) provides detailed insight into the structure and function of Hyd-2, the relevant details of HybA structure and function in relation to the likelihood that the HybA transmembrane domain is cleaved is summarized here.

5.1.2 HybA

Hyd-2 from *E. coli* is the most well studied *hyb* type [NiFe]-hydrogenase.^{10–14} It is theorized that other Hyd-2 orthologues have similar physiology and bioenergetics and the *hyb* type hydrogenases from *Salmonella enterica* and *Shigella* species have been consistent with this theory.^{15–18} As little is known about HybA and the physiological relevance of the protein in other organisms, the following description of HybA is based mainly on experiments from Hyd-2 in *E. coli*. The sequence for Hyd-2 like proteins, though, is highly conserved. For example, the sequence identity for HybA is 100% between *E. coli* and *Shigella flexneri*.

HybA contains four [4Fe-4S] clusters that facilitate electron transport from the subunit responsible for interaction with the quinone pool, HybB, to the catalytic core, HybOC (Figure 5.1). Without this relay, Hyd-2 is unable to link the redox status of the quinone pool to a driving force for the active site. Indeed, HybA has been shown to be necessary for in vivo H₂ oxidation and production by Hyd-2.¹⁴ Unsurprisingly, HybA is therefore also required for cells to grow with H₂ as the sole electron source and fumarate as the sole terminal electron acceptor in strains where Hyd-2 is the only membrane-bound hydrogenase.¹⁴ Original isolation of the catalytic core of Hyd-2 involved cleavage of the transmembrane domain of HybO. This technique resulted in purification of HybOC without any other Hyd-2 subunits.¹⁰ By contrast, when Hyd-2 was isolated by adding an affinity tag to the end of the TM domain of HybO, variable amounts of HybA were copurified with the Hyd-2 complex.¹⁹ These results suggest that interactions between HybO and HybA happen primarily through the TM domain.

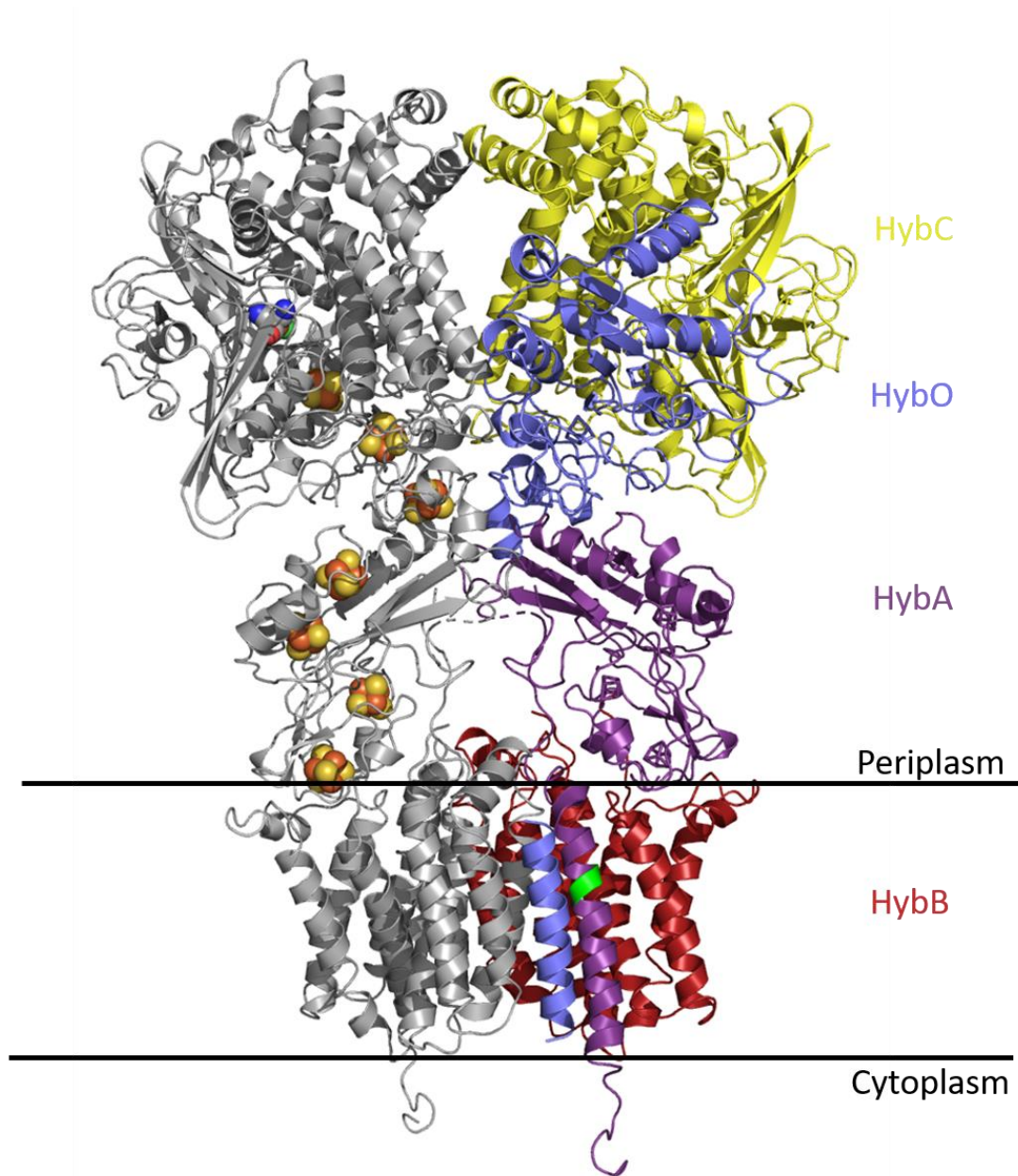


Figure 5.1: Proposed location of GlpG cleavage for HybA (purple) in green shown on the Hyd-2 quaternary model structure. The Fe-S clusters and active site are modelled in the dimer on the left showing the path of electron flow from the cytoplasmic membrane to the active site.

The structure of HybOC presented earlier in this thesis showed that there are hydrophobic patches on HybO where HybA likely interacts with the catalytic core of Hyd-2. These hydrophobic interactions between the surface of HybA and HybO are weak, and the close interaction of the TM domains within the cytoplasmic membrane likely provides the majority of the HybO/HybA interaction (Figure 5.1). If that is the case, the fact that

HybA copurifies with affinity tagged HybO would indicate that at least a portion of the HybA population has intact TM domains. These findings oppose the possibility of cleavage of the TM domain of the majority of HybA in the growth conditions thus far studied. However, cleavage of the HybA TM domain might partly explain the variable presence of HybA in Hyd-2 preps (Section 3.2.1).

Cleavage of the TM domain of HybA could result in either activation or inactivation of Hyd-2. It is common for proteins to experience a cleavage of peptides on either the C-terminus or N-terminus prior to becoming a fully functional protein.²⁰ However, there is only one known example of post-translocation cleavage of a transmembrane domain that results in activated protein in bacteria. Interestingly, the only known example of such cleavage is done by a rhomboid protease on TatA in *Providencia stuartii*, which is an essential protein in the Tat machinery of *P. stuartii*.²¹ Cleavage of the TM domain from TatA in *P. stuartii* allows TatA to properly associate with the other proteins in the Tat pathway.^{21,22} Similarly, it is possible that the HybA TM domain is simply for membrane targeting and that cleavage of the TM domain allows for structural shifts that connect HybB with the catalytic core.

By contrast, it is also possible that cleavage of the HybA TM domain inactivates Hyd-2 either by full disassociation from Hyd-2 or by causing structural shifts that prevent redox connection between the membrane and the active site of HybC. This mechanism could rapidly stop Hyd-2 activity and may be particularly useful when Hyd-2 is exposed to oxygen given the propensity for Hyd-2 to form reactive oxygen species (ROS) or waste reduction equivalents in the quinone pool on O₂ reduction.

However, the structure of the Hyd-2 catalytic core and the predicted quaternary structure of Hyd-2 raise questions about the effect of HybA TM cleavage. Because the

distal clusters of the copies of HybO are within acceptable distance for rapid electron transfer between them, only a single HybA may be necessary for Hyd-2 to function. The second HybA is predicted to contribute to protein stability and ideal electron transfer (Figure 5.1). Moreover, the quaternary structure shows that HybA and its TM domain could be shielded from interaction with proteases by the tight association with HybB and HybO. Biochemical analysis of HybA may therefore be misleading because affinity tags used for purification and analysis of HybA could cause a shift in the quaternary arrangement of Hyd-2 allowing a rhomboid protease easier access for cleavage.

5.1.3 *Shigella sonnei*

Like *E. coli*, *Shigella* is part of the Enterobacteriaceae family and therefore shares many important characteristics. They are both gram-negative bacteria that can survive in the human gut. However, *Shigella* is pathogenic and the cause of millions of infections and thousands of deaths each year, and *Shigella sonnei* is one of two species that account for the majority of infections.²³ The other prominent *Shigella* species, *Shigella flexneri*, is better studied in part due to the fact that it produces a more stable virulence plasmid.²⁴ While there are subtle differences between *S. flexneri* and *S. sonnei*, the two organisms are highly similar in their mechanisms of infection and metabolism.²³

Ingestion of as little as 100 bacterial cells can cause infection and after infection, *Shigella* cells can survive the highly acidic environment of the stomach, but they grow very little.^{25–27} Once *Shigella* cells enter the colon, they invade the colonic epithelium. They do so by being taken up by immune cells called M-cells that can cross the epithelial barrier after which *Shigella* uses Type III secretion machinery, of which the protein Spa33 is an

essential component,²⁸ that causes apoptosis in the M-cell and release of the bacterial cell.²⁶

The ability for *Shigella* to subsist in such acidic environments is remarkable and various studies have investigated the role that hydrogenases play in acid tolerance.^{15,27} Each of the four hydrogenases encoded by *E. coli* have an orthologue in *S. sonnei*, and they have been shown to have similar characteristics and physiological roles in *E. coli* i.e. Hyd-1 and Hyd-2 are primarily uptake hydrogenases and Hyd-3 and Hyd-4 are hydrogen producers.¹⁵ The majority of acid tolerance is attributed to Hyd-1, and evidence has shown that while Hyd-2 plays a role in short-term in vitro acid tolerance in *Shigella* (2h), it has little effect on long-term in vitro acid tolerance (4h+).¹⁵

This evidence points to an ambiguous importance of Hyd-2 in the survival of *Shigella* species in acidic conditions. However, these experiments were based on cells in stationary growth phase grown in media without supplemented fumarate or glycerol, which in *E. coli* has been shown to favour expression of Hyd-1 rather than Hyd-2.¹⁵ Because the exact timeline of colonization and infection is unknown, it is unclear what level of acid tolerance is necessary for successful infection and which growth conditions are the most analogous to those which *Shigella* experiences during the course of infection.

The similarity between *E. coli* and the *Shigella* species extends to their remarkable metabolic flexibility. *S. sonnei* can survive both anaerobic and aerobic growth utilizing very similar metabolic pathways, and previous experiments have shown that the [NiFe]-hydrogenases of *Shigella* are well expressed under similar anaerobic conditions.¹⁵ Without O₂ as a terminal electron acceptor, *Shigella* growing in minimal media supplemented with fumarate and H₂ will use fumarate as the terminal electron acceptor and H₂ as the source of reducing equivalents. By contrast, minimal media supplemented

with just glycerol will undergo fermentation in which glycerol is converted to glycerol 3-phosphate (G3P) followed by oxidation to dihydroxyacetone phosphate (DHAP). The conversion of G3P to DHAP results in the reduction of menaquinone. Typically, the reduced menaquinone is then used to reduce fumarate to succinate, but in the absence of fumarate, reducing equivalents are disposed of by H₂ production mainly by Hyd-2.^{14,29}

5.1.4 Rhomboids in *Shigella sonnei*

Work by Guangyu Liu showed that *Shigella sonnei* encodes two rhomboids. One of the rhomboids, GlpG, is an ortholog (99% sequence identity) of the rhomboid protease found in *E. coli* that has been structurally characterized but for which no natural substrate is known. GlpG has been shown to be beneficial for colonization of mouse gut with wild type *E. coli* outcompeting a Δ glpG strain of *E. coli* by 120 fold after a seven-day period after ingestion.³⁰ A second, unstudied rhomboid, Rhom7, was also found in *Shigella sonnei*. It contains the characteristic catalytic serine-histidine dyad and has no close homologue in any species.

A screen using an artificial substrate modified with the transmembrane domain from different proteins identified different substrates for both of the rhomboid proteases with HybO and HybA being the only substrates cleaved by both rhomboid proteases in *Shigella sonnei*. The correlation between GlpG and Hyd-2 is stronger when considering their observed phenotype. Both proteins have been shown to be crucial in the colonization of the gut of mice.^{17,18,30}

Further studies of the two rhomboid proteases showed that cleavage of HybA, but not HybO, was entirely dependent on the two rhomboid proteases with the majority of activity restored in the presence of GlpG (Figure 5.2). For ease of screening, the assay used

HybA and HybO with a 35 kDa attached tag (termed sfCherry-3xFLAG) on the end of each of the transmembrane domains. While this assay makes detection of a cleavage event easy, it could impact the assembly of the full quaternary structure of Hyd-2 exposing the putative cleavage site.

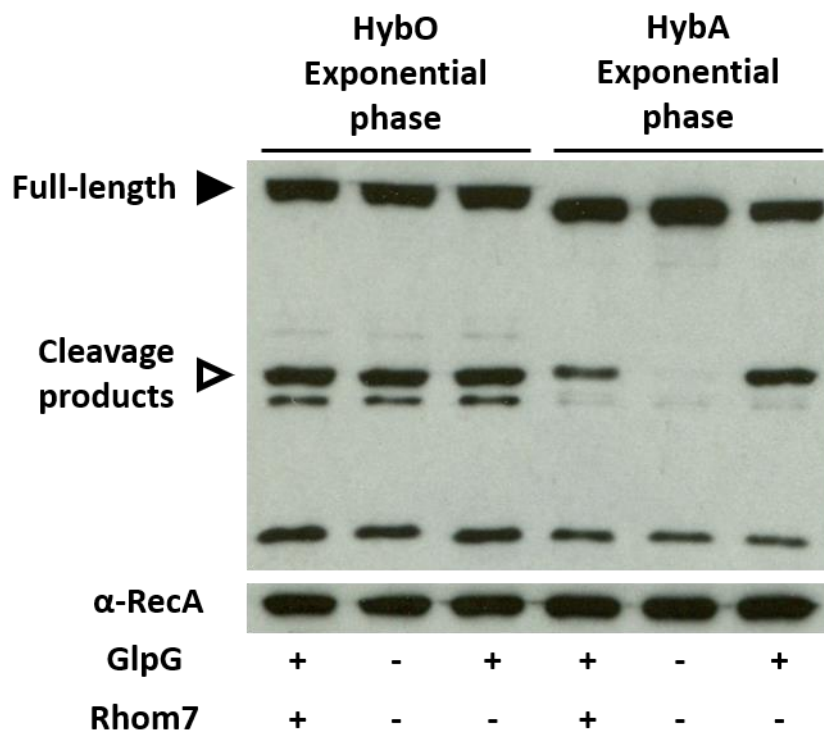


Figure 5.2: Western Blot of cleavage products of HybO and HybA. The transmembrane domain of HybO and HybA tagged with sfCherry 3xFLAG were cleaved showing that cleavage of HybA is dependent on GlpG while HybO is cleaved regardless of the expression of rhomboids. The cleavage product of low molecular weight is the sfCherry 3xFLAG by itself, and the α -RecA control show that approximately equal amounts of sample were added to each lane. All experiments were designed and conducted by Guangyu Liu.

Analysis of the sequence of HybA identified G296 as the most likely P1 residue in the cleavage motif of HybA (G296 is highlighted green as the cleavage site in Figure 5.1). Further, mutating G296 to phenylalanine inhibited cleavage by GlpG (Figure 5.3).



Figure 5.3: Western Blot of HybA cleavage products from *S. sonnei*. Cleavage of native HybA but not HybA G296F tagged with sfCherry 3xFLAG occurs in the presence of rhomboids. The cleavage product of low molecular weight is the sfCherry 3xFLAG by itself, and the α -RecA control show that approximately equal amounts of sample were added to each lane. SAHA stands for the double mutation that disables the catalytic dyad of the rhomboid (serine to alanine and histidine to alanine) and these controls show that the cleaved product is the direct result of an active catalytic dyad on the rhomboid. All experiments were designed and conducted by Guangyu Liu.

5.1.5 Purpose of this chapter

This chapter will explore the role of possible TM domain cleavage of HybA by an intramembrane protease GlpG in *Shigella sonnei*. The role of the TM domain cleavage was probed by measuring growth and H₂ uptake and production in vivo and has implications in understanding the in vivo structure and function of Hyd-2.

5.1.6 Contributions

Guangyu Liu (DPhil student, Sir William Dunn School of Pathology, University of Oxford) did all original screening for GlpG, constructed all the *S. sonnei* mutant strains, began all experiments (plating, inoculation, starter cultures), and measured cell death.

We collaboratively designed and executed the experiments to probe the effect of GlpG on growth on H₂, H₂ uptake, and H₂ production in *S. sonnei*. I performed all measurements using gas chromatography to measure H₂ uptake and production.

5.2 Mutant construction

All strains of *Shigella sonnei* used in these experiments were made avirulent by the deletion of *spa33*. In order to isolate the effect of Hyd-2, the genes encoding Hyd-1 (*hya* operon) and Hyd-3 (*hyc* operon) were also deleted. The three main test strains were one with *glpG* intact, *glpG* deleted (termed $\Delta glpG$), and a negative control with the *hyb* operon deleted (termed Δhyb). A fourth strain was also used as a more accurate control for GlpG activity in which HybA had been modified by site-directed mutagenesis to prevent GlpG cleavage (termed G296F for the site that had been mutated). All strains also had Rhom7 knocked out in order to assure there was no background cleavage by a rhomboid protease. As the sfCherry 3xFLAG tag present in initial experiments with GlpG and HybA could have changed the quaternary structure of Hyd-2, the strains used for H₂ production did not contain the sfCherry 3xFLAG tag on any Hyd-2 subunit.

5.3 Growth on minimal media and H₂

5.3.1. Growth on H₂

The easiest way to investigate whether HybA is activated by GlpG was to measure growth on minimal media during fumarate respiration in an anaerobic environment with H₂ as the sole electron source. Measurements done with large culture preps (100 mL) in flasks with attached cuvettes (see Methods) showed that GlpG was not required for active HybA as the growth during fumarate respiration was statistically equivalent between the

strain with and without GlpG (Figure 5.4). The starter cultures were grown aerobically which meant that even the strain without the *hyb* operon was able to use energy stored during aerobic growth to grow during the earliest portion of the anaerobic conditions.

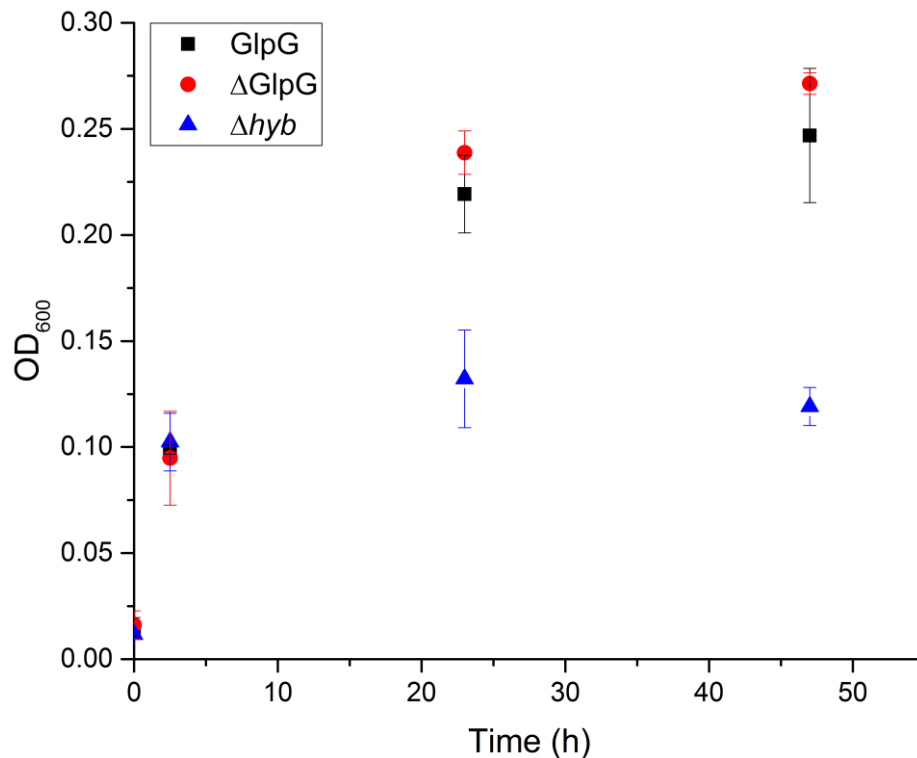


Figure 5.4: Growth of *Shigella sonnei* strains measured by the optical density ($\lambda=600$ nm) of the cultures on H_2 in minimal media supplemented with fumarate and casamino acids. *Shigella sonnei* were inoculated to the same OD ($OD_{600} = 0.01$) in M9 minimal media (150 mL) supplemented with 0.2% casamino acids 0.5% fumarate with 100% H_2 constantly flowing. Grown at 37°C. Error bars represent standard deviation of three measurements at each point for two trials.

These experiments also confirmed that Hyd-2 is necessary for growth in H_2 /fumarate respiration conditions for *S. sonnei* and that growth rates could be used to determine the activity of Hyd-2. However, high rates of error in the measurements meant that this experiment was unable to discern if there were slight differences in the ability of Hyd-2 to oxidize H_2 between strains with and without GlpG.

5.4 Hydrogen uptake and production

5.4.1 Hydrogen production

Hyd-2 is capable of H₂ production during glycerol fermentation, and results from Pinske et al suggest that H₂ production by Hyd-2 is driven by proton motive force.¹⁴ Since it was already established through growth curves that GlpG is not necessary for Hyd-2 activation, it was theorized that GlpG could cleave a population of the HybA TM domain to reduce the rate at which Hyd-2 dissipates proton motive force and reduction equivalents during glycerol fermentation.

A methodology similar to Pinske et al was used to measure H₂ production.¹⁴ Briefly, large culture volumes (250 mL) were concentrated into 5 mL (OD₆₀₀=35) and placed in glycerol fermentation conditions and the headspace was measured by gas chromatography (GC). In these conditions, strains of *S. sonnei* with Hyd-2 produced a significant amount of H₂ after a 20h lag, yet there was no reproducible trend in H₂ production between the strains that varied in the presence or efficacy of GlpG. Moreover, cell viability was measured at the end of various runs and showed that for every strain, more than 99% of the cells in the samples had died over the course of the experiment (data not shown). With so few living cells, there were large variations between different strains in the number of cells alive, yet there was no reproducible trend between strains as to which strain best survived glycerol fermentation conditions over multiple runs. Although there was no trend between the strains beyond Hyd-2 being necessary for H₂ production, there were large differences between runs concerning the scale and timeframe of H₂ production. Typically though, the two strains containing Hyd-2 produced between 3 and 10 μmol of H₂, and H₂ production began between 20 and 40 hours after

the beginning of glycerol fermentative conditions. Thus, only a representative graph of H₂ production is shown in Figure 5.5.

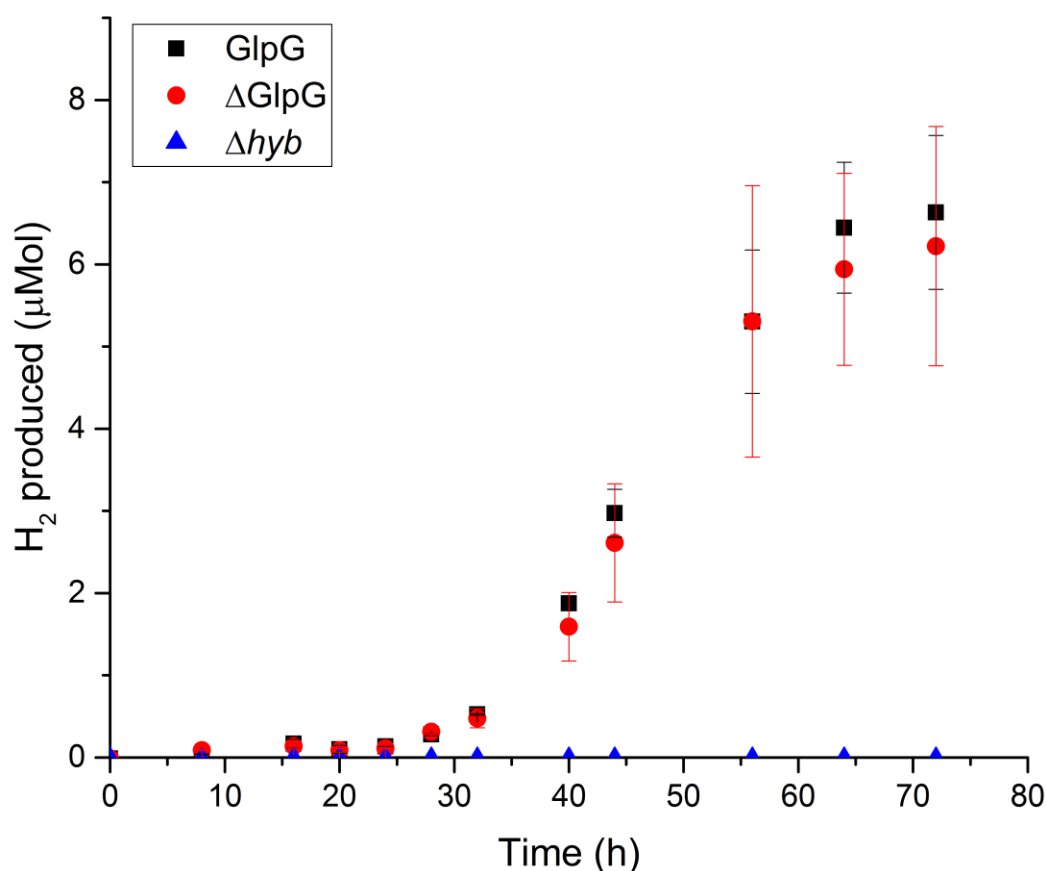


Figure 5.5: H₂ production by *S. sonnei* strains probing the effect of GlpG on H₂ production during glycerol fermentation. Strains of *Shigella sonnei* were grown anaerobically (approximately 2.5% H₂, 12.5% CO₂, 85% N₂) in LB supplemented with fumarate for 20 hours until stationary phase was reached. Cells were harvested and resuspended in 5 mL of PBS buffer with 170 mM glycerol. Cell solutions were then sparged with nitrogen for 20 min and incubated shaking at 37°C. Samples (15 μL) of the headspace were taken periodically which were then assayed with the GC. Error bars represent standard deviation on 2 measurements taken for each time point of one trial.

This result suggests that although Hyd-2 is capable of in vivo H₂ production as reported previously, H₂ production by Hyd-2 often coincides with cell death measured by the number of colony forming units (CFU). After 70 h of H₂ production there is typically a 100-fold decrease in the measured CFU count. The high death rate also made the results difficult to interpret, as it was impossible to separate the death rate from H₂ production

in order to calculate the amount of H_2 produced per mass of living cells. However, the results suggest that either GlpG does not cleave a significant portion of the HybA population in these conditions or HybA TM domain cleavage does not result in decreased H_2 production.

5.4.2 Hydrogen uptake

In an attempt to avoid the 20h wait time necessary for H_2 production in which the cells could be dying at variable and fast rates, hydrogen uptake during cell growth experiments were conducted. In these experiments, strains of *S. sonnei* were inoculated to an OD_{600} of 0.01 and allowed to grow in M9 minimal media supplemented with casamino acids and fumarate. In order to promote H_2 exchange between the headspace and media, the strains were grown in a shaking incubator, and samples from the headspace were taken periodically.

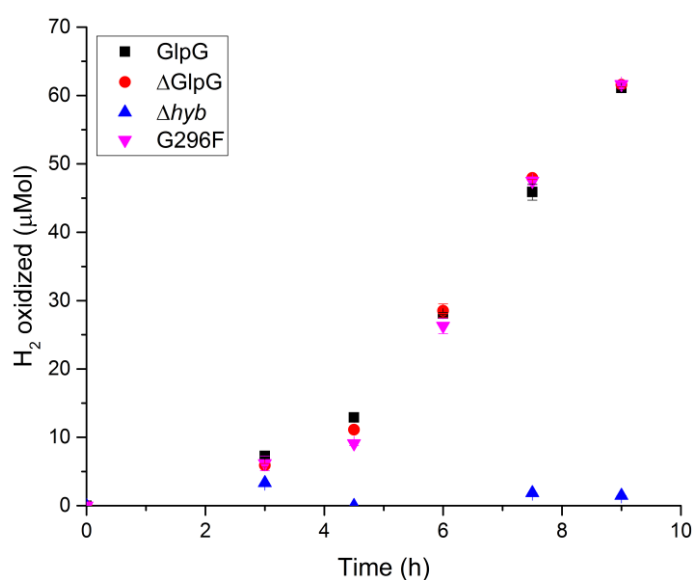


Figure 5.6: H_2 oxidation by the knockout strains probing the effect of GlpG. Strains of *Shigella sonnei* were inoculated to the same OD ($OD_{600}=0.01$) in M9 minimal media (5 mL) supplemented with 0.2% casamino acids, 0.5% fumarate, and purged for 10 min with 10% H_2 (Ar carrier gas) before shaking incubation at 37°C. Samples (15 μ L) of the headspace were taken periodically which were then assayed by gas chromatography. Error bars represent standard deviation on 2 measurements taken for each time point of one trial.

These experiments also showed very little variation in the amount of H₂ oxidation by the strains encoding Hyd-2 (Figure 5.6). The final CFU count after the growth also confirmed the findings from the growth experiments, as the CFU count revealed that the growth of the strains in anaerobic fumarate respiration conditions was nearly equivalent among all strains containing the *hyb* operon (Figure 5.7).

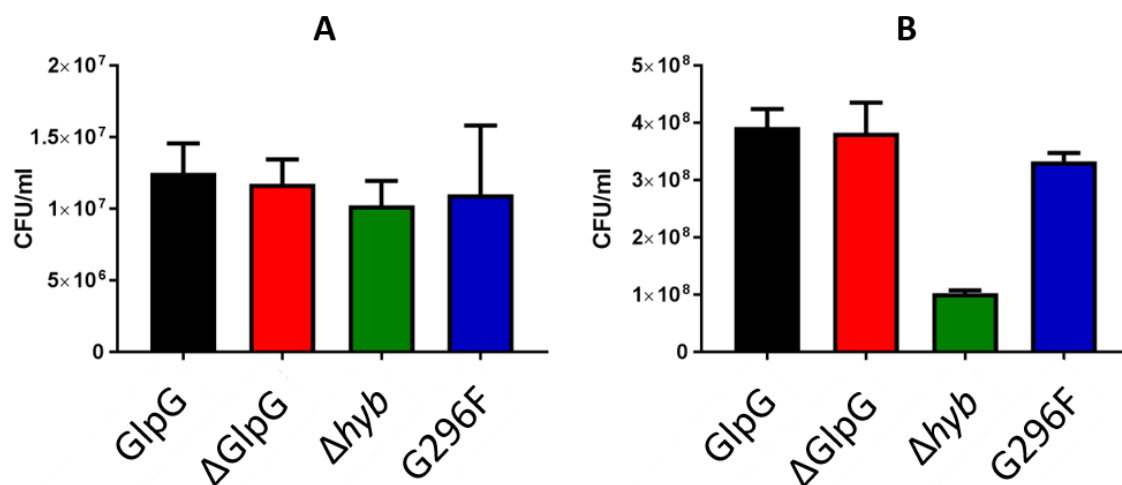


Figure 5.7: CFU of mutant strains of *S. sonnei* (A) before and (B) after measuring H₂ uptake during growth. Initial growth was done in aerobic conditions, and cultures were inoculated to approximately equivalent optical densities. These graphs compare the strains with GlpG, without GlpG, without the *hyb* operon, and with a point mutation G296F preventing cleavage of HybA.

5.4.2.1 Hydrogen uptake after O₂ exposure

In an attempt to induce conditions in which HybA TM domain cleavage would be favourable, O₂ was introduced to the growing cultures after 6h for both short (1h, Figure 5.8)) and long (4h, data not shown) periods and then the cultures were returned to anaerobic fumarate respiration conditions. Again, there was no reproducible difference between the strains in the rate of H₂ oxidation. These conditions also did not provide any differentiation between strains when returning to anaerobic conditions. Although Hyd-2

is oxygen sensitive *in vitro*, there is evidence from investigations of the [NiFe]-hydrogenases from *R. eutropha* that hydrogenases in their full quaternary *in vivo* structure are significantly less sensitive to oxygen.³¹

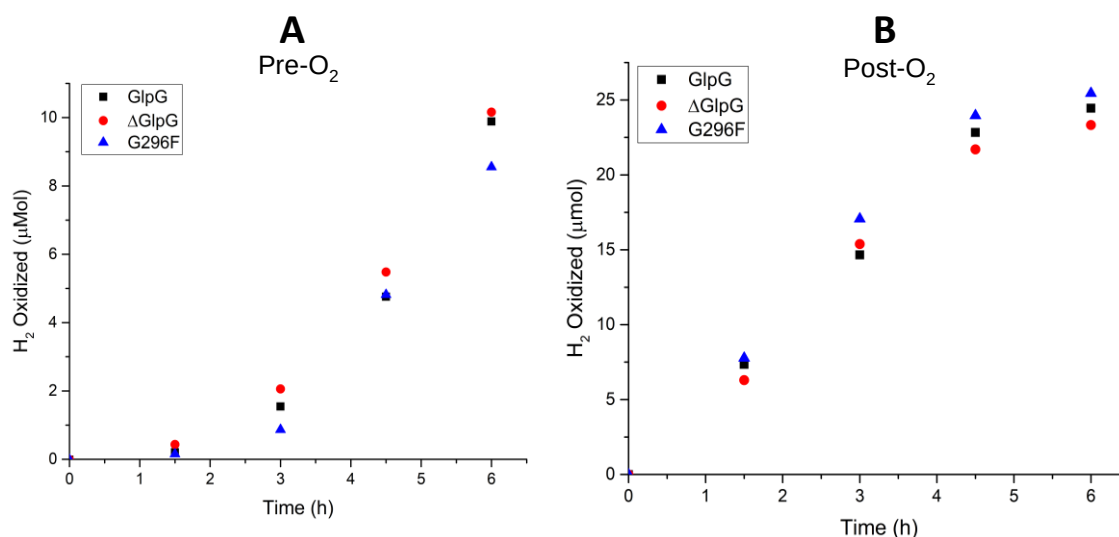


Figure 5.8: H₂ oxidation of the knockout strains growing on H₂ as the electron source **(A)** before and **(B)** after one hour of O₂ exposure. Strains of *Shigella sonnei* were inoculated to the same OD (OD₆₀₀=0.01) in M9 minimal media (5 mL) supplemented with 0.2% casamino acids, 0.5% fumarate, and purged for 10 min with 10% H₂ (Ar carrier gas) before incubation shaking at 37°C and purged for 10 min with 10% H₂ before incubation shaking at 37°C. After 6 h of incubation cultures were purged with air and left for 1 h before being purged for 10 min with 10% H₂ and returned to shaking incubation at 37°C.

5.5 Conclusion

Although preliminary tests clearly show that the HybA TM domain can be cleaved by the intermembrane protease GlpG, the physiological role of cleavage remains elusive. The two strains that should share a similar phenotype, *ΔglpG* and G296F, never present a phenotype that is measurably different than strains with GlpG and native HybA. Indeed, GlpG expressed at endogenous levels plays no obvious role in controlling H₂ production or oxidation by well-formed Hyd-2. While the impacts of these findings are limited, these results do however confirm that Hyd-2 plays a similar role in *S. sonnei* as it does in *E. coli*.

As reported earlier, Hyd-2 is predicted to be a complex structure with various TM domains that are in close proximity and therefore may protect each other from cleavage unless placed under currently unknown conditions in which structural changes allow greater access to HybA TM. This could indicate that GlpG is better at cleaving Hyd-2 in situations where the Hyd-2 is poorly formed. Moreover, all these strains were $\Deltahya\Delta hyc$ meaning that Hyd-2 is the only hydrogenase. Expression of the other two hydrogenases may upregulate *glpG* to cleave the HybA TM domain in conditions where Hyd-2 is vulnerable (i.e. O₂ attack) that other hydrogenases like the O₂-tolerant hydrogenase Hyd-1 are better suited to handle. Finally, the role of GlpG could be limited to maintaining the ideal stoichiometric ratio between subunits, which would be difficult to detect based on the other uncontrollable variables in experiments. More work must be done to verify the role that GlpG plays in Hyd-2 function and cell metabolism.

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Chapter 6

Establishing the importance of
outershell residues for hydrogen
activation in [NiFe]-hydrogenases

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6.1 Introduction

Because of the industrial importance of H_2 as well as its potential to be used as a clean fuel, activation of H_2 with non-Pt catalysts has been the focus of scientific enquiry for nearly a century. Currently, the various understood pathways to the activation of H_2 are divided into two groups: homolytic cleavage and heterolytic cleavage of H_2 . Homolytic cleavage was first observed in 1984 by Kubas et al. in solutions of $M(CO)_3(PCy)_2$ where the metal 'M' is Mo or W that, when exposed to a flow of pure H_2 , reacted to form metal dihydrides.^{1,2} This reaction occurs as the H_2 binds side-on to the metal centre primarily via donation of the two σ electrons on H_2 to a vacant d orbital. Back donation of metal d-electrons into the $H-H$ σ^* orbital disrupts the $H-H$ σ orbital.² Conversely, most early hydrogenase mimics employed heterolytic cleavage. In this reaction, the $H-H$ bond of the bound H_2 becomes polarized and is split by a process wherein a hydride (H^-) binds to the metal, and an intramolecular or intermolecular Lewis base removes a proton (H^+).² Although it has been shown that H_2 oxidation occurs by a heterolytic mechanism in [NiFe]-hydrogenases,^{3,4} there are conflicting theories as to how it occurs.

6.1.1 Active site architecture and H_2 activation

In all [NiFe]-hydrogenase structures solved to date, the active site is highly conserved.⁵ Beyond the primary coordination sphere described earlier (see Section 1.3.1), there is a group of highly conserved amino acids located directly above and in close proximity to the [NiFe] cofactor—frequently referred to as the active site 'canopy' (Figure 6.1).^{6,7} The canopy includes a pendant arginine with its guanidinium head group located above the bridging ligand (R479) as well as an aspartate positioned above the Ni (D103). Farther away from the [NiFe] cofactor is another aspartate (D544) capable of

hydrogen bonding with the pendant arginine. The canopy does not include E14 or V63 (Hyd-2 numbering), amino acids that have been shown to be crucial to proton transfer and gas pathways,^{8,9} respectively, but are located outside of the active site.

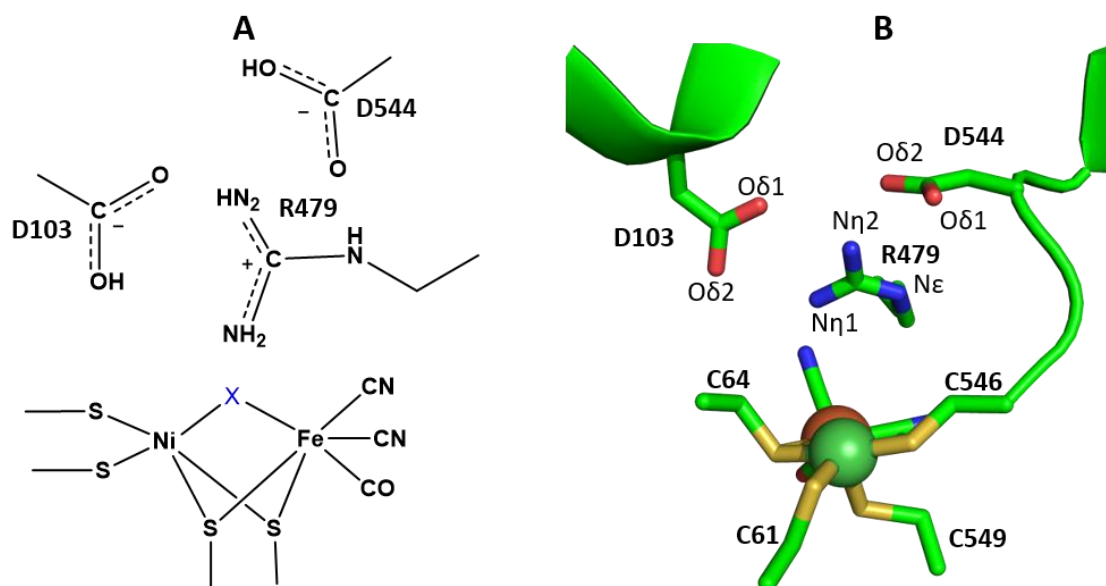


Figure 6.1: (A) Schematic and (B) crystal structure of the active site of as-isolated Hyd-2 (PDB 6EHQ) including the ‘canopy’ of conserved amino acids above the bimetallic cofactor. For as-isolated Hyd-2, X is a bridging hydroxide which has been omitted from the figure for clarity.

It is agreed that the canopy amino acids play an important role in shaping the electrostatics and structure of the active site,^{10,11} yet until recently, little attention has been given to the possibility that they play a direct role in the mechanism of H₂ oxidation or production.⁶ Instead, the H₂ oxidation reaction is typically depicted as proceeding via protonation of a terminal cysteine thiolate (C546 in Hyd-2 numbering).^{12–15} In this reaction scheme, the transient Michaelis-Menten complex between Ni-SI_a and Ni-R consists of H₂ bound side-on to the Ni in a Kubas-complex type arrangement polarizing the H₂ allowing abstraction of a proton by the sulphur of C546 and leaving a hydride to bridge the bimetallic active site.^{16,17} This mechanism of H₂ cleavage is supported by early

DFT calculations showing protonation of a terminal cysteine thiolate in both Ni-SI and Ni-R.¹⁵ A publication of the structure of the [NiFe]-hydrogenase from *D. vulgaris* MF claimed to resolve a proton bound to a terminal cysteine in the structure of the Ni-R state,¹² yet those claims were met with wide scepticism due to the difficulty of resolving a proton by X-ray diffraction given a proton's lack of electron density as well as the acute C-S-H bonding angle (77°) that violates normal rules of stereochemistry (private communication with Prof. Simon Phillips, Research Complex Harwell).¹² As the protein was reduced in crystalline form, approximately a third of the enzyme was in a reduced state and the remaining enzyme was in the as-isolated form.¹² The heterogeneity of the crystal likely confused interpretation of electron density of and around the terminal sulphur. Solving a structure of the crystal solely in Ni-R, though, would be extraordinary as a structure that could resolve both atoms of H₂ shortly after cleavage at the active site could provide clear insight into the pathway of proton transfer and the mechanism of heterolytic cleavage. While the complexity of hydrogenases makes resolving the mechanism difficult, biomimetic catalysts have served as a useful model for the active site.

6.1.2 Biomimetic catalysts

Early biomimetic catalysts were poor at replicating the efficiency of hydrogenase enzymes, often requiring high overpotentials and forcing conditions to activate H₂.^{18,19} Although these systems mimic the reactions of hydrogenase, their low turnover rates and complexity suggest a lack of fidelity in mimicking the true mechanism that hydrogenases employ to carry out the same reactions.

Catalysts have come closer to mimicking hydrogenase activity with the recent uncovering and use of the “Frustrated Lewis Pair” (FLP) mechanism (Figure 6.2).²⁰ In this reaction, a traditional Lewis acid and Lewis base are precluded from quenching either by steric hindrance, their positions relative to one another, or perhaps a combination of sterics and positioning.

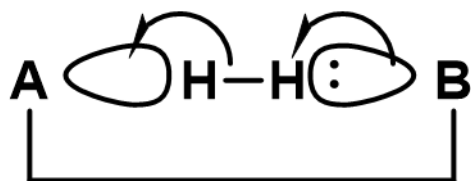


Figure 6.2: Diagram of Frustrated Lewis Pair Mechanism where A is a Lewis acid and B is a Lewis base.

Consequently, this pair splits small molecules by a reaction wherein both the Lewis base and the Lewis acid simultaneously form bonds with the favourable portion of a small molecule. This reaction is particularly relevant to heterolytic H₂ activation: the proton is capable of quenching a Lewis base and the hydride can quench a Lewis acid. Stephan and coworkers proved the utility of this mechanism with their creation of the first truly reversible hydrogenase mimic using a phosphine-borane system.²¹ Although this system did not require the help of strong external mediators, it did require temperatures above 150°C to liberate the bound hydrogen atoms.²¹

Recent work using FLP systems have improved the efficiency and application of these hydrogenase mimics.^{22–24} Specifically, the Dubois catalyst which uses a pendant amino group placed in the secondary coordination sphere adjacent to a vacant coordination site of Ni has shown the highest rates of H₂ production among homologous catalysts.²² Moreover, the Dubois catalyst can be tuned by extending the functional groups in the periphery to include amino acids like glycine and arginine.^{25,26} Inserting

these amino acids increases turnover rates as well as decreases the required overpotential (see Section 9.1.1 for explanation of overpotential in enzymes).²⁶

While the use of bioinspired structures has increased the efficiency of catalysts, they still require significant overpotentials, and they are irreversibly inactivated in the presence of oxygen.²⁷ Therefore, more must be done to understand the mechanism employed by nature to relay electrons and protons into and out of hydrogenases to achieve highly efficient reversible interconversion of molecular H₂ and protons while withstanding oxygen attack.

6.1.3 Hydrogenase active site ‘canopy’

6.1.3.1 Arginine as a base

Inspired in part by the success of catalysts with pendant amino groups, recent investigations of [NiFe]-hydrogenases have focused on the role of the amino acids in the outer coordination shell of the bimetallic cofactor.⁶ Early efforts to mutate residues around the active site focused on residues believed to be involved in stabilizing the carboxyl and nitriles bound to the active site Fe.¹⁰ These studies successfully established the importance of amino acids proximal to the active site in moderating the electronics at the [NiFe] cofactor.¹⁰

These same studies identified the pendant arginine that is strictly conserved above the [NiFe] cofactor as possibly important to the stabilization of the active site, and mutating the pendant arginine to isoleucine in hydrogenases from *Thiocapsa roseopersicina* or *Desulfovibrio fructosovorans* resulted in immature enzyme or lack of expression.^{10,11} Consequently, it was theorized that arginine possibly played a dual role in both proton transfer as well as structure stabilization.¹¹ Fortunately, a variant in which the pendant arginine, R509, in *E. coli* Hyd-1 was mutated to a lysine (R509K) is stable and

active—although two orders of magnitude slower at H₂ oxidation (see Section 3.2.6 to Section 3.2.8 for introduction on enzymatic characteristics).⁶ The structure of Hyd-1 R509K shows that the lysine resides in a similar position as the pendant arginine, but temperature factors around the active site imply greater flexibility of the lysine.⁶ The sidechain amino of K509 is at nearly the same position as the Nη1 of R509 in native Hyd-1, and it is still within appropriate distance (4.4 Å) for an FLP-like mechanism.^{6,28} Because both amino acids' functional group contain nitrogen capable of acting as a base, it was theorized that if the R509 is directly involved in the mechanism, then the R509K variant should be capable of hydrogen activation at less than ideal rates (free lysine has a pKa of 10.5 and free arginine has a pKa of 12.4).⁷

Further, experiments showed that not only did the mutation change the turnover rate, but it also had a substantial effect on the energy of activation and the H₂ to D₂ kinetic isotope effect (KIE) of the hydrogen oxidation reaction.⁷ The KIE is a measure of the kinetic effect of replacing hydrogen with bulkier deuterium and is an important metric in all reactions in which proton exchange is the rate-limiting step. The decrease in KIE from native Hyd-1 (1.37 ± 0.02) to R509K (1.14 ± 0.02) suggests that the arginine to lysine mutation changed the dynamics of the proton abstracted during the rate limiting step.⁷ This finding was supported by the change in the energy of activation for R509K, which also suggested that the arginine to lysine mutation altered the thermodynamics of the catalytic intermediate in the rate-limiting step.⁷ Electrochemical experiments confirmed that the H₂ oxidation reaction in the R509K variant has a lower standard enthalpy. These results were used with the H₂ turnover rates for the variants measured by solution assays to calculate that there is a large entropic barrier for the H₂ oxidation reaction in R509K, perhaps driven by the flexibility of the lysine.⁷

The change in enzymatic parameters for Hyd-1 R509K is more compelling given that mutating residues that are also in close proximity to the arginine, like D118 and D574 in Hyd-1, slow the rate of turnover while maintaining similar parameters for the standard enthalpy and KIE.⁷ Taken together, this evidence supports the theory that the arginine could act as a base in a Frustrated Lewis Pair reaction with the bimetallic cofactor acting as an acid.

However, recent predictions by QM/MM calculations have cast doubt on the pendant arginine as a base.²⁹ The QM/MM models of the active site of [NiFe]-hydrogenases are mainly concerned with the population of protonated vs. unprotonated R479, and with a pKa of approximately 12, it is unlikely that a significant population of R479 is unprotonated. Moreover, the study predicts that the energy required to deprotonate the arginine (approximately 154-158 kcal/mol) would make the arginine less favourable than the terminal cysteines as a primary base for H₂ activation.²⁹ However, this study postulates that the arginine plays an important role in both structurally stabilizing the active site and polarizing the H-H molecule as it binds to the Ni.²⁹ There are two significant limitations to this theoretical investigation though: (1) the cysteines are in part deemed more favourable as an initial base because of their interactions with E14,²⁹ which recent studies have shown is not necessary for the initial steps of H₂ activation in Hyd-1³⁰ (see Chapter 8) and (2) the paper does not consider the pendant arginine's role in the reverse reaction, H₂ production, which could give further insight into the pendant arginine's role.

6.1.3.2 Proton transfer

After cleavage of the H-H bond, the resulting proton is transported away from the active site. Previous mutagenic studies have identified a glutamate (E14 in Hyd-2 numbering, discussed in detail in Chapter 8) located close to the active site as essential for catalytic activity likely due to its role in shuttling protons after breaking of the H-H bond.⁹ The proximity between the sulphur of C546 and the E14 essential for proton transfer has typically been another reason to favour the theory that the sulphur is directly involved in the mechanism at the active site.^{14,15,29} The hydride at the active site is then released either by sequential steps of proton release followed by electron transfer or by a concerted proton-coupled electron transfer (PCET) step. Results obtained by Dyer and coworkers for the soluble [NiFe]-hydrogenase (Group 3) shows that Ni-C to Ni-SI_a likely follows a PCET pathway,¹⁴ yet these results are inconsistent with most findings from Group 1 O₂-tolerant hydrogenases that favour proton transfer away from the active site followed by electron transfer to the proximal cluster.³¹ If the pendant arginine were involved in hydrogen activation in an FLP-type mechanism, candidates for proton transfer away from the pendant arginine would include ordered water molecules at the active site as well as the aspartates in the canopy.

6.1.3.3 Aspartate fine tuners

Asp 103 (in Hyd-2 numbering, D118 in Hyd-1 numbering) is located close to the pendant arginine (2.8 Å) and could play an important role in transferring protons away from the active site if arginine were the initial base for H₂ activation (see Figure 4.9 in Section 4.3.2 for overlay of the active site of Hyd-1 and Hyd-2). Moreover, it is sufficiently close to the terminal cysteines (7.4 Å to C546), that it could be the second or third step

along the path of proton transfer for a terminal cysteine thiolate. Thus, D103 is well-positioned to facilitate proton transfer near the active site. In work by Brooke et al., mutations to aspartic acid residues (D118 and D574 in Hyd-1 which are equivalent to D103 and D544 in Hyd-2, respectively) in the canopy of the active site also helped elucidate the importance of those residues for enzymatic activity.^{6,7} In the D118A, D574N, and D118N/D574N Hyd-1 variants, decreased hydrogen oxidation activity is observed, although the decrease is not as drastic for the aspartate variants as it is for R509K.⁶ These results show that although the aspartate residues are essential for optimized catalytic activity, the canopy aspartates are likely not necessary for proton transfer to and from the [NiFe] cofactor for Hyd-1.⁷

Conserved water molecules around the active site may further complicate mapping proton transfer routes, as mutations around the active site often cause the addition of conserved water molecule(s) that may assist in proton transfer, as has been reported in other redox enzymes.^{32,33} In Hyd-1 D118A, there are no additional waters, yet there are noticeable shifts of 1.3 Å of two ordered water molecules in the active site that could help stabilize the variant (Figure 6.3). One shift brings an ordered water molecule closer to the site of Oδ1 of where D118 resides in the native enzyme and within 3.0 Å of both D574 and R509 suggesting D118 may play a role in stabilizing other canopy residues in the active site.

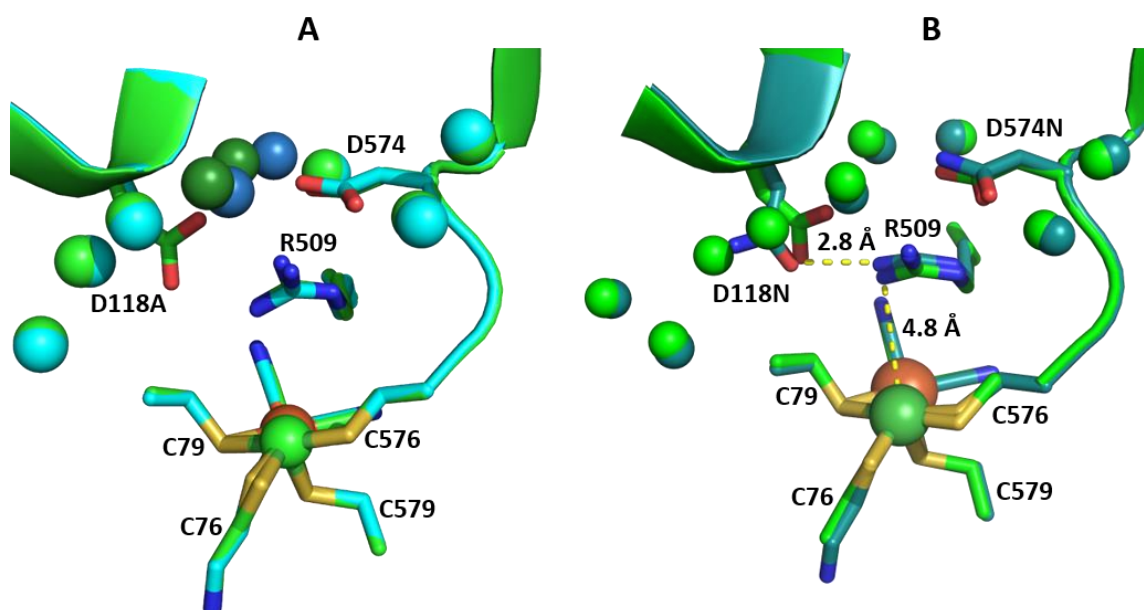


Figure 6.3: (A) Overlay of native Hyd-1 (PDB 5A4M) (green) and Hyd-1 D118A (PDB 5A4F) (blue) including ordered water molecules at the active site. The water molecules with significant shifts are darker in colour. (B) Overlay of native Hyd-1 (green) and Hyd-1 D118N/D574N (PDB 5ADU) (dark teal) including ordered water molecules. Measurements indicate distances between R479 N η 1 and Ni and the O δ 1 of D118N.

Mutating the aspartates also causes a significant increase in the $K_M^{H_2}$ of Hyd-1 that is potential dependent (Figure 6.4).⁷ It was reasoned that the unusually large $K_M^{H_2}$, which for the sake of simplicity can be reduced to $(k_{-1}+k_{cat})/k_1$ where k_1 and k_{-1} are the on and off rates, respectively, and k_{cat} is the turnover frequency, was likely driven by a very small k_1 .⁷ This was theorized because a high k_{-1} would likely lead to less product inhibition and therefore enhanced H_2 production, but D118A is incapable of H_2 production under all conditions.⁷ The high $K_M^{H_2}$ likely impacts perceived O_2 tolerance as a given partial pressure of H_2 is less able to reactivate hydrogenase variants with high values for $K_M^{H_2}$.⁷ Similarly, the aspartate mutations lower the $\Delta H^\ddagger$ in a potential dependent manner, yet because the $K_M^{H_2}$ is so high, the activity used to calculate $\Delta H^\ddagger$ may represent the catalysis of substrate limited hydrogenase, rendering the calculated $\Delta H^\ddagger$ less accurate.⁷

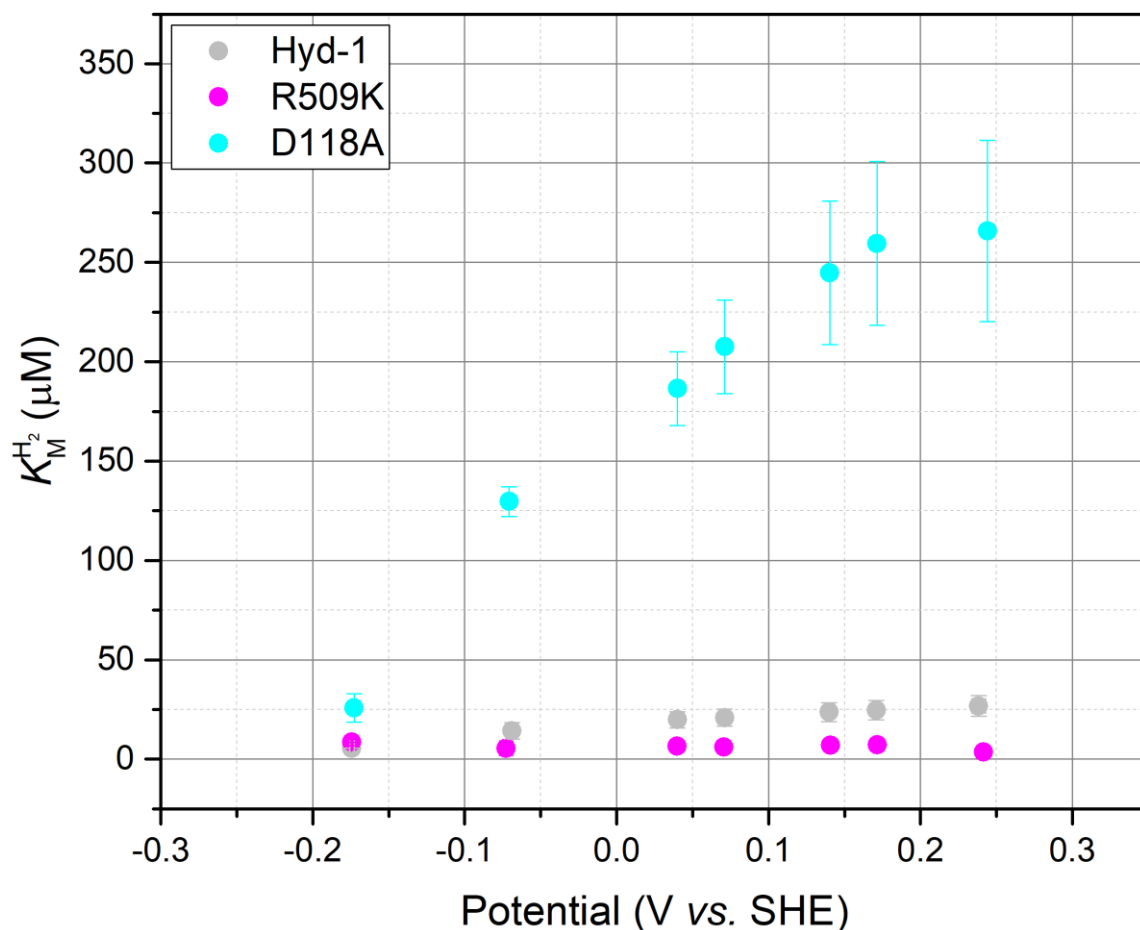


Figure 6.4: Reported $K_M^{H_2}$ of native Hyd-1 and selected Hyd-1 variants (R509K, D118A). Figure adapted from reference.⁷

All three variants with mutations to the aspartates in the canopy also have decreased values for E_{SWITCH} , which suggests that the charge of the active site is important in the stabilization of Ni-B and that the mutations making the active site more positive by the removal of the aspartates help stabilize the negative OH^- ligand in Ni-B.⁷ Notwithstanding, rates of Ni-B formation between the variants are similar.⁷

All three aspartate variants (D118A, D574N, D118N/D574N) have values for $\Delta H^\ddagger$ that decrease with increasing potentials.⁷ This effect is likely due in part to the potential dependent increase in $K_M^{H_2}$ which signifies that the variants require increasing substrate concentrations at higher potentials. For D118N/D574N and D574N, the effect is mild, and

because the values for $\Delta H^\ddagger$ for these two variants start higher than the $\Delta H^\ddagger$ for the native enzyme, these variants do not have a $\Delta H^\ddagger$ that is significantly different than the $\Delta H^\ddagger$ of native Hyd-1.⁷ The trend for $\Delta H^\ddagger$ of D118A variant is different than the other two aspartate variants in that the $\Delta H^\ddagger$ values at high potentials are significantly lower than those for native Hyd-1.⁷ Nevertheless, at lower potentials, where the values are likely less complicated by high values of $K_M^{H_2}$, the $\Delta H^\ddagger$ values are in a similar range as $\Delta H^\ddagger$ for native Hyd-1.⁷

The most noticeable shift in activity from the mutations of the aspartates in the canopy is the loss of proton reduction activity of the D118A variant.⁷ Because the D118A variant shows no observed H_2 evolution (native Hyd-1 is capable of minimal H_2 evolution at pH 3), it was hypothesized that a polar or charged residue at that position is necessary for proton reduction.⁷ The reason for loss of observable H_2 production in D118A is unclear, but given that the Hyd-1 variant with a D118N mutation designed to inhibit proton transfer is capable of H_2 production, the importance of the polar or charged residue at position 118 is likely limited to electrostatic interactions with the surrounding molecules.⁷ Native Hyd-1, however, is capable of H_2 production only under acidic conditions,³⁴ and to better test the effect of the mutation on H_2 production, the homologous mutation should be made in a reversible hydrogenase like Hyd-2.

6.1.4 The power of comparative systems

Although there are currently significant efforts to probe the mechanism of [NiFe]-hydrogenases, the variability among [NiFe]-hydrogenases makes observations for one hydrogenase difficult to generalize to the field thereby limiting the utility of observations.^{5,17} Recent studies and classifications of spectroscopic signatures have

presented further evidence that there may be slight differences in the mechanism of the reaction in O₂-tolerant [NiFe]-hydrogenases and standard [NiFe]-hydrogenases. For example, standard, O₂-sensitive hydrogenases like Hyd-2 have energetically higher spectroscopic signatures for Ni-R and Ni-L than their O₂-tolerant counterparts which could signify that the two groups of hydrogenases have slightly altered proton pathways (see Figure 1.2 in Section 1.3.2 for the states of the catalytic cycle in [NiFe]-hydrogenases).¹⁷

Because the two membrane-bound hydrogenases in *E. coli* share high sequence similarity and cellular environments, they serve as excellent model enzymes to help elucidate the mechanism of H₂ activation and oxygen tolerance through comparative studies. Furthermore, Hyd-2 is capable of sustained H₂ production, and measuring the impact of canopy mutations on H₂ production could give valuable insight into catalyst design. Mutating the equivalent residues in Hyd-2 is therefore important to investigate the different roles the canopy could play in different types of hydrogenases.

6.1.5 Aims of this chapter

This chapter presents the enzymatic and structural characterization of Hyd-2 R479K-OP and D103A-OP, and they are compared to the homologous mutations in Hyd-1. The implications of the changes in enzymatic parameters on the mechanistic theory are also discussed.

6.2 Results

6.2.1 Variant production, purification, and structural characterization

Canopy mutations were successfully incorporated into the overexpression strain HJ001-hyp, and the strains were then transformed with pO^c (see Table 2.5, Table 2.6, and Table 2.7 for a list of strains, plasmids, and primers used in this thesis). Hyd-2 R479K overproduced and Hyd-2 D103A overproduced (termed Hyd-2 R479K-OP and Hyd-2 D103A-OP, respectively). Both give similar yields to Hyd-2 NOP at approximately 0.15 mg protein per gram of cell. The two protein variants also give a similar purification profile when eluted from a Ni column with a linear gradient of imidazole (data not shown). As expected, the variants purify as the catalytic core HybOC, and do not copurify with any other additional subunit (Figure 6.5).

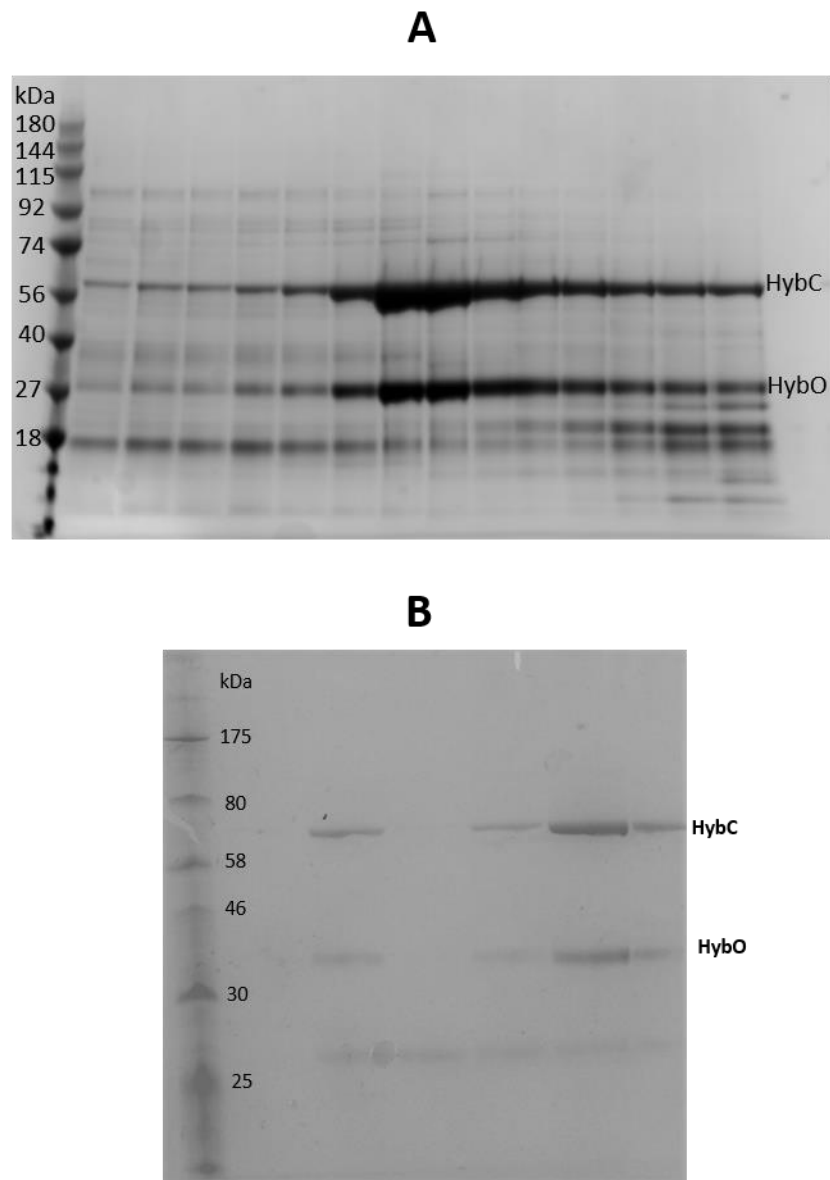


Figure 6.5: SDS-PAGE denaturing gel of **(A)** Hyd-2 R479K-OP after purification by size exclusion chromatography and **(B)** D103A-OP after purification on Ni^{2+} column. A sample (10 μL) was mixed with an equal amount of SDS-PAGE loading dye, boiled, and loaded. SDS-PAGE gel was run at 200 V for approx. 55 min. HybC (61 kDa) and HybO (33 kDa) are present. Molecular weight measured by comparison with RunBlue™ Prestained Molecular-Weight Markers (NXA6050) for **A** and Protein Standard, Broad Range (NEB #P7706) for **B**.

The ability for the overproduction system to increase the yield of an active site canopy variant further proves that the system is robust and capable of withstanding site-directed mutagenesis without affecting Hyd-2 production levels.

6.2.1.1 Hyd-2 D103A-OP

As-isolated Hyd-2 D103A-OP crystallized well in similar conditions as were initially discovered for Hyd-2 NOP, a mixture of Bis-tris (100 mM, pH 5.3-5.7), MgCl₂ (200 mM) and PEG 3350 (19-22% w/v) (see Section 4.2.1). The structure of as-isolated Hyd-2 D103A-OP was solved to a maximum resolution of 1.93 Å. Unfortunately, H₂-reduced D103A-OP did not crystallize under the same conditions even after trying to seed the crystals with the streak seeding method (see Section 4.2.2).

Table 6.1: X-ray data collection and refinement statistics for as-isolated Hyd-2 D103A-OP.

Sample	As-Isolated D103A
Data Collection	
Space group	$P2_12_12_1$
Unit cell dimensions (Å)	a=99.92, b=100.29, c=168.88
Resolution (Å)	100.3 – 1.9 (1.93-1.90)
Total reflections	580,543 (29,210)
Unique reflections	130,093 (6,535)
Completeness (%)	97.7 (99.4)
Multiplicity	4.5 (4.5)
$\langle I/\sigma \rangle$	6.2 (1.3)
R _{merge} (%)	15.1 (113)
R _{pim} (%)	11.2 (83.3)
CC _{1/2}	0.989 (0.49)
Refinement	
R _{work} /R _{free} (%)	16.8/19.6
No. of atoms	13,467
Macromolecule	12,739
Solvent	735

Average B-factors	
Macromolecule	21.7
Solvent	24.3
RMSD bond lengths (Å)	0.0176
RMSD angles (°)	1.75
Ramachandran plot (%) favoured / outliers	96.4/0
Clashscore	1.51

Values in parenthesis refer to data in the highest resolution shell. See Section 2.8 for more information about R_{merge} , R_{meas} , R_{pim} , R_{work} , R_{free} , and $CC_{1/2}$.

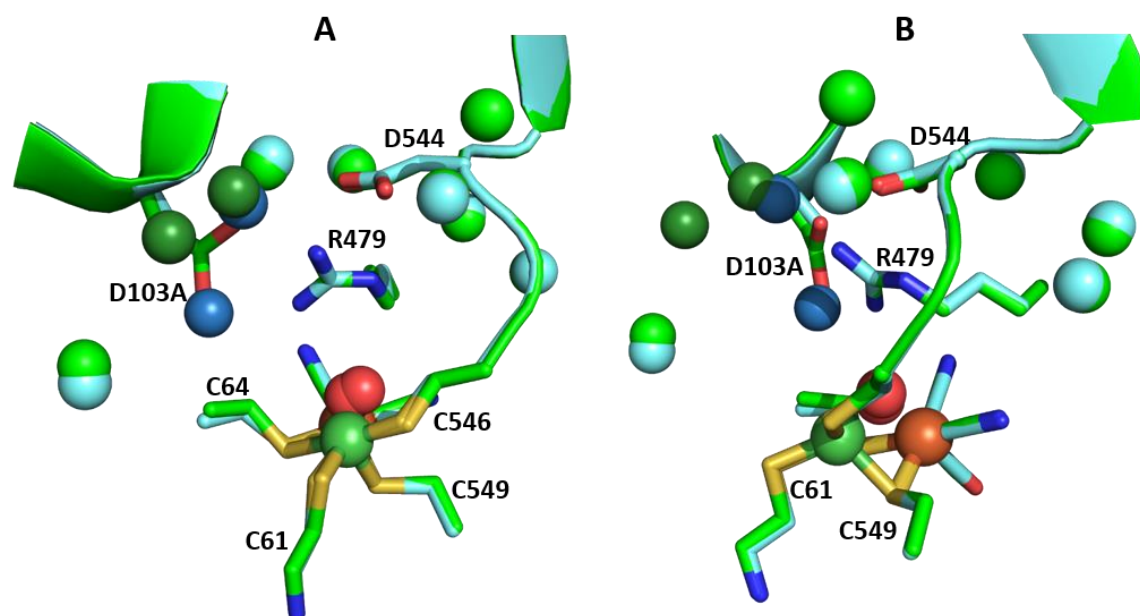


Figure 6.6: Overlay of the structure of Hyd-2 NOP (green) and Hyd-2 D103A-OP (cyan) active sites including the additional conserved water molecule from different angles. The figure in panel **A** is then rotated 90° in panel **B** to highlight the positioning of the water molecules in relation to D103/A103 of the enzyme. Waters experiencing significant shifts are darker.

The structure reveals that Hyd-2 D103A-OP has an active site that is consistent with Hyd-2 NOP. Although the D103A mutation was predicted to open a space near R479, two ordered water molecules near the active site of the variant shift to occupy positions similar to where the oxygen atoms of the aspartate reside in the native enzyme. The most drastic movement is by an ordered water molecule, which is typically approximately 5 Å

from the O δ 2 of the aspartate headgroup in the native enzyme. In Hyd-2 D103A-OP, that water molecule shifts to fill nearly the same position occupied by O δ 2 in the native enzyme (Figure 6.6 and Table 6.2). This shift allows the water molecule to move within 2.88 Å of the N η 1. Another important shift, although subtler, occurs with a water molecule that shifts 0.9 Å closer to the position of where O δ 1 resides in the native enzyme. While such a movement might be typically insignificant, it is important because it brings the water molecule from 3.62 Å of N η 2 to within 2.96 Å of N η 2, putting the water molecule into moderate hydrogen bonding distance of the arginine. The latter shift is similar to the shift seen in Hyd-1 D118A, yet importantly for Hyd-2 D103A, the water molecule is farther from D544 (4.5 Å) than the water molecule in Hyd-1 D118A is from D574 (2.9 Å). This difference could signify that the shift in the water molecule in Hyd-2 is stabilizing R479 as opposed to interacting with D544.

The fidelity of the replacement of O atoms in the native enzyme with ordered water molecules in D103A-OP is particularly true for the distances between the water molecules and R479 and D544, with increasing differences for the distance to the bimetallic cofactor and coordinating CO, yet even these latter shifts are limited (Table 6.2). The ordered water molecules allow other residues to remain mostly static, with no other single atom moving more than 0.25 Å when compared to Hyd-2 NOP (see Table 4.2 for comparison to as-isolated Hyd-2 NOP).

Table 6.2: Table of distances of active site atoms for Hyd-2 D103A-OP in both subunits (termed L and M).

Hyd-2 D103A-OP As-Isolated										
	Residue	A118	H ₂ O*	D544			Ni	Fe	CN	OH
	Atom	Cb	H ₂ O*	Od1	Od2	-CO				
R479	Nη1 (L)	4.71	2.88	4.57	4.06	5.15	4.68	4.46	3.32	3.25
	Nη1 (M)	4.75	2.87	4.57	4.09	5.13	4.71	4.42	3.14	3.14
	Average	4.73	2.88	4.57	4.08	5.14	4.70	4.44	3.23	3.20
	Nη2 (L)	4.92	3.97	2.81	3.14	4.48	5.68	6.26	5.44	4.51
	Nη2 (M)	4.86	3.78	2.85	3.16	4.50	5.70	6.23	5.33	4.29
	Average	4.89	3.88	2.83	3.15	4.49	5.69	6.25	5.39	4.40
	Ne (L)	6.45	5.13	3.04	3.20	2.83	5.14	5.00	4.33	3.46
	Ne (M)	6.45	5.06	3.06	3.20	2.83	5.19	5.01	4.29	3.41
	Average	6.45	5.10	3.05	3.20	2.83	5.17	5.01	4.31	3.44
		A118	Cb (L)	6.81	5.61	8.84	8.67	8.61	6.63	7.82
			Cb (M)	6.81	5.35	8.83	8.72	8.63	6.50	7.63
			Average	6.81	5.48	8.84	8.70	8.62	6.57	7.73
		H ₂ O*	H ₂ O (L)	6.63	5.95	7.92	5.81	5.97	4.71	5.25
			H ₂ O (M)	6.53	5.94	7.83	5.75	5.97	4.71	4.96
			Average	6.58	5.95	7.88	5.78	5.97	4.71	5.11
					D544	Od1 (L)	7.66	8.02	6.26	7.13
						Od1 (M)	7.69	8.04	6.15	7.12
						Average	7.68	8.03	6.21	7.13
						Od2 (L)	8.17	7.85	6.17	6.56
						Od2 (M)	8.27	7.98	6.23	6.55
						Average	8.22	7.92	6.20	6.56
						-CO (L)	7.03	6.87	6.53	5.42
						-CO (M)	7.06	6.89	6.52	5.45
						Average	7.05	6.88	6.53	5.44
	S-Cys 61	S-Cys 546	S-Cys 64	S-Cys 549						
	2.13	2.57	2.41	2.06		Ni (L)		2.92	5.14	1.88
	2.1	2.59	2.39	2.04		Ni (M)		2.93	5.13	1.88
	2.12	2.58	2.4	2.05		Average		2.93	5.14	1.88
			2.29	2.34		Fe (L)			2.99	2.24
			2.36	2.30		Fe (M)			2.98	2.43
			2.33	2.32		Average			2.99	2.34
									CN (L)	3.77
									CN (M)	3.79
									Average	3.78

6.2.1.2 Hyd-2 R479K-OP

Hyd-2 R479K-OP consistently crystallized in the same conditions that were optimized for Hyd-2 NOP (see above). The structure of Hyd-2 R479K-OP was originally solved in the as-isolated (oxidized) state. The reported as-isolated structure of Hyd-2

R479K-OP was solved to a resolution of 1.3 Å (Table 6.3). All enzyme was crystallized at approximately 20°C, frozen in N₂(l), and the data was collected at 100 K.

Table 6.3: X-ray data collection and refinement statistics for as-isolated and reduced Hyd-2 R479K-OP.

Sample	As-Isolated R479K	H ₂ -reduced R479K
Data Collection		
Space group	<i>P</i> ₂ ₁ <i>2</i> ₁ <i>2</i> ₁	<i>P</i> ₂ ₁ <i>2</i> ₁ <i>2</i> ₁
Unit cell dimensions (Å)	a=99.66, b=100.43, c=169.01	a=99.30, b=100.28, c=168.55
Resolution (Å)	86.3 – 1.3 (1.32-1.30)	100-1.5 (1.53-1.50)
Total reflections	4,061,443 (190,489)	1,137,751 (54,830)
Unique reflections	413,411 (20,304)	262,942 (12,626)
Completeness (%)	100 (100)	97.8 (95.7)
Multiplicity	9.8 (9.4)	4.3 (4.3)
<I/σ>	10.5 (1.1)	8.4 (1.1)
R _{merge} (%)	9.0 (148)	8.6 (109)
R _{pim} (%)	3.2 (53.5)	5.3 (67.0)
CC _{1/2}	0.999 (0.73)	0.997 (0.515)
Refinement		
R _{work} /R _{free} (%)	13.2/16.2	15.4/17.7
No. of atoms	13,928	13,866
Macromolecule	12,807	12,811
Solvent	1121	1055
Average B-factors		
Macromolecule	18.9	19.2
Solvent	26.9	23.9
RMSD bond lengths (Å)	0.0181	0.016
RMSD angles (°)	1.82	1.83

Values in parenthesis refer to data in the highest resolution shell. See Section 2.8 for more information about R_{merge} , R_{meas} , R_{pim} , R_{work} , R_{free} , and $CC_{1/2}$.

As-Isolated Hyd-2 R479K-OP										
K479	Residue	D103		D544			Ni	Fe	CN	XX*
	Atom	Od1	Od2	Od1	Od2	-CO				
	Nη1 (L)	2.71	3.60	3.34	2.95	4.61	5.33	5.58	4.28	2.99
	Nη1 (M)	2.71	3.59	3.41	2.98	4.58	5.29	5.51	4.20	3.01
	Average	2.71	3.60	3.38	2.97	4.60	5.31	5.55	4.24	3.00
		D103	Od1 (L)	4.82	4.00	7.04	7.24	7.53	5.72	4.92
			Od1 (M)	4.86	3.98	6.99	7.18	7.46	5.64	4.90
			Average	4.84	3.99	7.02	7.21	7.50	5.68	4.91
			Od2 (L)	6.56	5.83	8.20	6.35	6.57	4.95	4.57
			Od2 (M)	6.59	5.80	8.16	6.34	6.55	4.91	4.57
			Average	6.58	5.82	8.18	6.35	6.56	4.93	4.57
					D544	Od1 (L)	7.49	8.07	7.16	5.22
						Od1 (M)	7.50	8.07	7.17	5.27
						Average	7.50	8.07	7.17	5.25
						Od2 (L)	7.94	7.87	6.18	5.69
						Od2 (M)	7.94	7.88	6.18	5.74
						Average	7.94	7.88	6.18	5.72
						-CO (L)	6.83	6.95	6.70	5.43
						-CO (M)	6.83	6.97	6.71	5.45
	S-Cys 61	S-Cys 546	S-Cys 64	S-Cys 549		Average	6.83	6.96	6.71	5.44
	2.36	2.26	2.29	2.29	Ni (L)			2.64	4.81	2.55
	2.35	2.25	2.29	2.29	Ni (M)			2.63	4.80	2.52
	2.36	2.26	2.29	2.29	Average			2.64	4.81	2.54
			2.24	2.27	Fe (L)				3.01	3.92
			2.24	2.28	Fe (M)				3.01	3.93
			2.24	2.28	Average				3.01	3.93
									CN (L)	4.43
									CN (M)	4.45
									Average	4.44

The as-isolated structure of Hyd-2 R479K-OP revealed that the enzyme did not have many of the important elements of as-isolated Hyd-2 NOP. Specifically, minimal oxidation of any of the ligating thiolate-S atoms was observed. The distance between the Ni and the Fe atom is 2.64 Å, which is a distance that is typical of reduced rather than as-isolated Hyd-2 (see Section 4.2). The structure also reveals significant shifts in the

orientation of the pendant lysine from the expected position. Without the sterics of the guanidinium of the arginine, the lysine rotates around the C γ -C δ bond to take up a non-standard configuration in which the lysine head group is significantly farther from the active site than the arginine in the native enzyme. Moreover, D103 shifts inwards slightly to allow for hydrogen bonding between O δ 1 and the lysine N ζ . Although it was originally theorized that the pendant lysine might get closer upon reduction, all the structures solved of Hyd-2 R479K-OP under various gas treatments show the pendant lysine in a single conformation 5.2 Å from the Ni (see Chapter 7).

In the as-isolated enzyme, that distance between the Ni atom and the lysine allows unexpected electron density to occupy the area adjacent to the Ni in a similar configuration to the CO position seen in the structure of *D. vulgaris* MF.³⁵ The identity and significance of the molecule that was unexpectedly found at the active site is explored in depth in the following chapter (Chapter 7). Importantly, the majority of that molecule is cleared by activation under H₂. After a 20 h purge with H₂, most of the enzyme is cleared of the molecule that could inhibit activity and is instead replaced with an additional ordered H₂O molecule (numbered 3 in Figure 6.7). This ordered water molecule is in the same position off the Ni as the diatomic found in the as-isolated enzyme and is one of three water molecules found in the active site of reduced Hyd-2 R479K-OP that are not found in Hyd-2 NOP (Figure 6.7).

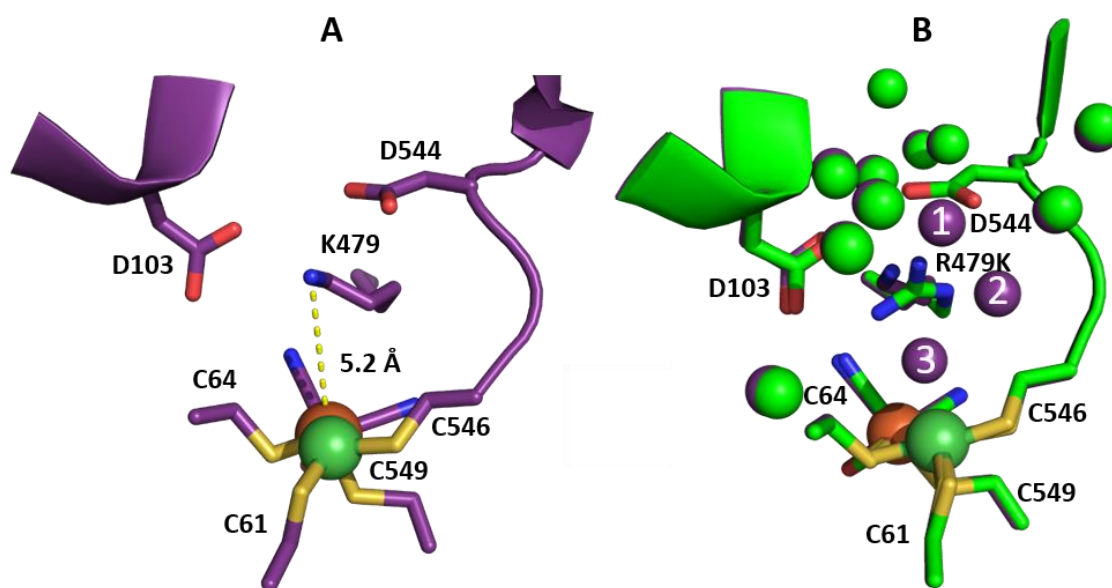


Figure 6.7: Structure of the **(A)** active site of reduced Hyd-2 R479K-OP and **(B)** an overlay of the active sites of Hyd-2 NOP (green) and Hyd-2 R479K-OP (purple) including ordered water molecules. The occupancy from the bridging hydride in Hyd-2 NOP is omitted from the figure for clarity.

One of the water molecules is positioned above the N ζ that was previously observed in Hyd-1 R509K (Figure 6.7).⁶ The second ordered water molecule is positioned 4.2 Å above the sulphur of a terminal cysteine (C546) proposed to be involved in the mechanism of H₂ activation.^{13–15} Additionally, the water molecule is 3.6 Å away from the N ζ of K479. This water molecule was not modelled in the structure of Hyd-1 R509K, yet upon analysis of the electron density maps of Hyd-1 R509K there is evidence that there is an ordered water molecule that resides in the same position in Hyd-1 R509K (private communication with Dr. Steve Carr, Research Complex at Harwell). A third water molecule replaces the diatomic found in as-isolated Hyd-2 R479K-OP and could indicate that the structure of reduced R479K-OP is of an enzymatic state that has only been characterized by IR-spectroscopy: (Ni-Si_r)_{II} (see Section 1.3.2). In the reduced structure, the third water molecule appears to be loosely coordinating the Ni atom (2.4 Å) with minimal interactions to the Fe atom (3.8 Å). In the structure of H₂-reduced Hyd-2 R479K-

OP, there is no oxidation of any of the coordinating sulphur atoms. The Ni-Fe distance in reduced R479K-OP showed no change from as-isolated Hyd-2 R479K-OP (2.6 Å) and thus aligns well with the Ni-Fe distance seen in reduced Hyd-2 NOP.

Table 6.5: Distance between residues in the active site of Hyd-2 R479K-OP after 20h of reduction with H₂.

Reduced Hyd-2 R479K-OP										
K479	Residue	D103		D544			Ni	Fe	CN	OH
	Atom	Od1	Od2	Od1	Od2	-CO				
	N η 1 (L)	2.72	3.68	3.27	2.84	4.58	5.40	5.69	4.35	2.96
	N η 1 (M)	2.72	3.63	3.37	2.95	4.61	5.32	5.60	4.33	3.19
	Average	2.72	3.66	3.32	2.90	4.60	5.36	5.65	4.34	3.08
		D103	Od1 (L)	4.93	3.99	7.08	7.23	7.53	5.67	4.85
			Od1 (M)	4.93	4.02	7.06	7.18	7.49	5.71	5.09
			Average	4.93	4.01	7.07	7.21	7.51	5.69	4.97
			Od2 (L)	6.65	5.82	8.24	6.35	6.59	4.92	4.54
			Od2 (M)	6.64	5.83	8.23	6.32	6.55	4.98	4.67
			Average	6.65	5.83	8.24	6.34	6.57	4.95	4.61
					D544	Od1 (L)	7.48	8.05	7.13	5.14
						Od1 (M)	7.50	8.09	7.20	5.49
						Average	7.49	8.07	7.17	5.32
						Od2 (L)	7.91	7.85	6.13	5.58
						Od2 (M)	7.94	7.87	6.18	5.93
						Average	7.93	7.86	6.16	5.76
						-CO (L)	6.82	6.94	6.68	5.37
						-CO (M)	6.86	6.98	6.69	5.60
						Average	6.84	6.96	6.69	5.49
	S-Cys61	S-Cys546	S-Cys64	S-Cys549		Ni (L)		2.61	4.76	2.64
	2.31	2.24	2.27	2.31		Ni (M)		2.61	4.75	2.28
	2.32	2.23	2.26	2.3		Average		2.61	4.76	2.46
	2.32	2.24	2.27	2.31						
			2.24	2.27		Fe (L)			3.01	3.98
			2.23	2.26		Fe (M)			2.96	3.71
			2.24	2.27		Average			2.99	3.85
									CN (L)	4.38
									CN (M)	4.35
									Average	4.37

6.2.2 Enzymatic profile

6.2.2.1 Voltammogram shape

A cyclic voltammogram provides a snapshot of the activity of the enzyme, and for Hyd-2 D103A-OP, the voltammogram reveals that Hyd-2 D103A-OP is a typical, reversible

enzyme that cuts sharply through the equilibrium potential showing minimal overpotential for both H_2 oxidation and production.

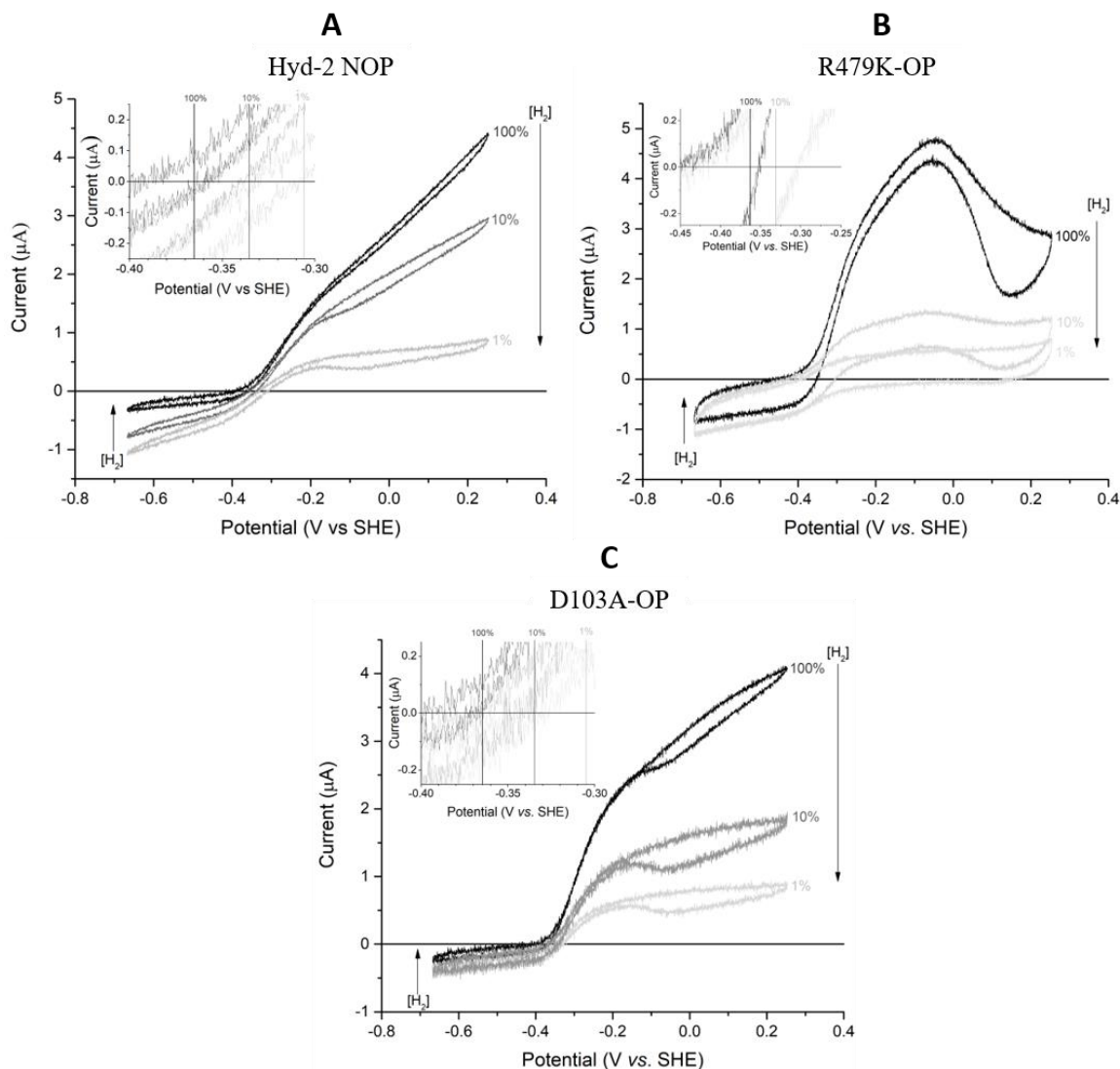


Figure 6.8: Measurements of activity under different H_2 concentrations for **(A)** Hyd-2 NOP, **(B)** Hyd-2 R479K-OP, and **(C)** Hyd-2 D103A-OP. Increasing activity corresponds with increasing H_2 concentration, and the zero-current potential corresponds well to the values predicted by the Nernst equation (inset). Other conditions: scan rate 5 mv/sec, 30°C, pH 6, carrier gas Ar, $\omega=3000$ rpm.

The shape and scale of activity of the CV changes little during the course of activation, and the enzyme shows a slight increase in enzymatic activity during activation on an electrode. This is likely because the rate of activation and film loss are approximately equivalent during activation as is typically the case for Hyd-2 NOP. Moreover, Hyd-2 D103A-OP shows little inactivation at high potentials and the corresponding reactivation

of Ni-B on the backwards scan is minimal. Overall, the CV for Hyd-2 D103A-OP is similar to the CV for Hyd-2 NOP.

Hyd-2 R479K-OP exhibits a drastically different voltammogram from that of Hyd-2 NOP. First, Hyd-2 R479K-OP experiences significant growth in current during the initial activation protocol (Figure 6.9). The maximum current from H₂ oxidation typically increases by almost an order of magnitude over the course of activation for Hyd-2 R479K-OP, which is likely a reflection of the unexpected inhibitor being reduced and/or cleared from the active site (see Chapter 7). During the activation, the shape of the voltammogram also changes. The forward scans of early voltammograms show a sharp rise in current, a plateau of current, and finally near total inactivation at oxidizing potentials even at a scan rate of 30 mV/sec with the reverse scan showing rapid reactivation of the enzyme (Figure 6.9). Later scans better resemble native Hyd-2 voltammograms, yet the CVs of Hyd-2 R479K-OP consistently exhibit more inactivation and cut more sharply through the X-axis at the potential predicted by the Nernst equation (Figure 6.10).

Inactivation occurring at oxidizing potentials in an anaerobic environment typically indicates formation of Ni-B (see Figure 1.2 in Section 1.3.2), but given the evidence of the possible stabilization of a second state outside of the catalytic cycle in Hyd-2 R479K-OP (see below), it is unclear what portion of anaerobic inactivation in Hyd-2 R479K-OP represents formation of Ni-B. Thus, in this thesis, the inactive state(s) formed at oxidizing potentials in an anaerobic environment for Hyd-2 R479K-OP are not referred to as simply Ni-B.

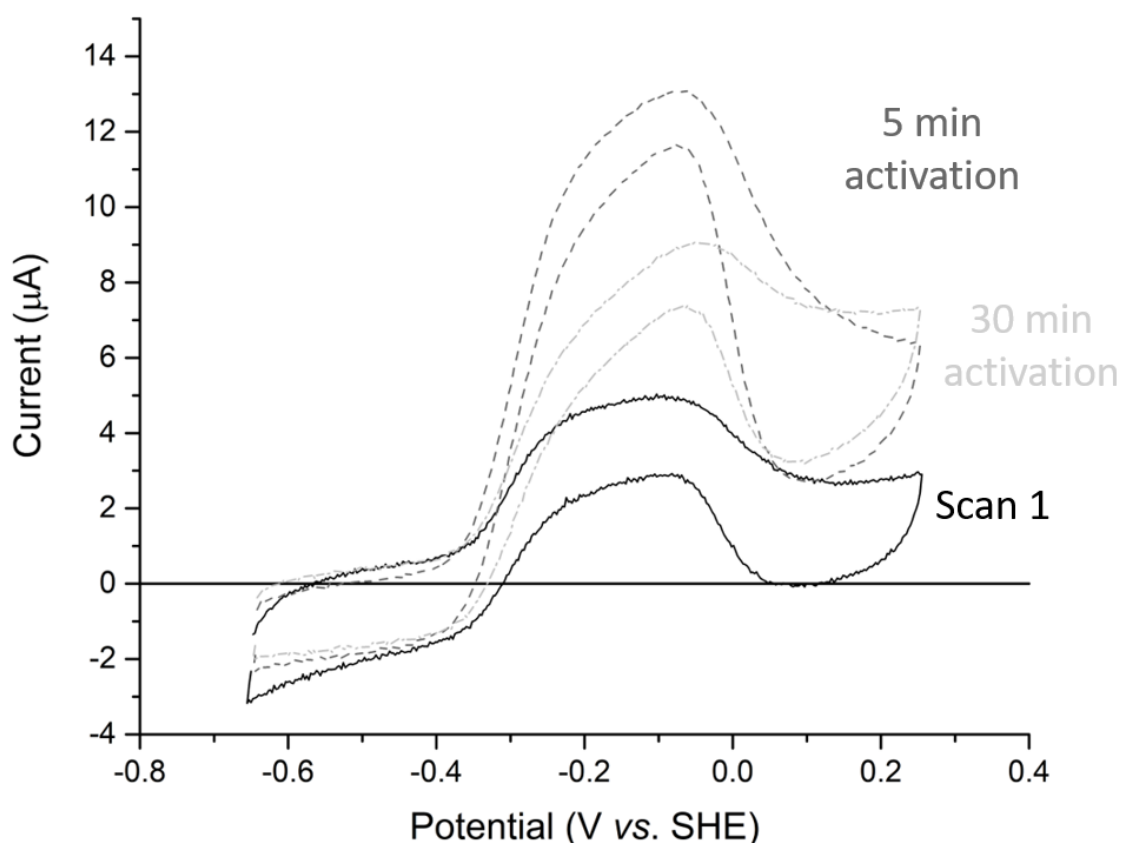


Figure 6.9: Time-series cyclic voltammograms of Hyd-2 R479K-OP with earlier scans depicted as darker. The first scan of a Hyd-2 R479K-OP film shows little activity (dark solid line), rising sharply after 5 min of cycling potentials (grey dotted line), and slowly changing shape after >30 min of activation protocol (dotted and dashed light grey line). Other conditions: scan rate 30 mv/sec, 30°C, pH 6, 100% H₂, ω =1000 rpm.

The sharper rise in current for Hyd-2 R479K-OP allows for accurate measurement of enzymatic parameters, such as $K_M^{H_2}$ and $\Delta H^\ddagger$, at potentials only slightly more oxidizing than the equilibrium potential (Figure 6.10). This makes the direct comparison of Hyd-2 R479K-OP and Hyd-2 NOP challenging because at potentials close to the equilibrium potential, Hyd-2 NOP activity is too low for accurate measurement of enzymatic parameters, and at higher potentials, oxidative inactivation in Hyd-2 R479K-OP interferes with accurately measuring enzymatic parameters under steady-state turnover conditions (Figure 6.10). Notwithstanding these limitations to direct comparison, the measured enzymatic parameters of Hyd-2 R479K-OP differ significantly from Hyd-2 NOP.

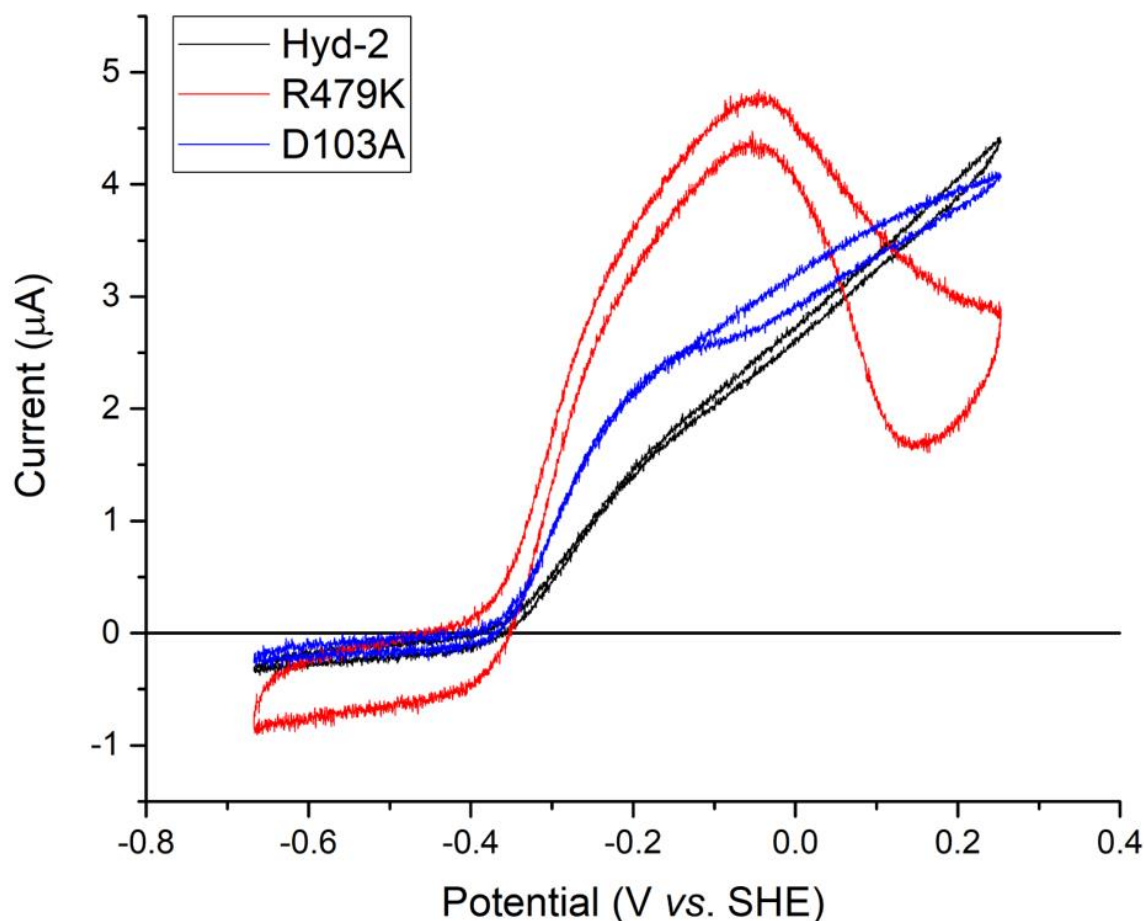


Figure 6.10: Overlay of cyclic voltammograms of Hyd-2 NOP and canopy variants. The overlay highlights the different current profiles of the enzymes and the quick rise in current shown by Hyd-2 R479K-OP after the H_2/H^+ equilibrium potential. Other conditions: pH 6.0, 30°C, 5 mV/sec, 100% H_2 , and $\omega=3000$ RPM.

6.2.2.2 $K_M^{H_2}$ and $K_i^{H_2}$

The same methods for determining the $K_M^{H_2}$ and $K_i^{H_2}$ of Hyd-2 NOP (Section 3.2.6) were used for the canopy variants. In short, to calculate $K_M^{H_2}$ slow (5 mV/sec) scans at varying concentrations of H_2 were used to populate a Lineweaver-Burke plot. The $K_i^{H_2}$ for the variants was calculated by holding the enzyme film at -660 mV and varying the concentration of H_2 and plotting the resulting data on a Hanes-Wolfe plot. For Hyd-2 NOP and Hyd-2 D103A-OP, the same H_2 concentrations were sufficient to capture the relevant portions of the Michaelis-Menten curve (Section 2.10.3.4) for $K_M^{H_2}$ and $K_i^{H_2}$. Initial results

revealed that the $K_M^{H_2}$ and $K_i^{H_2}$ of Hyd-2 R479K-OP are much higher and required a different set of H_2 concentrations for calculation of $K_M^{H_2}$ and $K_i^{H_2}$.

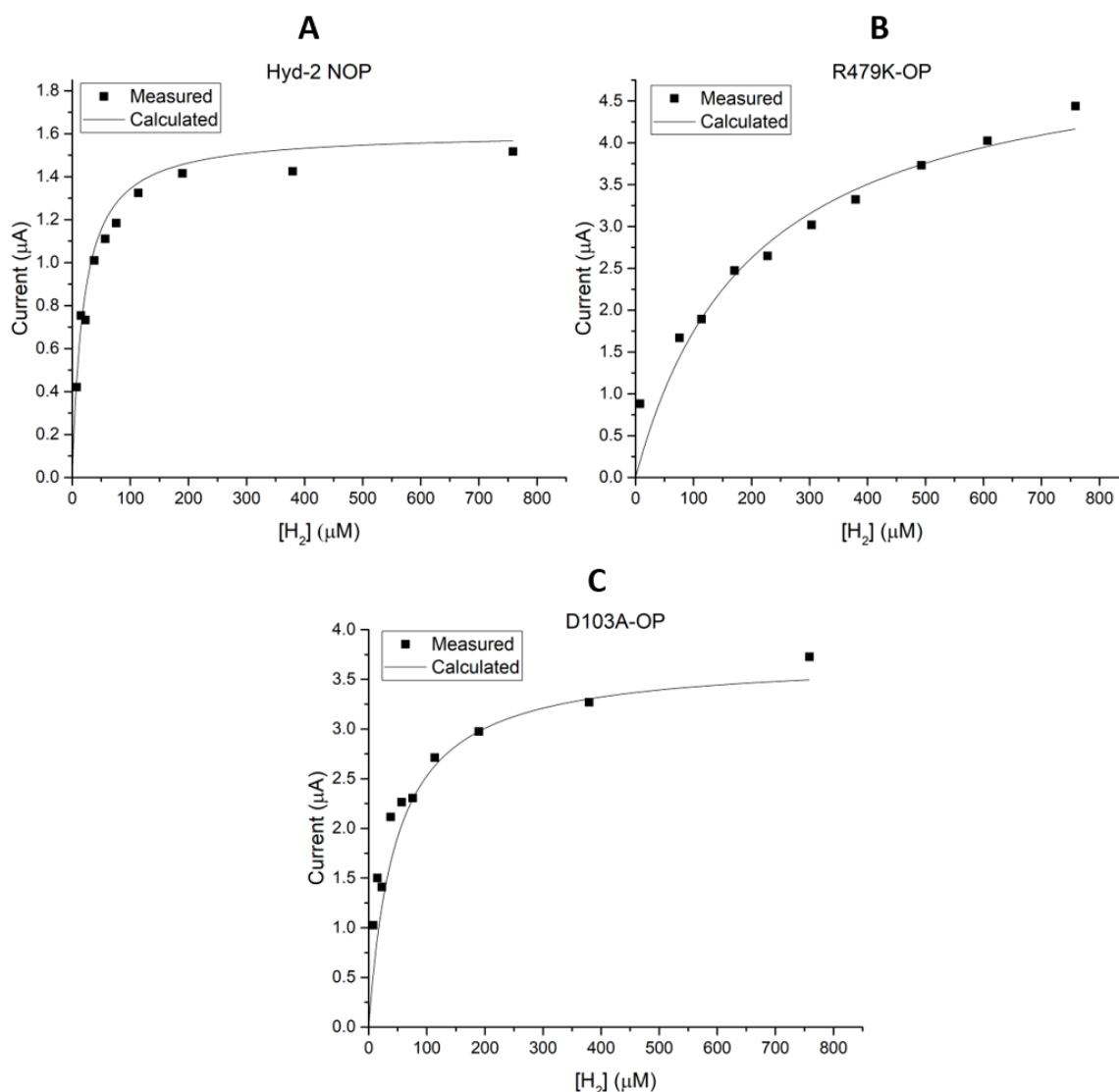


Figure 6.11: Comparison of the activity at -0.175 V with the calculated curve based on the K_M and v_{max} for (A) Hyd-2 NOP, (B) Hyd-2 R479K-OP, and (C) D103A-OP. Other conditions for measurement of the activity: pH 6.0, $30^\circ C$, 5 mV/sec, 100% H_2 , and $\omega=3000$ RPM.

The activity at ten different H_2 concentrations were still used to calculate both enzyme parameters in Hyd-2 R479K-OP, but rather than being primarily clustered between 1-200 μM H_2 like the concentrations used for Hyd-2 NOP and Hyd-2 D103A-OP, the concentrations for determining the $K_M^{H_2}$ of Hyd-2 R479K-OP evenly spanned 10-750

$\mu\text{M H}_2$ (Figure 6.11). Even at the highest possible concentration of H_2 at 1 atmosphere and 30°C , the data range is insufficient to approximate V_{max} , unlike the data from other enzymes (Figure 6.11). Although plotting this data in the Lineweaver-Burk plot gives the V_{max} and $K_{\text{M}}^{\text{H}_2}$, the lack of data points where the activity nears the V_{max} makes those calculated parameters for R479K-OP less accurate.

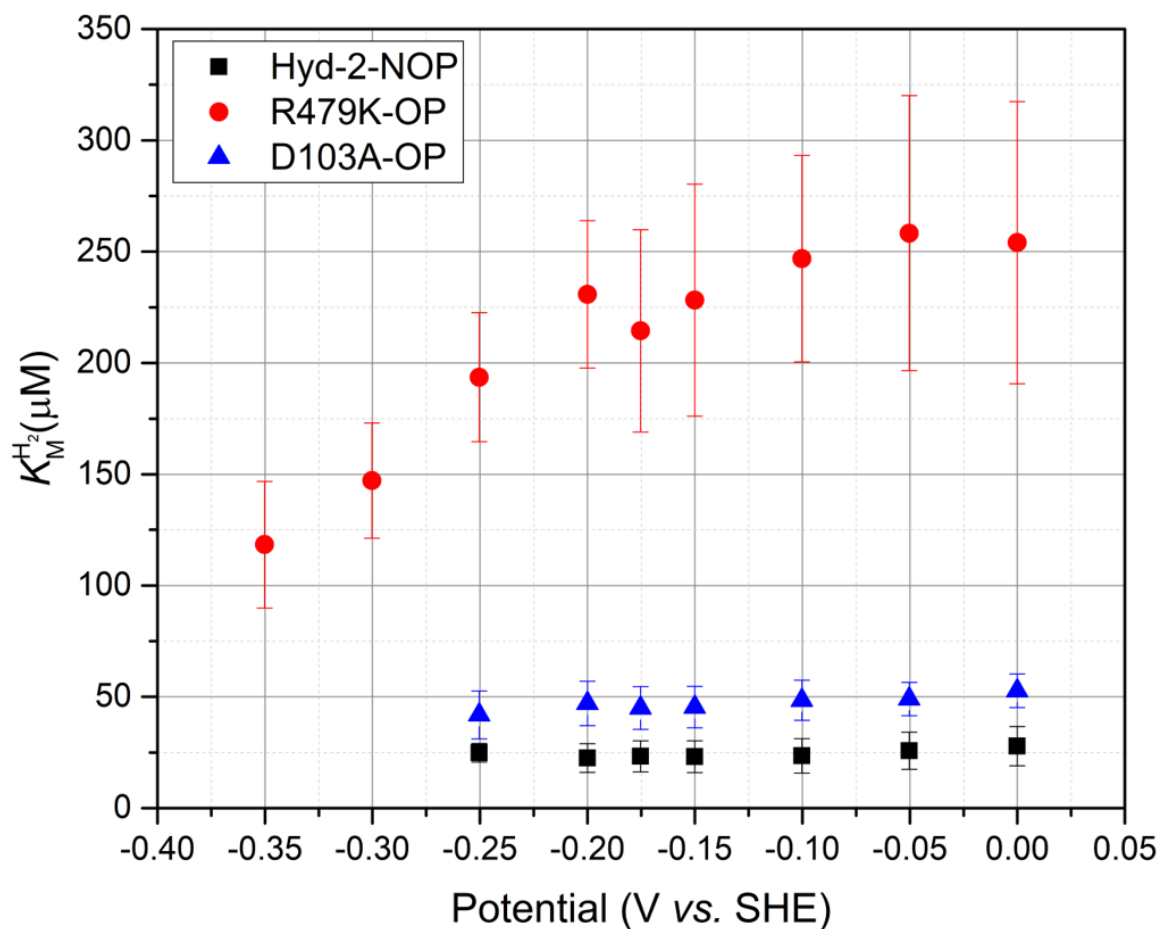


Figure 6.12: Michaelis constant measured for Hyd-2 NOP, Hyd-2 R479K-OP, and Hyd-2 D103A-OP measured at different potentials. Other conditions: pH 6.0, 5 mV/sec, 100% H_2 , and $\omega=3000$ RPM.

The resulting calculations showed that Hyd-2 D103A-OP has a slightly raised $K_{\text{M}}^{\text{H}_2}$ (Figure 6.12). Although the $K_{\text{M}}^{\text{H}_2}$ for Hyd-2 D103A-OP is only slightly increased, it shows slight dependence increasing with the potential. The $K_{\text{M}}^{\text{H}_2}$ of Hyd-2 R479K-OP is significantly higher than the $K_{\text{M}}^{\text{H}_2}$ of Hyd-2 NOP and heavily potential dependent (Figure

6.12). Again, rapid oxidative inactivation impairs precise measurement of the $K_M^{H_2}$, and the measured $K_M^{H_2}$ at higher potentials is likely only an estimate because with a $K_M^{H_2}$ that large, the data does not capture the full window of activity. At -0.175 V, the measured $K_M^{H_2}$ for Hyd-2 R479K-OP is 214.4 μM .

Moreover, Hyd-2 R479K-OP experiences significantly less product inhibition than Hyd-2 NOP. While Hyd-2 NOP has an $K_i^{H_2}$ of $240 \pm 10.6 \mu\text{M}$, Hyd-2 R479K-OP has a $K_i^{H_2}$ of $1.08 \pm 0.096 \text{ mM}$ (Figure 6.13).

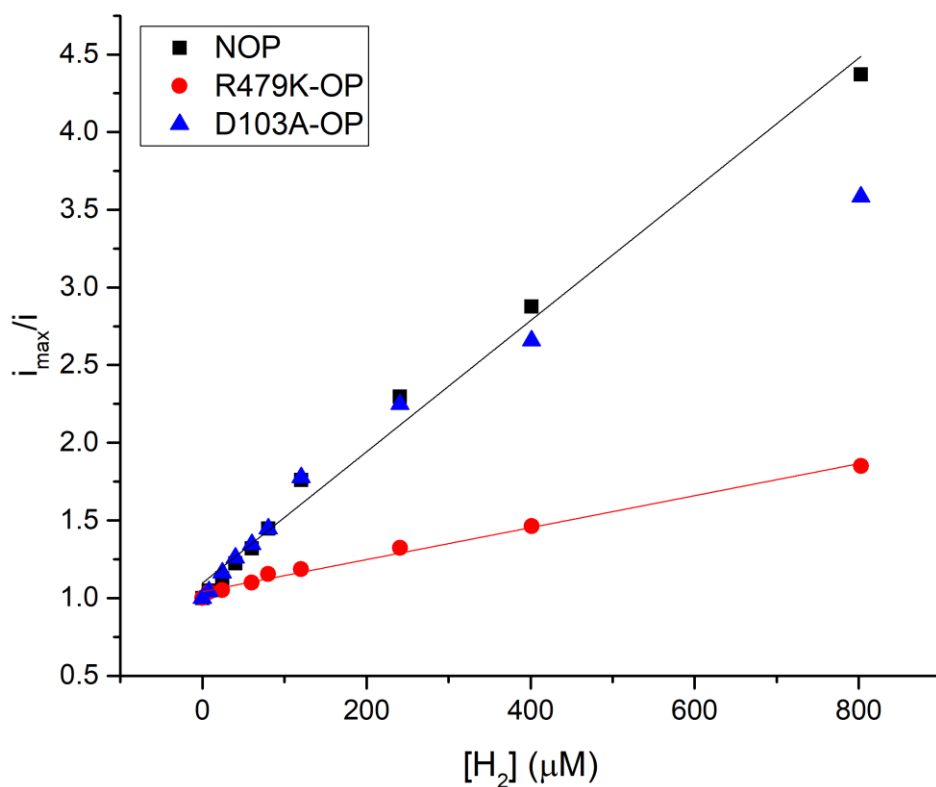


Figure 6.13: Measurements for $K_i^{H_2}$ for H_2 for Hyd-2 NOP, Hyd-2 R479K-OP, and Hyd-2 D103A-OP.

Hyd-2 D103A-OP exhibits a similar inhibition to H_2 in the low range of H_2 concentration ($<250 \mu\text{M}$), but in the higher range, Hyd-2 D103-OP exhibits less inhibition than Hyd-2 NOP (Figure 6.14). For Hyd-2 D103A-OP, $K_i^{H_2}$ is therefore calculated using H_2 concentrations less than $250 \mu\text{M}$ because the inhibition experienced at higher

concentration of H_2 heavily affects the slope of the line. Moreover, graphing the activity against the concentration of the inhibitor for Hyd-2 D103A-OP shows that the activity is halved at approximately 200 μM H_2 (Figure 6.14B).

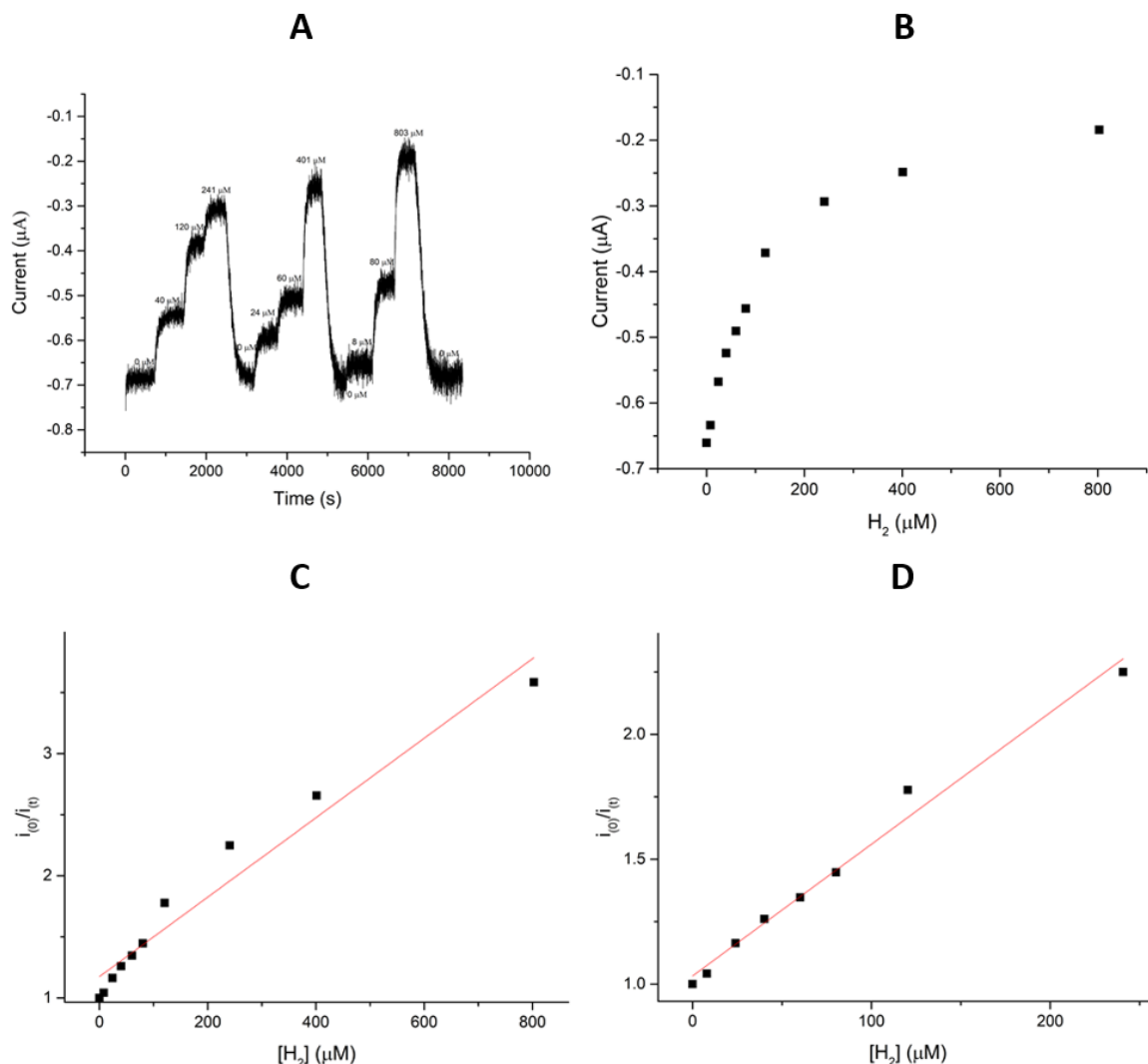


Figure 6.14: Measurements for inhibition constant for H_2 for Hyd-2 D103A including the (A) chronoamperometry measuring H_2 production activity as a function of H_2 , (B) plot of the current from H_2 production, (C) Hanes-Wolfe plot over the entire range of H_2 concentrations tested, and (D) Hanes-Wolfe plot between 0-250 μM H_2 . Potential was held at -0.660 V and the order of concentrations tested were 0 μM , 40 μM , 120 μM , 241 μM , 0 μM , 24 μM , 60 μM , 401 μM , 0 μM , 8 μM , 80 μM , 803 μM , 0 μM . The return to 0 μM H_2 every fourth scan allowed correction for film loss, and the plot shown is corrected for film loss. Other conditions: pH 6.0, 20°C, 100% Ar, and $\omega=3000$ RPM.

6.2.2.3 E_{SWITCH}

The potential at which the protein reactivates under specific parameters is known as E_{SWITCH} and is defined as the minimum of the first derivative of the backwards scan

after complete anaerobic inactivation of the enzyme. For Hyd-2 D103A-OP, the E_{SWITCH} is -0.022 V, which is approximately equivalent to the E_{SWITCH} for Hyd-2 NOP (Figure 6.15). This measurement has typically been a proxy measurement of the stability of Ni-B. Thus, the similarity of the values for E_{SWITCH} likely reflects that the D103A mutation does not have a large effect on the stability of Ni-B.

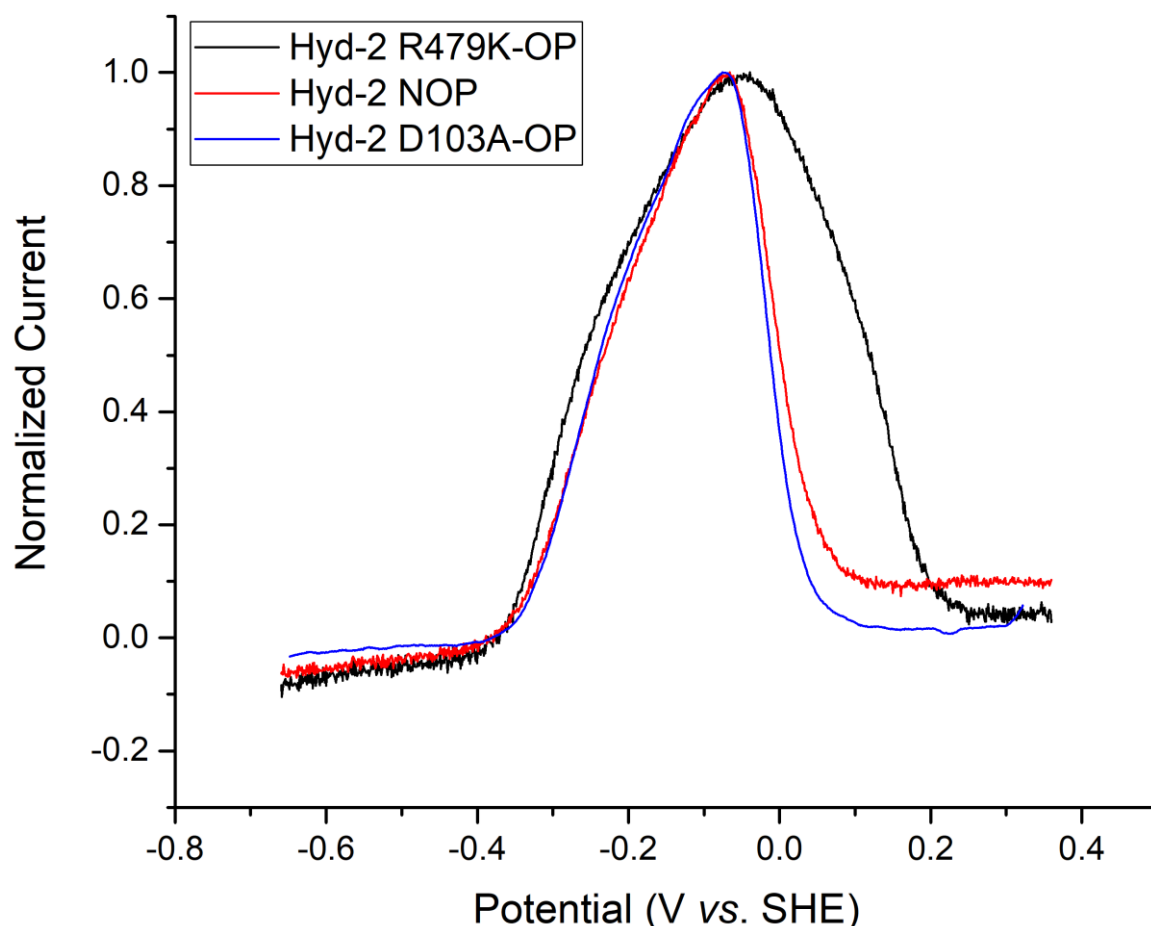


Figure 6.15: Overlay of backwards scans following anaerobic inactivation at high potential for Hyd-2 NOP, Hyd-2 R479K-OP, and Hyd-2 D103A-OP. The driving force required to reactivate Ni-B gives insight into the stability of the resting inactive state. Data have been normalized to max current for each experiment for easier comparison. Hyd-2 data for E_{SWITCH} has been smoothed with the Savitzky-Golay method, 41 points per window. Other conditions: pH 6.0, 30°C, 0.1mV/sec, 100% H_2 , and $\omega=1000$ RPM.

The measured E_{SWITCH} for Hyd-2 R479K-OP is 0.158 V, but is complicated by the shape of the reactivation profile. Typically, there is a single inflection point, which is reported as E_{SWITCH} . For Hyd-2 R479K-OP, the reactivation profile shows a strong

inflection point at the potential reported above (0.158 V) with a second weaker pseudo-inflection point at a potential more typical for E_{SWITCH} of Hyd-2 NOP—around 0 V (Figure 6.16).

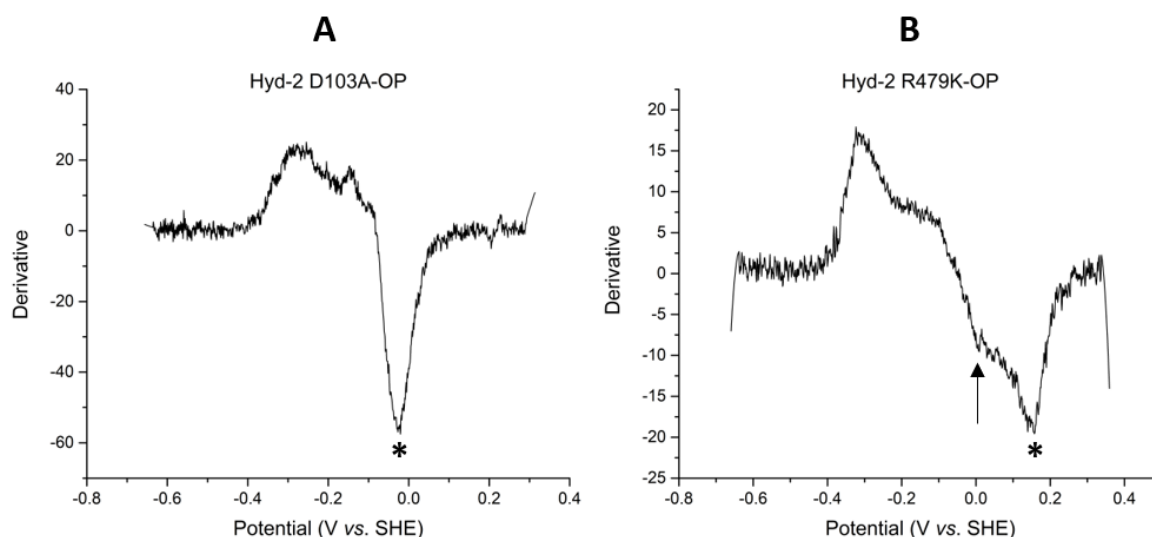


Figure 6.16: Comparison of the first derivative of the backwards scan for **(A)** Hyd-2 D103A-OP and **(B)** Hyd-2 R479K-OP. The asterisked minimums are the reported values for E_{SWITCH} , and the arrow in the graph for Hyd-2 R479K-OP indicates the presence of a second pseudo inflection point close to the typical potential of E_{SWITCH} of Hyd-2 NOP. Other conditions: pH 6.0, 30°C, 0.1mV/sec, 100% H_2 , and $\omega=1000$ RPM.

6.2.2.4 Turnover rate

According to spectrophotometric activity assays, the activity of Hyd-2 D103A-OP has decreased by an order of magnitude for both H_2 oxidation and H_2 production compared with Hyd-2 NOP (Figure 6.17). These measurements confirm what is observed in the cyclic voltammograms of D103A-OP in that the apparent bias of the enzyme did not change. Hyd-2 R479K-OP has a rate of H_2 oxidation of $4.97 \mu\text{mol H}_2 \text{ s}^{-1}$ showing a 50-fold decrease in the activity from Hyd-2 NOP (Figure 6.17). Because the potential of benzyl viologen is -0.359 V, this assay is better suited for enzymes like Hyd-2 R479K-OP that cut extremely sharply through the equilibrium potential. Thus, a direct comparison

of oxidation turnover rates between Hyd-2 NOP and Hyd-2 R479K-OP with benzylviologen as an electron acceptor understates the turnover rate for Hyd-2 NOP.

Hyd-2 R479K-OP has a rate of H₂ production of 0.030 $\mu\text{mol H}_2 \text{ min}^{-1} \text{ mg}^{-1}$ which equates to an approximately 150-fold decrease in H₂ production activity. Thus, in Hyd-2 the R479K mutation seemingly has more of an effect on the rate of H₂ production than the rate of H₂ oxidation.

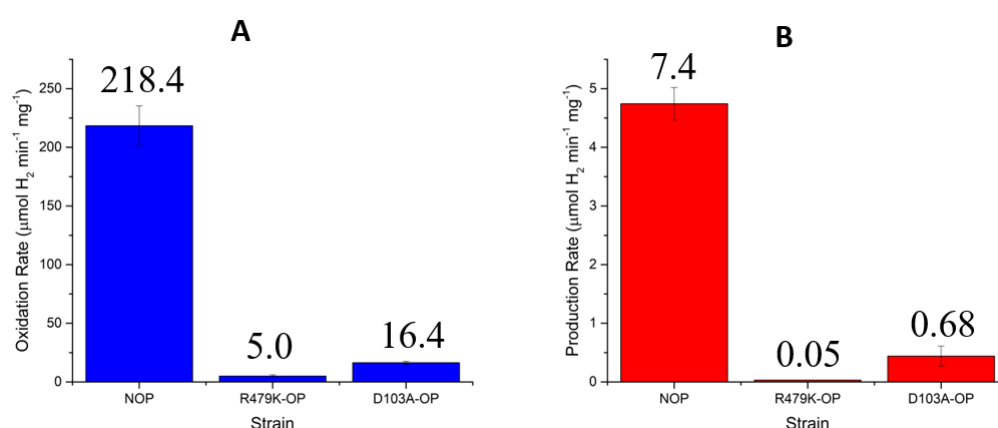


Figure 6.17: Rate of **(A)** H₂ oxidation and **(B)** production for Hyd-2 NOP, Hyd-2 R479K-OP, and Hyd-2 D103A-OP.

6.2.2.5 The effect of changing temperature on Hyd-2 variants

Figure 6.18 shows voltammograms of Hyd-2 NOP and the Hyd-2 canopy variants at different temperatures. Hyd-2 D103A-OP exhibits slightly more inactivation at high potentials than Hyd-2 NOP and shows a similar trend of increasing inactivation at higher temperatures. As shown earlier, Hyd-2 R479K-OP has a high rate of oxidative inactivation. Hyd-2 R479K-OP follows a more dramatic increase of inactivation at higher temperatures, and over 20°C, oxidative inactivation becomes so rapid that increasing the temperature appears to decrease Hyd-2 R479K-OP activity at most potentials. However, Hyd-2 R479K-OP shows increased activity corresponding to increased temperatures at lower potentials (-0.35 V to -0.20 V) where oxidative inactivation is less prevalent suggesting that the decrease

in H₂ oxidation could partially be attributed to rapid inactivation rather than decreased activity.

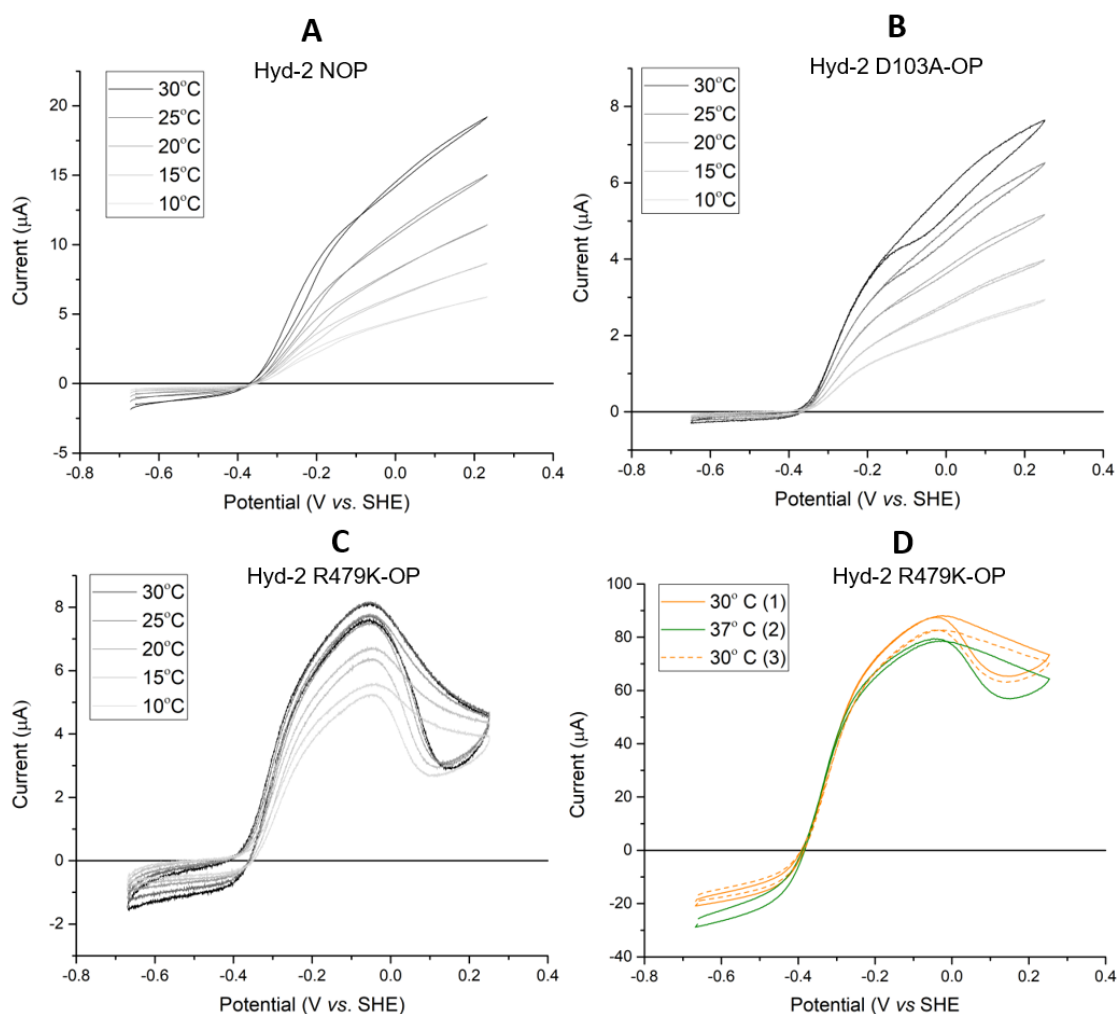


Figure 6.18: Cyclic voltammograms at varying temperatures for (A) Hyd-2 NOP, (B) Hyd-2 R479K-OP, and (C) Hyd-2 D103A-OP. (D) Overlay of scans of Hyd-2 R479K-OP at different temperatures. The film of Hyd-2 R479K-OP was taken to a higher temperature and then lowered again to 30°C to show that the activity appears to decrease at higher temperatures. These CVs are not corrected for film loss and are on a PGE electrode with multi-walled carbon nanotubes increasing the coverage and stability of a Hyd-2 R479K-OP film (see Section 2.10.4). Other conditions: pH 6.0, 5 mV/sec, 100% H₂, and $\omega=3000$ RPM.

The standard enthalpy of reaction for the formation of the transition state, $\Delta H^\ddagger$, was calculated by incorporating data from Figure 6.18 into Eyring plots (Figure 6.19). The electrochemistry of hydrogenase on PGE does not allow for calculation of the turnover rate which is required to calculate standard entropy, $\Delta S^\ddagger$. The calculated $\Delta H^\ddagger$ was

therefore used in combination with the turnover rates obtained from the spectrophotometric solution assays to calculate $\Delta S^\ddagger$. The rate of H_2 oxidation was measured with benzyl viologen (-0.359 V) and ideally the $\Delta H^\ddagger$ used to calculate the $\Delta S^\ddagger$ would be from that potential as well.

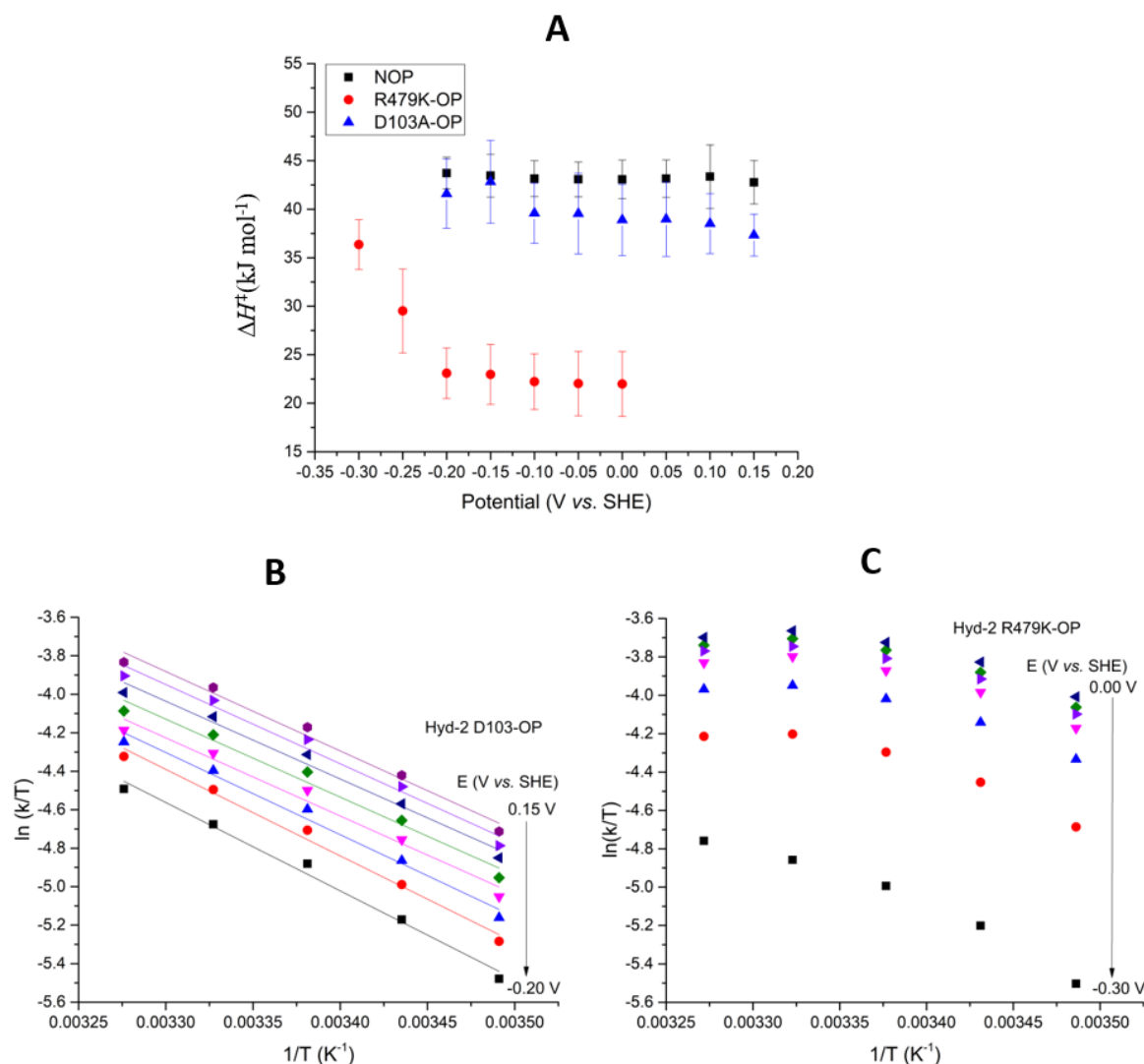


Figure 6.19: Panel A contains values for $\Delta H^\ddagger$ of H_2 oxidation activity (at -0.200 V vs. SHE) at varying temperatures for Hyd-2 NOP, Hyd-2 D103A-OP, and Hyd-2 R479K-OP calculated from Eyring plots (panels B and C). Each set of points represents the activity at a single potential over a series of temperatures. See Figure 3.16 in Chapter 3 for the Eyring plots of Hyd-2 NOP. Other conditions: pH 6.0, 5 mV/sec, 100% H_2 , and $\omega=3000$ RPM.

Unfortunately, the activity of Hyd-2 NOP and Hyd-2 D103A-OP is too low at that potential for reliable calculation of $\Delta H^\ddagger$. Thus, for Hyd-2 NOP and Hyd-2 D103A-OP, the $\Delta H^\ddagger$

calculated at -0.200 V was used, and for Hyd-2 R479K-OP the $\Delta H^\ddagger$ calculated at -0.300 V was used. The effect of this difference is likely minimal as the calculated $\Delta H^\ddagger$ for Hyd-2 NOP and Hyd-2 D103A-OP does not vary widely with potential.

The value of $\Delta H^\ddagger$ for Hyd-2 D103A-OP is slightly lower over most potentials than the $\Delta H^\ddagger$ of Hyd-2 NOP, but the two $\Delta H^\ddagger$ figures are within error of each other. However, the calculated $\Delta S^\ddagger$ for Hyd-2 D103A-OP is $-84.7 \text{ J K}^{-1} \text{ mol}^{-1}$, $35.7 \text{ J K}^{-1} \text{ mol}^{-1}$ lower than Hyd-2 NOP, which means that the decrease in turnover rate measured by solution assays is likely the effect of an increase in the entropic barrier for the reaction. This finding is consistent with an active site that has lost stability and has therefore experienced a range of small shifts that must be ordered for the reaction to proceed. Although the ordered water molecules replacing the O δ atoms of the native enzyme help stabilize the active site, they lack covalent linkage to the proteinaceous scaffolding that would provide greater stability for the amino acids essential to the reaction at the active site, and therefore could help explain the entropic barrier in D103A-OP.

Hyd-2 R479K-OP has a calculated $\Delta H^\ddagger$ of 23 kJ mol^{-1} at -0.20 V which likely reflects a decrease in the activation energy of the rate limiting step. The significantly lowered rate of oxidation indicates that there must be a large entropic barrier for the reaction as the $\Delta H^\ddagger$ has been lowered. Indeed, the calculated $\Delta S^\ddagger$ is $-111.7 \text{ J K}^{-1} \text{ mol}^{-1}$, which is $62.7 \text{ J K}^{-1} \text{ mol}^{-1}$ lower than the calculated value for Hyd-2 NOP.

However, these calculations are complicated by a convex curved Eyring plot for Hyd-2 R479K-OP (Figure 6.19). A convex curved Eyring plot could be indicative of two overlapping processes with a different rate-limiting step. In Hyd-2 R479K-OP, the rapid inactivation, perhaps due to the stabilization of a water molecule at the active site seen in the structure, likely affects measurements of the $\Delta H^\ddagger$. For most moderately oxidizing

potentials, Ni-B formation is sufficiently slow in native Hyd-2 under 100% H₂ that the effect of formation of and reactivation from Ni-B are negligible on the calculations of $\Delta H^\ddagger$ (Figure 6.18). However, the increase in the rate of oxidative inactivation with temperature for Hyd-2 R479K-OP is so high that only measurements taken below 25°C can be used as they are pseudolinear. Thus, the $\Delta H^\ddagger$ for Hyd-2 R479K-OP was calculated by taking the slope from the current measured at the four lowest temperatures (10°C, 15°C, 20°C, and 25°C) for comparison to Hyd-2 NOP.

The $\Delta H^\ddagger$ measurements for Hyd-2 R479K-OP at higher potentials are also more affected by anaerobic inactivation, thus Eyring plots at -0.30 V and -0.25 V should be more linear. This observation is consistent with the data (Figure 6.19), though there is still significant curvature for the Eyring plots at -0.30 V and -0.25 V. Moreover, using data from 30mV/s scans should render more linear Eyring plots than those populated with the activity from 5 mV/s scans if the second overlapping rate-determining step is formation of a Ni-B like state. However, a comparison of the Eyring plots from these two conditions shows that they are not substantially different (Figure 6.20). The calculated $\Delta H^\ddagger$ from these two scan rates are within error of each other.

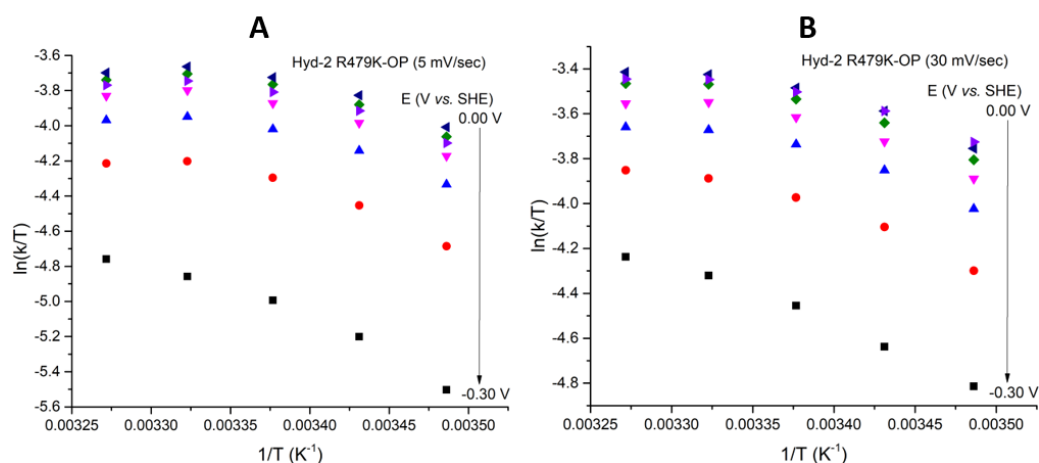


Figure 6.20: Comparison of Eyring plots for Hyd-2 R479K-OP at (A) 5 mV/sec and (B) 30 mV/sec. Other conditions: pH 6.0, 100% H₂, and $\omega=3000$ RPM.

6.2.2.6 Sensitivity to O₂

Hyd-2 NOP exhibits a high but reversible sensitivity to O₂. When the enzyme is exposed to 16 µM (final concentration) of O₂ at 0.05 V during the forward scan, Hyd-2 NOP is fully, but reversibly, inhibited (Figure 6.21). Hyd-2 NOP does not recover from the O₂ attack until below 0 V in the reverse scan. The phenomenon seen in Hyd-2 R479K-OP of rapid anaerobic inactivation with a high E_{SWITCH} complicates O₂ injection experiments as the potential at which the O₂ should be injected has typically been based on E_{SWITCH} .^{36,37} The potential at which the enzyme reactivates is used to benchmark O₂ injection to deconvolute anaerobic Ni-B formation and aerobic Ni-A formation in the enzyme's response to O₂. For Hyd-2 R479K-OP, E_{SWITCH} is so high and anaerobic inactivation so rapid that injecting O₂ around the value of E_{SWITCH} (0.175V) would result in injecting O₂ at a point where the hydrogenase is almost inactivated entirely from anaerobic inactivation. On the other hand, injecting O₂ at potentials at which anaerobic inactivation has not become excessively rapid could test aerobic attack at a potential sufficiently reducing to conceal the enzyme's true sensitivity to O₂.

Balancing these limitations, the O₂ sensitivity of Hyd-2 R479K-OP was tested by various means. At first, the O₂ sensitivity of Hyd-2 R479K-OP was tested by injecting O₂ to a final O₂ concentration of 16 µM at 0.05 V resulting in almost no inactivation (Figure 6.21). A second subsequent injection of the same amount of O₂ was done at 0.10 V with a similarly small impact on the activity of Hyd-2 R479K-OP. These results show that Hyd-2 R479K-OP is significantly less sensitive to O₂, requiring 130 µM O₂ to achieve only half the inactivation as seen with 16 µM for Hyd-2 NOP (Figure 6.21). However, Hyd-2 R479K-OP does not recover from the O₂ attack until it reaches very reducing potentials, and the

decrease in current from the O_2 attack is not recovered until the following scan regardless of the direction of the scan during which O_2 is injected (Figure 6.21).

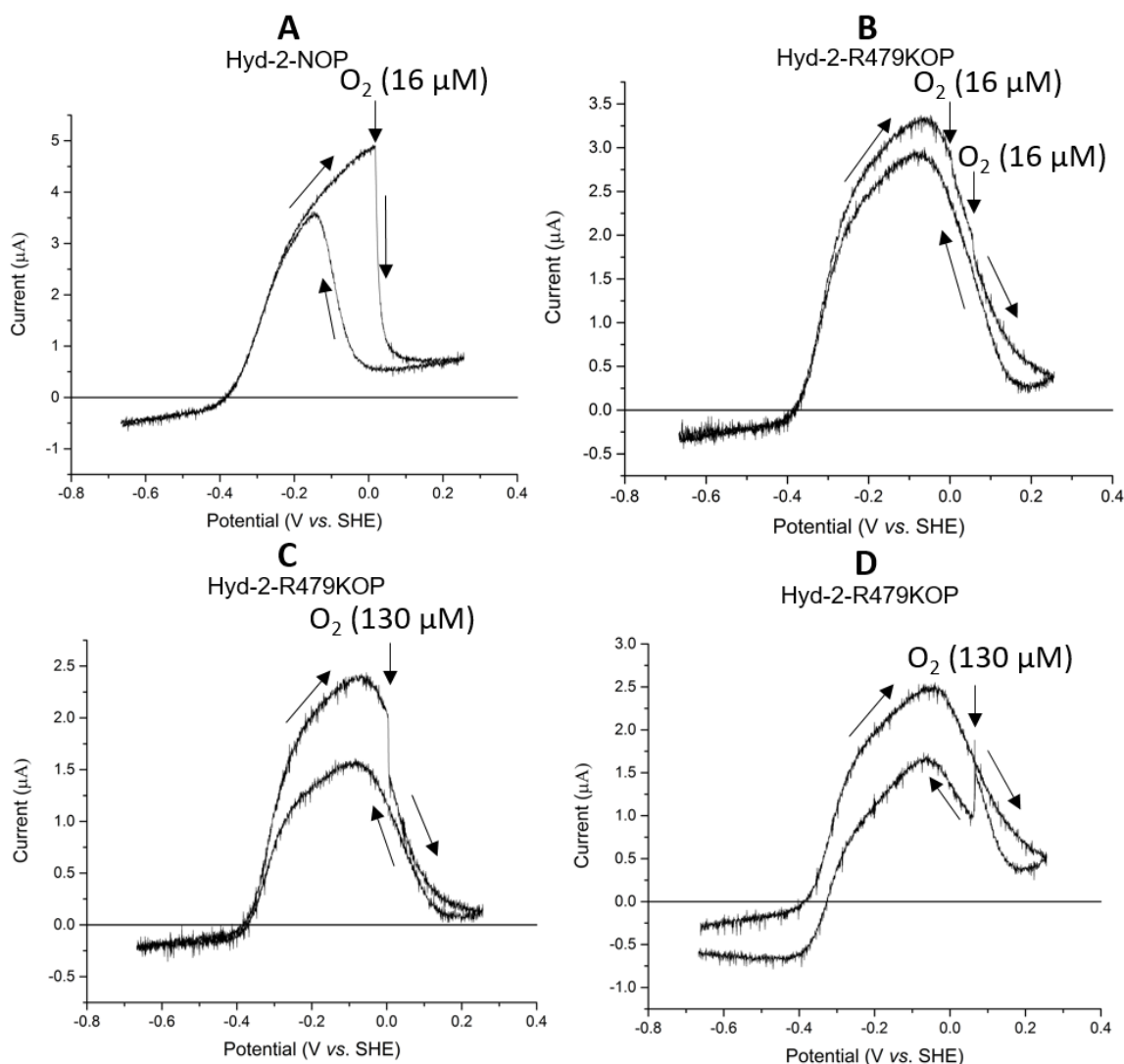


Figure 6.21: Hyd-2 response to injection of O_2 in H_2 atmosphere. **(A)** Injection of O_2 (16 μM final concentration) during the forwards scan of a fully-activated film of Hyd-2 NOP at 0.05 V. **(B)** Dual injection of O_2 (16 μM) during the forwards scan of a fully-activated film of Hyd-2 R479K-OP first at 0.05 V and then at 0.10 V. **(C)** Injection of O_2 (130 μM) during the forwards scan of a fully-activated film of Hyd-2 R479K-OP. **(D)** Injection of O_2 (130 μM) during the backwards scan of a fully-activated film of Hyd-2 R479K-OP. Other conditions: pH 6.0, 0.5 mV/sec, 100% H_2 , and $\omega=3000$ RPM.

6.2.2.7 Inhibition by CO

Like most standard, reversible hydrogenases, Hyd-2 is severely inhibited in the presence of CO. The canopy variants are more sensitive to CO at the same concentration

of H_2 , but that can mostly be attributed to the difference in $K_M^{H_2}$ between the variants (Figure 6.22). The two canopy variants have larger $K_M^{H_2}$ values and are therefore less able than Hyd-2 NOP to reactivate from CO inhibition at a given concentration of H_2 . Accounting for the larger values for $K_M^{H_2}$ in the equation for K_i^{CO} (see section 2.10.3.5), the native and two variant proteins all have values for K_i^{CO} that are within error of each other.

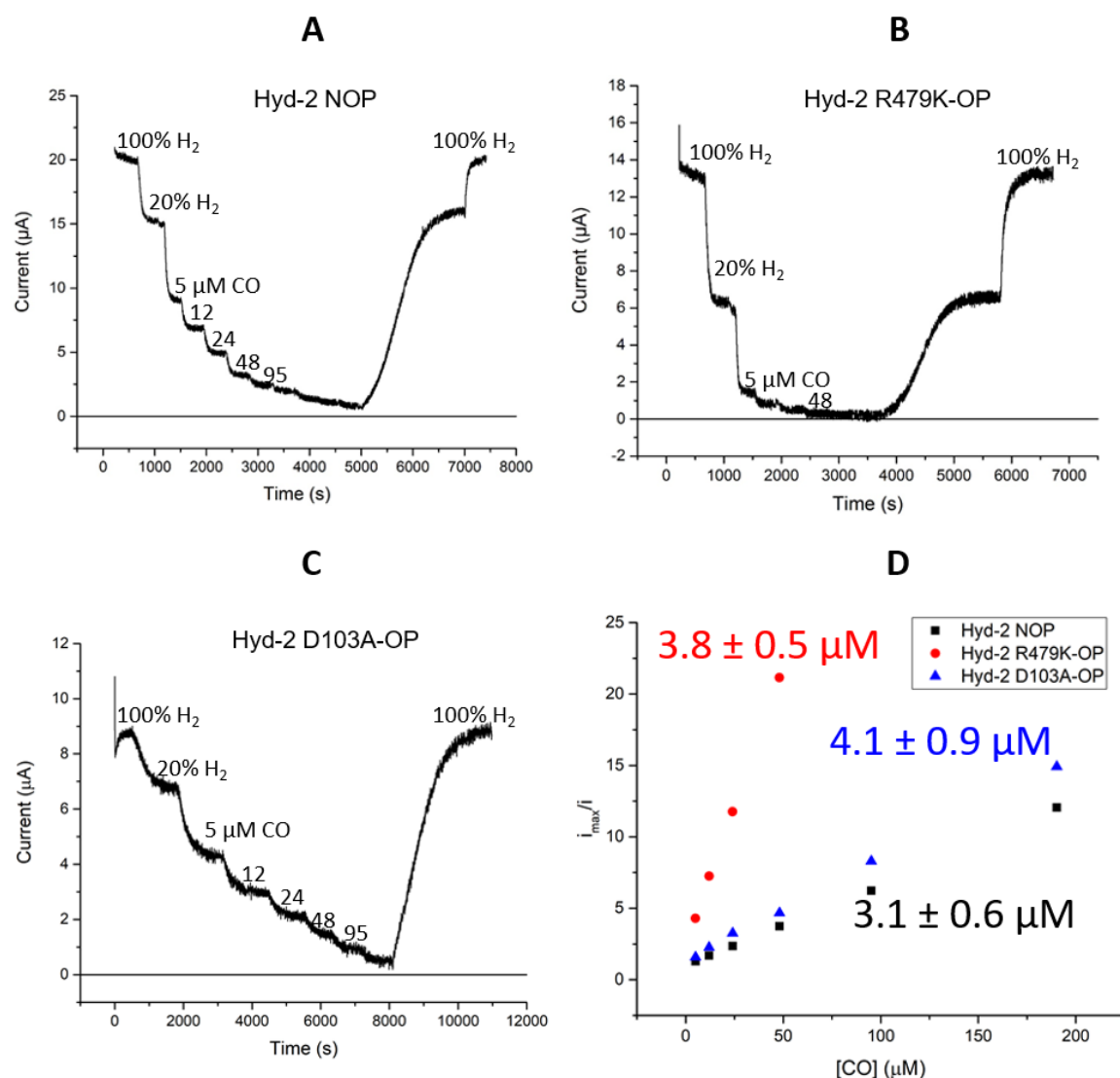


Figure 6.22: Profile of inactivation in 20% H_2 in varying concentrations of CO for (A) Hyd-2 NOP, (B) Hyd-2 R479K-OP, and (C) Hyd-2 D103A-OP as well as (D) the plot used to calculate K_i^{CO} . The experiment begins and ends with 100% H_2 though the CO is added with only 20% H_2 . Other conditions: pH 6.0, 5 mV/sec, 20°C, 20% H_2 , Ar carrier gas, and $\omega=3000$ RPM.

6.3 Discussion

6.3.1 The (Ni-SI_r)_{II} state in reduced Hyd-2 R479K-OP

The structure of reduced Hyd-2 R479K-OP revealed that there was a water molecule in a position coordinating the Ni atom. This conformation strongly resembles one of the structures proposed for the (Ni-SI_r)_{II} state (Section 1.3.2).^{15,38} If this observation is confirmed by IR-spectroscopy, the structure of reduced Hyd-2 R479K-OP could be the first time the structure of an intermediate in the reactivation pathway of Ni-B has been solved (Figure 6.23). It is likely that the R479K mutation stabilizes the (Ni-SI_r)_{II} state because of the increased space between the [NiFe] cofactor and the pendant lysine, which would allow a water molecule to coordinate the Ni atom without the steric strain of the guanidinium headgroup. Just as R479 is theorized to stabilize the H₂O-bound state,³⁹ the pendant lysine could further enhance the stability of that state leading to its capture in the R479K-OP variant. The correlation between the trapped intermediate in the reactivation pathway from Ni-B in the reduced enzyme and the identification of the diatomic in the as-isolated enzyme (see Chapter 7) shows that the effect of the R to K mutation has been to trap small molecules at the active site.

Without the guanidinium group of the Arg479 impeding access to the active site, it would clearly be more favourable for H₂O to enter the active site when it is in a state like Ni-SI_a in which there is no ligand bridging the [NiFe] cofactor. The different CV shapes seen in the activation profile would also be explained by the stabilization of (Ni-SI_r)_{II} (Figure 6.9). The as-isolated enzyme starts almost totally inactive due to the diatomic at the active site (see Chapter 7). Initial scans reduce that diatomic to a water molecule and activity begins as the water molecule is cleared from the active site. The high rate of

inactivation during the first active scans could be due to the fact that the cleared water molecule stays local to the active site and easily inactivates the enzyme at higher potentials. Final scans show that $(\text{Ni-SI}_r)_{\text{II}}$ can still form readily in Hyd-2 R479K-OP, but the formation of $(\text{Ni-SI}_r)_{\text{II}}$ is less prominent because the water molecule is cleared farther away from the active site.

Table 6.6: Summary of enzymatic parameters for Hyd-2 NOP, Hyd-2 R479K-OP, and Hyd-2 D103A-OP. Error represents the standard deviation from three trials.

	Hyd-2 NOP	Hyd-2 R479K-OP	Hyd-2 D103A-OP
solution assay oxidation turnover frequency (s^{-1})	218 ± 17^a	5 ± 1^a	16 ± 1^a
solution assay H_2 production turnover frequency (s^{-1})	7 ± 1^b	0.03 ± 0.002^b	0.4 ± 0.2^b
$K_{\text{M}}^{\text{H}_2}$ (μM)	23 ± 7^c	214 ± 45^c	44 ± 4^c
$\Delta\text{H}^\ddagger$ (kJ mol^{-1})	47 ± 2^d	23 ± 2.6^d	42 ± 4^d
$K_{\text{i}}^{\text{H}_2}$ at -0.670 V (μM)	240 ± 10	1077 ± 96.4	203 ± 16
K_{i}^{CO} at -0.175 V (μM)	3.1 ± 0.6	3.8 ± 0.5	4.1 ± 0.9

^ameasured with benzyl viologen ($E = -358$ mV); ^bmeasured with methyl viologen ($E = -446$ mV);

^cmeasured at -0.175 V; ^dmeasured at -0.200 V;

It is also likely that $(\text{Ni-SI}_r)_{\text{II}}$ would form at any potential where Ni-SI_a is present, and the increased stability could explain many of the different parameters seen in Table 6.6. Thus, the curvature of the Eyring plots even at mildly oxidizing potentials like -0.30 V can be explained by the presence of a second rate-determining step which could be the $(\text{Ni-SI}_r)_{\text{II}}$ to Ni-SI_a transition by the clearing of the water molecule. However, the effect of the stabilization of $(\text{Ni-SI}_r)_{\text{II}}$ should be lower at more reducing potentials where the enzyme is more likely to be in more reduced states like Ni-R and Ni-C. The formation of Ni-B has always been known to be sensitive to temperature with higher rates of Ni-B formation at higher temperatures,^{37,38} but in R479K-OP it would appear that the lower temperatures help stabilize the water molecule at the active site. Thus, measuring the activity of Hyd-2 R479K-OP at increasing temperatures would be expected to register two

phenomena: (1) an increase in activity due to the increase in temperature and (2) an increase in activity with decreasing stability of $(\text{Ni-SI}_r)_{\text{II}}$. The latter effect would likely diminish above temperatures at which the water can more freely leave the active site. Thus, at temperatures above 25°C, the activity of Hyd-2 R479K-OP increases only very slightly while formation of Ni-B also increases with temperature. These two trends would explain the increasing inactivation at higher temperatures.

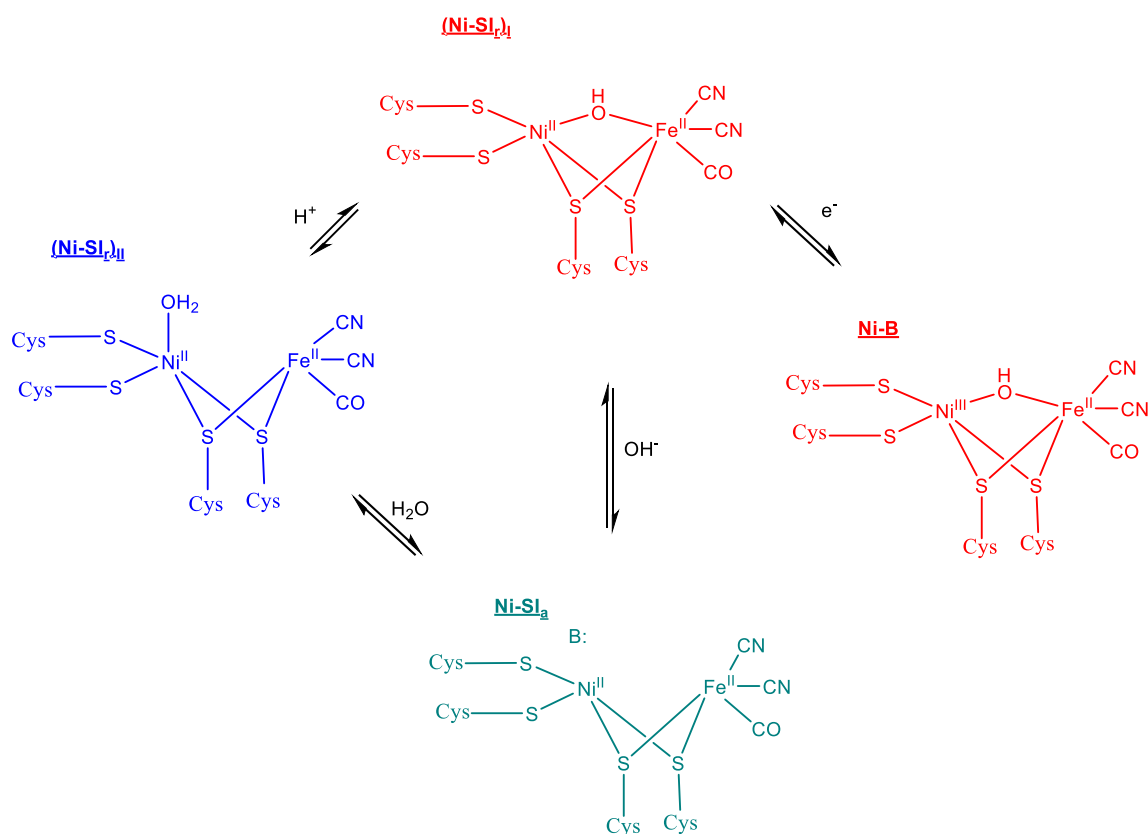


Figure 6.23: Schematic of the proposed intermediates involved in the anaerobic inactivation for Hyd-2 R479K-OP.

The increased stability of the $(\text{Ni-SI}_r)_{\text{II}}$ state would also explain most of the large changes in the measured enzymatic parameters of Hyd-2 R479K-OP. For example, the two inflection points in the reactivation scan for E_{SWITCH} suggests that there are either two resting inactive states or two reactivation pathways from a single resting inactive state.

If the formation of $(\text{Ni-SI}_r)_{\text{II}}$ is much more stable in R479K-OP than in the native enzyme, then the inactivation procedure E_{SWITCH} could convert the enzyme film predominantly to $(\text{Ni-SI}_r)_{\text{II}}$ with only small amounts of Ni-B. Such a distribution of states would give the reactivation profile seen for Hyd-2 R479K-OP. Alternatively, if the inactivation protocol converts the enzyme solely to Ni-B, the bound hydroxide in Ni-B could leave in two pathways: (1) as a hydroxide or (2) as a water molecule through $(\text{Ni-SI}_r)_{\text{II}}$. The reactivation profile of E_{SWITCH} is also consistent with this explanation. Either way, the transition from $(\text{Ni-SI}_r)_{\text{II}}$ to Ni-SI_a as it is depicted in Figure 6.23 would not require reduction of Ni(III) to Ni(II), and it would therefore be expected that the E_{SWITCH} would shift to more oxidizing potentials.

Moreover, enzyme in the $(\text{Ni-SI}_r)_{\text{II}}$ state has been shown to be less reactive to H_2 , O_2 , and CO as the water molecule bound at the Ni atom has to be removed before the active site becomes available to interact with other small molecules.^{38,40} Thus, enzyme variants with a stable $(\text{Ni-SI}_r)_{\text{II}}$ state would be expected to have a much smaller on rate, k_1 . With the simplified equation for $K_{\text{M}}^{\text{H}_2}$, $(k_{-1}+k_{\text{cat}})/k_1$ (see Section 6.1.3 under 'Aspartate Fine Tuners'), it is obvious that a small k_1 equates to a large $K_{\text{M}}^{\text{H}_2}$. The prediction of a small k_1 bears out in the large $K_{\text{M}}^{\text{H}_2}$ of the R479K-OP variant. As Ni-SI_a is the most oxidized state of the catalytic cycle, there would be a greater opportunity for the enzyme to shift to the $(\text{Ni-SI}_r)_{\text{II}}$ state at higher potentials at which more of the enzyme is in Ni-SI_a . Thus, the increasing $K_{\text{M}}^{\text{H}_2}$ with potential can also be explained with the stability of $(\text{Ni-SI}_r)_{\text{II}}$.

The presence of the H_2O molecule at the active site could also explain the ability for the variant enzyme to block O_2 from entering the active site making Hyd-2 R479K-OP less susceptible to aerobic attack. What is clear is that if there is an H_2O molecule at the

active site, it does not change the sensitivity to CO in Hyd-2 R479K-OP as the K_i^{CO} is comparable between the native Hyd-2 and the R479K variant.

The stabilization of $(\text{Ni-SI}_r)_{\text{II}}$ might also partially explain the lower product inhibition measured in the R479K-OP variant. Product inhibition can be understood as the persistence of a Michaelis complex between Ni-R and Ni-SI_a in which H₂ is at the active site. Such a state would be in equilibrium with both Ni-R and Ni-SI_a, and the rapid formation of $(\text{Ni-SI}_r)_{\text{II}}$ from Ni-SI_a could drive that equilibrium towards Ni-SI_a and away from the product inhibited state. Together these findings show that the arginine in native Hyd-2 appears to have a role beyond H₂ activation: controlling access to the active site which has profound implications on the activity of the enzyme.

6.3.2 Ordered waters in aspartate variants

While the minor shift in the water molecule close to Oδ1 is similar to the shift that occurs in Hyd-1 D118A, the large shift of the ordered water molecule to the position of where Oδ2 resides in Hyd-2 NOP is unique to Hyd-2 D103A-OP (Figure 6.24). As Hyd-2 D103-OP is also uniquely capable of proton reduction, these findings refine the theory that a polar residue in the D103 position is necessary for proton reduction, as a conserved water molecule in the position where Oδ2 resides in Hyd-2 NOP appears to be sufficient to mediate proton reduction for Hyd-2 D103A-OP. While it has been previously observed that ordered water molecules are conserved across a range of hydrogenases in various species, comparing the active sites of Hyd-1 D118 and Hyd-2 D103-OP gives strong evidence that an ordered water molecule is necessary for catalysis in this instance. Except for the ordered water molecules, the two active sites are nearly identical. The inability for Hyd-1 D118A to catalyse measurable proton reduction is therefore likely to be an

effect of the lack of a polar molecule at the site occupied by the O δ 2 position of the aspartate in the native enzyme—conveniently within hydrogen bonding distance of N η 1 of the pendant arginine.

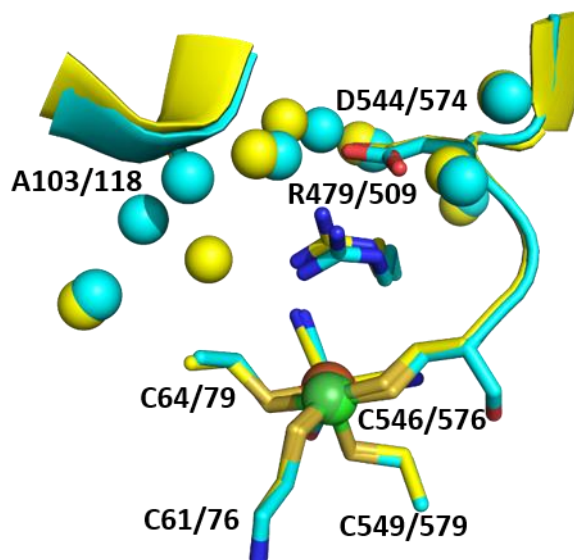


Figure 6.24: Overlay of Hyd-1 D118A (PDB 5A4F, cyan) and Hyd-2 D103A-OP (yellow) including the water molecules conserved at the active site. Numbering is listed with Hyd-2 numbering first.

6.3.3 $K_M^{H_2}$

Previous work on Hyd-1 D118A led to the conclusion that the large and potential dependent $K_M^{H_2}$ of the aspartate variant was caused by a decrease in the on rate, k_1 (see 6.1.2). This was concluded because Hyd-1 D118A is incapable of H_2 production which was assumed to be the result of very tight product inhibition and therefore an off rate that is at least equal to the off rate of the native enzyme. This evidence pointed to the aspartate in that position as controlling substrate access to the active site.

Considering the effect of the mutation on Hyd-2 D103A-OP, it is unlikely that D103 directly controls the gas channel in [NiFe]-hydrogenases. The $K_M^{H_2}$ of Hyd-2 D103A-OP is only slightly raised, and if the D to A mutation truly impairs the H_2 gas channel, that would

likely also be an effect seen in Hyd-2 D103A-OP. Lastly, the $K_M^{H_2}$ profile for Hyd-1 D118A and Hyd-1 D118N/D574N closely resembles the $K_M^{H_2}$ profile of Hyd-2 R479K-OP while being drastically different from that of Hyd-2 D103A-OP.

For Hyd-2 R479K-OP, the large $K_M^{H_2}$ suggests that Hyd-2 R479K-OP has a very small k_1 , likely caused by the stabilization of the $(Ni-SI_r)_{II}$ state. Interestingly, the three variants with large, potential-dependent $K_M^{H_2}$ are the three variants with the largest space opened around the [NiFe] cofactor. While the double aspartate variant is the Hyd-1 variant with the longest distance between the [NiFe] cofactor and the pendant arginine (4.8 Å for the double mutant compared to 4.5 for native Hyd-1), Hyd-1 D118A has a large space left from the D to A mutation that is not occupied by an ordered water molecule in the as-isolated enzyme. Similarly, the orientation of Hyd-2 R479K-OP allows a large space between the [NiFe] cofactor. As the resulting $K_M^{H_2}$ profile is similar between three different variants, perhaps the effect of decreasing steric hindrance around the [NiFe] cofactor explains the converging enzymatic parameters.

In fact, it is possible that the reduced Hyd-1 D118A contains a water molecule in a similar position as D103A—a water molecule found in the position occupied by Oδ2 in the native enzyme. If this water molecule were closer to the Ni atom than the one found in Hyd-2 D103A-OP, it may have a similar effect as the water molecule loosely coordinating the Ni atom in Hyd-2 R479K-OP. The water molecule could impair access to the active site by H_2 and because Hyd-1 is already O_2 -tolerant, a similar effect on impairing access to O_2 molecules as seen in Hyd-2 R479K-OP would be unnoticed. However, if the D118A variant does stabilize the $(Ni-SI_r)_{II}$ state, it does so to a lesser degree as the structure of the as-isolated D118A variant clearly showed Ni-B formation.

The hydroxide at the active site of as-isolated D118A, though, could be formed from the water molecule close to the Ni which would explain why the active site of as-isolated Hyd-1 D118A appears to lack a conserved water molecule when compared to the active site of Hyd-2 D103A-OP (Figure 6.6). If this theory were correct, then the water molecule that is postulated to exist at the active site of reduced Hyd-1 D118A would have to be mispositioned and therefore incapable of performing H₂ production to remain consistent with the enzymatic parameters measured for Hyd-1 D118A.

6.3.4 Flexibility of R479K

Hyd-2 R479K-OP is the canopy variant with the longest distance between the [NiFe] cofactor and the pendant headgroup. The active site of Hyd-1 R509K has higher temperature factors than the active site of Hyd-1 implying a less stable active site, yet the lysine was always crystallized in the conformation in which the N ζ remains in nearly the same position as N η 1 in Hyd-1.

Importantly, neither the D118A variant in which a large space above the Ni is opened, nor the D118N/D574N variant in which both negative charges around the arginine are removed result in a significant shift of the pendant arginine in Hyd-1.⁶ In both variants, the arginine remains in its pendant position within 4.8 Å or less of the Ni of the bimetallic cofactor.⁶ Although the negative charges of the aspartates likely help stabilize the positively charged guanidinium of the arginine and tune the electrostatics of the active site, the long and possibly mobile R509 appears to be more stable in the pendant position in Hyd-1 than the pendant group in Hyd-2.

6.3.5 Arginine as the base

While the evidence from Hyd-2 R479K-OP shows the importance of the pendant arginine in [NiFe]-hydrogenase, the total shift in enzymatic parameters between Hyd-2 NOP and Hyd-2 R479K-OP makes a direct comparison difficult. Similar evidence in Hyd-1 that arginine plays an important role in the mechanism also exists in Hyd-2, specifically the large difference in turnover rate and $\Delta H^\ddagger$. For the potentials at which $\Delta H^\ddagger$ of Hyd-2 R479K-OP are more accurate, the values for $\Delta H^\ddagger$ for Hyd-2 R479K-OP are still significantly lower than Hyd-2-NOP. Indeed, the values given for $\Delta H^\ddagger$ in Hyd-2 R479K-OP at -0.30 V and -0.25 V are well aligned with the reported $\Delta H^\ddagger$ of Hyd-1 R509K. This distinction is important, as the $\Delta H^\ddagger$ measured for the aspartate variants of Hyd-1 share a similarly potential dependent $\Delta H^\ddagger$ profile, yet the value for $\Delta H^\ddagger$ for these Hyd-1 variants started within a similar range as native Hyd-1 and measurements at higher potentials are complicated by high $K_M^{H_2}$ values. By contrast, the $\Delta H^\ddagger$ at low potentials show that Hyd-2 R479K-OP likely has a lower $\Delta H^\ddagger$ regardless of the complications presented by inactivation and high $K_M^{H_2}$ values. Moreover, a similar two orders of magnitude decrease in turnover rate for Hyd-2 R479K-OP is observed. Of importance, this decreased turnover rate extends to H_2 production, which is the first evidence that arginine could play a role in H_2 production as well as H_2 oxidation.

However, these findings are complicated by the structure of Hyd-2 R479K-OP, and for the lysine to be directly involved as a base, the pendant lysine would likely have to take on a different conformation bringing it closer to the [NiFe] cofactor, as distances closer to 4.2-4.4 Å would correspond better with ideal distances for an FLP-like mechanism.^{20,28} While temperature factors in both Hyd-1 R509K and Hyd-2 R479K-OP suggest flexibility, K479 was only crystallized in a single position even when it was

crystallized in ostensibly turnover conditions where the enzyme is nearly fully reduced. The position of the diatomic/ordered water molecule would also likely inhibit ideal positioning of the pendant lysine for catalysis, and this water is likely displaced during turnover conditions.

The ordered water molecule first observed in Hyd-2 R479K-OP (labelled with the number 2 in Figure 6.7) and later confirmed to exist in Hyd-1 R509K is in close proximity to the pendant lysine (3.6 Å) and the sulphur of the terminal cysteine C546 (4.2 Å) and could therefore mediate proton transfer between the residues.⁴¹ If K479 were to mediate hydrogen activation, subsequent proton transfer to the terminal thiolate would allow access to the proton transfer pathway gated by E14 (see Chapter 8).

6.4 Conclusion and Outlook

In conclusion, the effects of the mutations on the canopy variants for Hyd-2 were surprising and clarify the conclusions drawn for the cause of the phenomenon observed in Hyd-1. Hyd-1 D103A-OP possibly illustrates the importance of ordered water molecules in catalysis, particularly H₂ production. The variant also illustrates that the D103A mutation in Hyd-2 has little direct effect on substrate access as was theorized for Hyd-1. Hyd-2 R479K-OP also helps clarify some of the effects, like high $K_M^{H_2}$, seen in Hyd-1 that were thought to be exclusive to aspartate variants. While Hyd-2 R479K-OP offers unique evidence that the pendant arginine could play an important role in H₂ production, the large differences in the structure and enzymatic parameters make conclusions from Hyd-2 R479K-OP difficult. Most of these different parameters in Hyd-2 R479K-OP can be explained by the prominence of an intermediate with a water molecule that occupies the area next to the Ni termed (Ni-SI_r)_{II}. Characterization by IR-spectroscopy would be

required to confirm this observation, yet this state may be the intermediate between Ni-SI_a and Ni-B presenting a unique opportunity to characterize a key intermediate involved in anaerobic inactivation. However, further experimentation is necessary to verify the role of the pendant arginine in the mechanism of H₂ activation.

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Chapter 7

A putative Ni-O₂ adduct in an outershell
variant of Hyd-2

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7.1 Introduction

[NiFe]-hydrogenases differ from [FeFe]-hydrogenases in that they have a greater ability to withstand O₂ attack.¹ While some [NiFe]-hydrogenases are classified as O₂-tolerant due to their ability to sustain H₂ oxidation in the presence of O₂, even standard O₂-sensitive [NiFe]-hydrogenases are only reversibly inactivated upon O₂ exposure.² Most catalysts with similar turnover rates and low overpotential requirements are irreversibly destroyed in the presence of O₂.³ The ability to design O₂-tolerant hydrogen catalysts is therefore of great interest because any industrial application of H₂ activation catalysts will benefit from O₂ tolerance as full anaerobic systems are prohibitively costly. Notwithstanding, little is known about the atomic-level mechanism of inactivation for hydrogenases and catalysts in the presence of O₂. Typically, reactions with O₂ are rapid, and intermediates are unstable. With further insight into the mechanism of aerobic inactivation, it might be possible to rationally design O₂-tolerance into enzymes and catalysts.

7.1.1 Mechanisms of O₂ tolerance

Upon exposure to O₂, [NiFe]-hydrogenases typically form two states: Ni-B which can be reactivated in seconds and Ni-A which requires up to hours to fully reactivate. These two species have been the subject of intense investigation and have well defined spectroscopic and EPR signatures.^{1,4} Under standard conditions, O₂-tolerant hydrogenases almost exclusively form Ni-B upon O₂ attack, which allows rapid reactivation. Original explanations for this O₂ tolerance pointed to mechanical access of O₂ to the active site.⁵ The most O₂-tolerant [NiFe]-hydrogenases contain bulky residues in the hydrophobic gas channels that limit access of larger molecules like O₂ while still

allowing H₂ to reach the active site.⁵ Mutations to gas channels in the regulatory [NiFe]-hydrogenase from *Rhodobacter capsulatus* and *Ralstonia eutropha* that replaced bulky amino acids like isoleucine and phenylalanine with amino acids like valine and leucine, respectively, increased O₂ sensitivity in the enzyme *in vitro*.^{6,7}

More recently, explanations for O₂ tolerance have focused on the supply of electrons to the active site. While hydrogenase in the Ni-C state only needs an additional two electrons to fully reduce attacking O₂ and form Ni-B, hydrogenase in Ni-SI_a must be rapidly supplied with 3 electrons to fully reduce the attacking O₂ to H₂O.⁸ Lacking sufficient electrons, the active site only partially reduces O₂, forming reactive oxygen species that are trapped at the active site and react with the sulphur from coordinating cysteines.⁹ Investigations revealed the importance to O₂ tolerance of two cysteine residues bordering the proximal cluster that are conserved in O₂-tolerant hydrogenases but replaced by glycine in standard O₂-sensitive hydrogenases.^{7,10–12} Calculations suggested that this special proximal cluster in O₂-tolerant hydrogenases is capable of rapid, sequential two-electron transfer.¹³ After the structure of O₂-tolerant [NiFe]-hydrogenase was solved, it revealed that the O₂-tolerant [NiFe]-hydrogenases contain a special [4Fe-3S]6cys proximal cluster capable of rapidly releasing two electrons (see Section 4.1.3 for more discussion of the special [4Fe-3S]6cys cluster found in O₂-tolerant [NiFe]-hydrogenases).^{14–16}

Spectroscopic investigations of the distribution of states in active hydrogenases also points to another explanation for O₂ tolerance that involves the identity of the majority resting state of the hydrogenase. It is theorized that O₂-tolerant hydrogenases, when under H₂ turnover conditions, exist mainly in more reduced states (Ni-C) rather than the more oxidized state (Ni-SI_a) which makes full reduction of O₂ at the active site

more favourable (see Section 1.3.2 for a diagram of the states in [NiFe]-hydrogenases). For example, *E. coli* Hyd-1 in H₂ oxidation turnover conditions typically has a small percentage of enzyme in the Ni-SI_a state whereas standard, O₂-sensitive [NiFe]-hydrogenases like the [NiFe]-hydrogenase from *D. vulgaris* Miyazaki F are predominantly in the Ni-SI_a state.^{17–19} Because Ni-SI_a is the most oxidized state of the active cycle, aerobic attack is more likely to form Ni-A in hydrogenase that predominantly reside in the Ni-SI_a state.

7.1.2 O₂ attack in [NiFe]-hydrogenases—the known

Investigations of the active site have focused on identifying the physical meaning of Ni-A and Ni-B. While the ligand occupying the bridging position for Ni-B has long been theorized to be an OH[–],²⁰ the chemical nature of Ni-A has been disputed. Initially, the bridging species was identified as a sulphur-containing species²¹ or a hydroperoxide.^{22,23} Later it was discovered that the sulphur bridging species were products of purification from sulphur-reducing bacteria rather than a naturally occurring product of aerobic attack on the active site.^{24,25} It was also shown that it is unlikely that Ni-A contains a peroxide given its resilience to UV exposure²⁶ and the ability to attain Ni-A under moderately oxidizing potentials in anaerobic conditions.²⁷ By using isotopically labelled H₂¹⁷O, it was also shown with ENDOR that the bridging ligand contained an oxygen atom that could come from solvent.²⁸ Other studies with isotopically labelled ³⁴O₂ showed that the oxygen atom in the bridging ligand of both Ni-A and Ni-B could come from air as well.²⁹ In variants designed to investigate the bridging species that exists upon oxidation, Volbeda et al. were able to isolate the effects of photoreduction by X-ray and obtained convincing structural evidence that the bridging species of Ni-A is a hydroxide.³⁰ They also

showed that some of the debate around the partial occupancy of certain oxidized species at the active site was an effect of the photoreduction that occurs upon X-ray diffraction, and with that information they refined electron density maps of the previously solved [NiFe]-hydrogenase from *A. vinosum* to show it contained a bridging hydroxide.³⁰ There is therefore growing consensus that [NiFe]-hydrogenases have a hydroxide at the bridging position of Ni-A,³⁰ just as with Ni-B.

As-isolated structures also reveal oxidative damage at one or more of the sulphurs coordinating the Ni atom. In most [NiFe]-hydrogenases in which the oxidative damage of a coordinating cysteine is observed and modelled, it occurs at the sulphur of the bridging C64.^{22,23,30,31} However, both Hyd-2 from *E. coli* and the [NiFe]-hydrogenase from *D. desulfuricans* show oxidative damage on the terminal sulphur of C546 (the [NiFe]-hydrogenase from *H. marinus* is excepted from both these descriptions as excess electron density is observed on all the cysteines besides C549 in its structure).^{15,32} Density functional theory (DFT) calculations of the oxidized active site have suggested that the sulfenate created by oxidation of the sulphur of a cysteine is the most stable element of Ni-A regardless of whether the sulfenate occurs at a bridging or terminal position of the bimetallic cofactor.^{33,34} The recalcitrant nature of Ni-A is therefore likely an effect of the need to reduce the sulfenate prior to Ni-SI_a formation.

These DFT calculations also offer valuable insight into the probable mechanism of O₂ attack at the active site. Using a comprehensive model of the active site that explicitly includes all the amino acids in the primary and secondary coordination sphere to include those located in the canopy, a detailed DFT model has provided a possible pathway for O₂ attack.³⁵ In these calculations, the pathway to Ni-A and Ni-B starting from both Ni-SI_a and Ni-C are modelled using the [NiFe]-hydrogenase from *A. vinosum*. For Hyd-2 from *E.*

coli, this model is not perfectly homologous as the damage to the coordinating sulphur differs. Moreover, the model frequently uses a biologically unlikely protonated D544 because a negatively charged molecule at the edge of the model gives unrealistic outputs.³⁵ While this decision may have minimal effects on the model as a whole, it could distort the positioning and role of the closest neighbouring amino acids to D544 in the canopy. Nevertheless, because there are no reported structural or spectroscopic insights for the reaction mechanism of aerobic oxidation at the active site, the DFT calculations are a good starting point for understanding the initial steps of inactivation by O₂.

It is also important to note that the model excludes the Fe-S clusters present in [NiFe]-hydrogenases. These clusters are also vulnerable to aerobic oxidation, and Ni-C typically corresponds to reduced Fe-S clusters.^{20,36,37} Thus, O₂ could prefer reacting with the reduced Fe-S clusters over the Ni(III) of Ni-C. Upon aerobic oxidation, the Fe-S clusters would likely pull electrons from the neighbouring Fe-S clusters as well as the active site, which would result in conversion of the hydrogenase into more oxidized states like Ni-SI_a. Moreover, it is more likely that O₂ would react with the electron rich hydride over the Ni atom in the 3+ oxidation state, and previously it has been theorized that O₂ is unreactive with enzyme in the Ni-C state.³⁷ While O₂ attack at the active site when the hydrogenase is in the Ni-C state is therefore unlikely, for the sake of considering all possibilities, it is worth considering the mechanism for O₂ attack on Ni(III) in the Ni-C state that has been proposed in the literature.

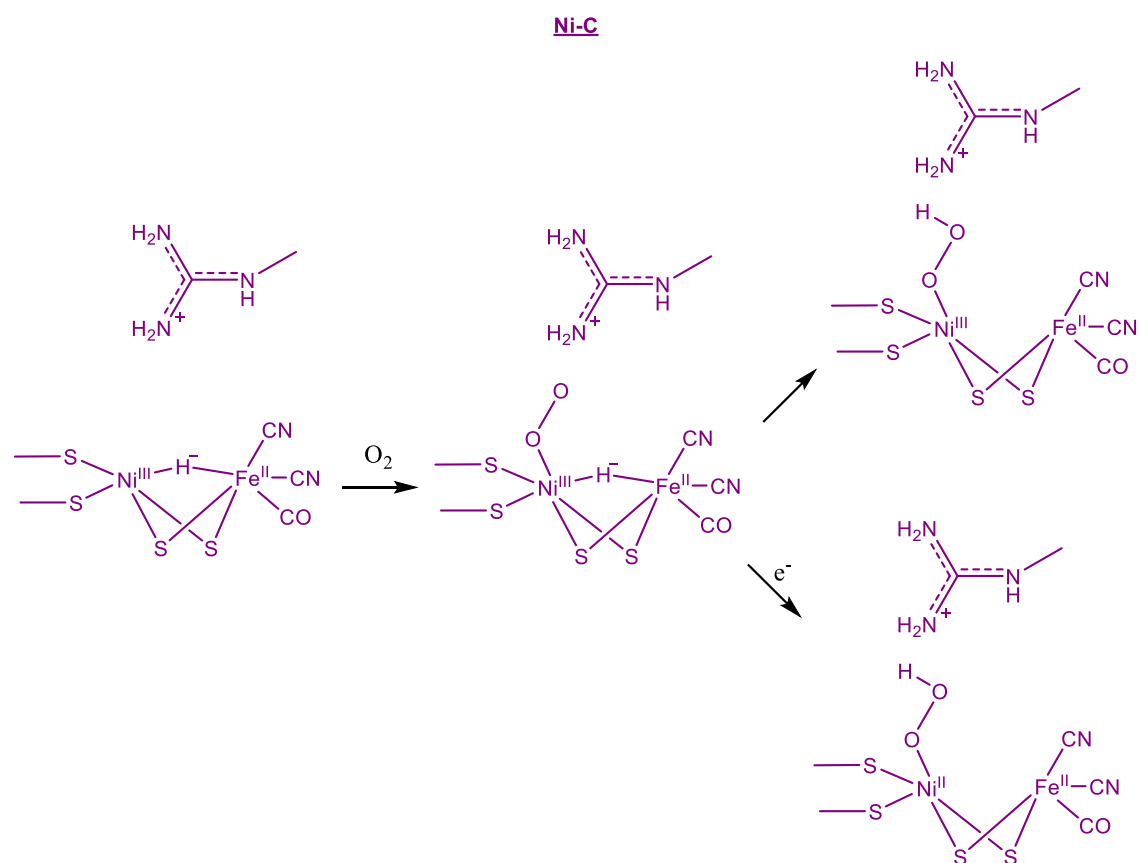


Figure 7.1: Proposed initial steps of O₂ attack at the active site of [NiFe]-hydrogenase if the incident state of the hydrogenase is Ni-C. Adapted from reference.³⁵

In short, after O₂ attacks Ni-C, both Ni-A and Ni-B are likely formed by going through an intermediate in which O₂ is terminally bound to the Ni (Figure 7.1). The O₂ is modelled binding in a pseudo-octahedral geometry. Although the O₂ could bind to the Ni in either a low-spin (LS) or a high-spin (HS) configuration (Figure 7.2), the model highly favours the LS system.³⁵

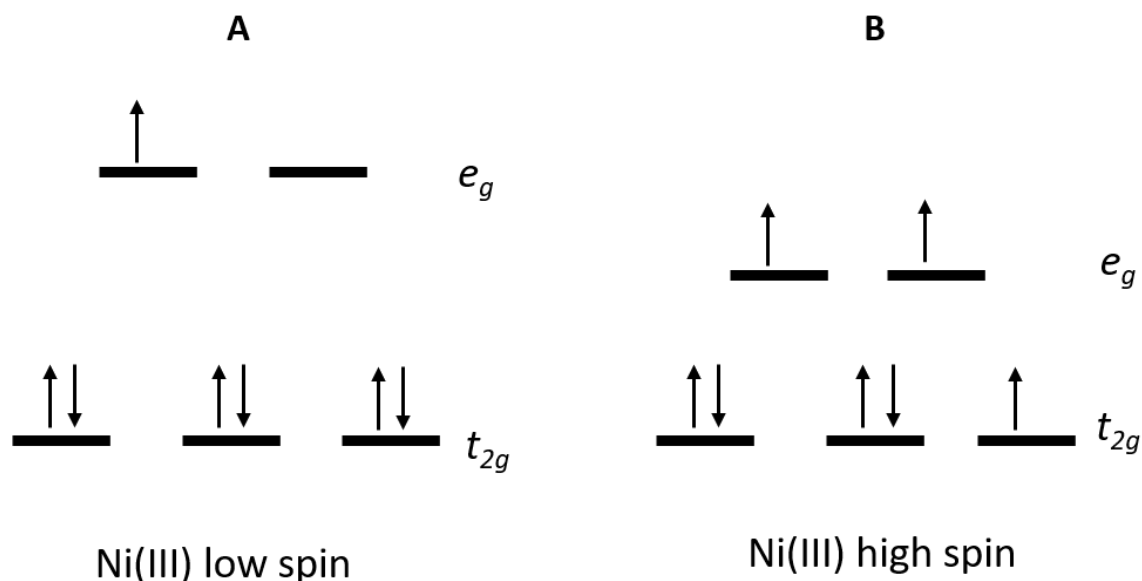


Figure 7.2: Ni(III) in an octahedral geometry with the electrons arranged either in the (A) low-spin or (B) high-spin arrangement.

After the steps shown in Figure 7.1, the pathway to Ni-A is relatively simple: the bound OOH must go through a series of rearrangements before forming the sulfenate and bridging hydroxide. For Ni-B, the active site must undergo two separate single electron reductions. The first reduction in Figure 7.1 is shown happening during the second step in a concerted step with the isomerization, though the reduction could occur as a single step between 4 different isomerization events in the pathway to Ni-B. Crucially, this reduction is calculated to make the intermediate diamagnetic Ni(II) more likely than the paramagnetic Ni(III) of the incident Ni-C state. The DFT calculations predict that in the native enzyme hydrogen bonding between the guanidine of the pendant arginine and the H of the OOH is crucial to stabilizing the intermediate, contributing more than 11 kcal/mol to the stabilization.³⁵

Another pathway of aerobic attack is one in which O₂ attacks Ni-SI_a (Figure 7.3). In this scheme, O₂ could also bind terminally as it does with Ni-C, yet with the bridging

position of Ni-SI_a empty, the O₂ could also coordinate side-on. There is debate over whether Ni-SI_a exists in a LS square planar geometry or a HS distorted tetrahedral (trigonal bipyramidal missing a ligand)^{38,39} and both of the possibilities were modeled.³⁵ Again, the LS Ni-O₂ adduct was favoured in their models by approximately 6 kcal/mol.³⁵ DFT calculations predicted that in the native enzyme the O₂ side-on binding is likely the most stable form by approximately 10 kcal/mol, though the pendant arginine has a large effect on the DFT calculations.³⁵ In a similar manner to the O₂ from aerobic oxidation of Ni-C, the bound O₂ then undergoes subsequent isomerization events, and in the case of Ni-B reduction (3e⁻) is required, to form the final inactive states of Ni-A and Ni-B.

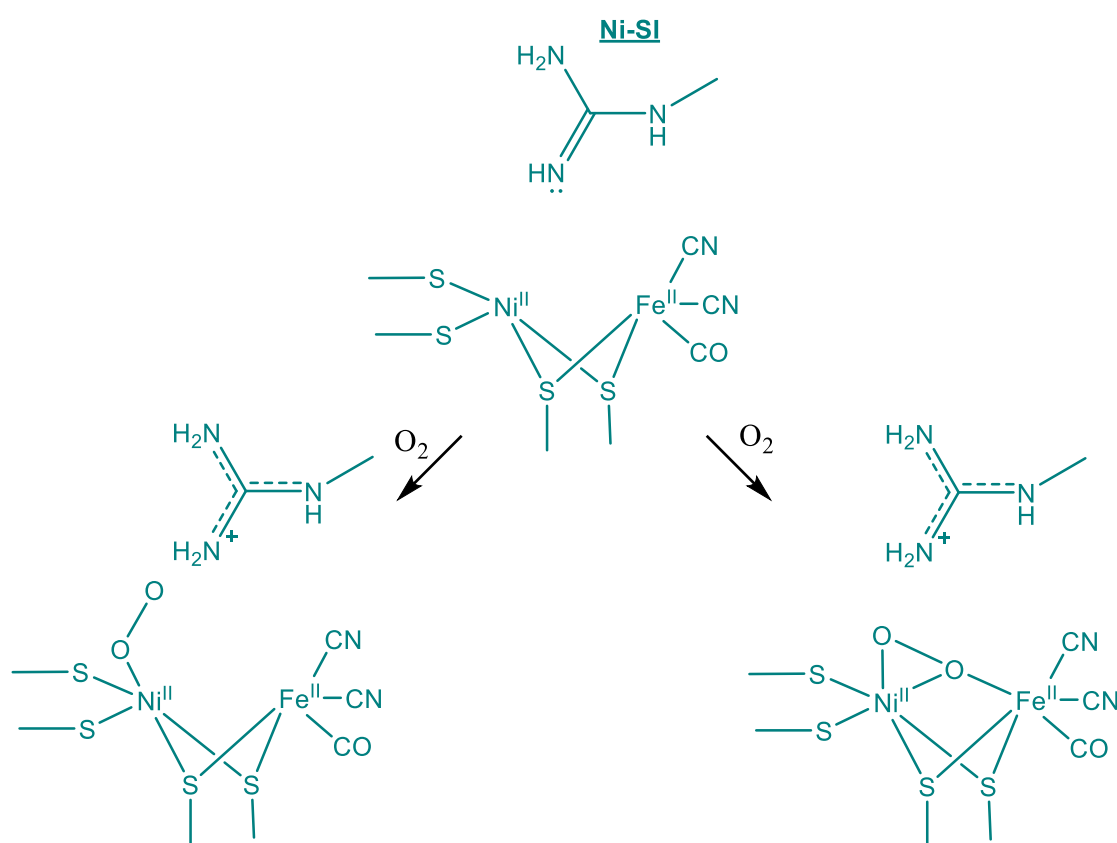


Figure 7.3: Proposed initial steps of O₂ attack at the active site of [NiFe]-hydrogenase if the incident state of the hydrogenase is Ni-SI_a. Adapted from reference.³⁵

7.1.3 CO at the active site of [NiFe]-hydrogenase

In contrast to O₂, which reacts with the active site to form an inactive state, CO is a competitive inhibitor of [NiFe]-hydrogenase and simply blocks access to the active site. The inhibition of [NiFe]-hydrogenase by CO is a well-studied phenomenon and provides further insight into the interaction of the active site and small molecules.^{19,21,40–42} [NiFe]-hydrogenase is susceptible to inhibition by CO in states that do not contain a bridging species (see Section 1.3.2). Two states can be formed upon inhibition by CO depending on the incident state of CO exposure. The first, Ni-SCO, is EPR silent and is formed when CO reacts with Ni-SI_a.¹⁹ A second state, Ni-CO, is formed when CO binds Ni-L and results in an EPR-active species. Both species can be photolysed at near cryogenic temperatures (100 K).^{21,42}

Although CO typically binds to metals in a colinear orientation,⁴³ the CO-inhibited structures of [NiFe]-hydrogenase from *D. vulgaris* Miyazaki F revealed a bond angle of approximately 160°. ²¹ One of the CO-inhibited structures even displayed a significantly more bent arrangement with a Ni-C-O angle at 135°. ²¹ The Ni-C bond distance varied less widely than the bond angle and was measured at approximately 1.76-1.78 Å while the C-O bond ranged from 1.02-1.14 Å. The occupancy of the CO also ranged from 80% to 95% among the different gas treatments of the crystals. This range of bonding angles and bond length is likely indicative of the various binding modes of CO at the active site. However, in that study, the protein was treated with CO in crystalline form which is more likely to lead to a heterogeneous sample than if the [NiFe]-hydrogenase was treated prior to crystallization, and heterogeneity in crystals sometimes confuses the interpretation of the electron density (Section 6.1.1). Later spectroscopic studies of CO inhibition in [NiFe]-

hydrogenases in solution revealed that rebinding of the CO is biphasic which supports the previous evidence that there are various binding modes of CO to Ni.¹⁹

7.1.4 Spectroscopic signature of O₂-tolerant R to K variants

As Hyd-1 R509K is the only pendant arginine variant that has been investigated by EPR, it is worth noting details about the EPR spectrum of native Hyd-1. In short, EPR investigations of as-isolated Hyd-1 have always been complicated by the effect of an interaction between the high-potential proximal cluster and the active site. Indeed, as-isolated Hyd-1 at 80 K is not as clean as the clear Ni-A and Ni-B signals in the spectrum of as-isolated Hyd-2.⁴⁴ At lower temperatures, the signal from the oxidized medial cluster dominates the spectrum, whereas at higher temperatures the Fe-S clusters relax too quickly allowing measurements at high temperatures to be specific to the active site (for complete introduction of EPR of hydrogenase see Section 3.2.7).^{2,44} Upon reduction, native Hyd-1 has Ni-C and Ni-L signals and subsequent oxidation using chemical oxidizing agents or simply purging with Ar produces Ni-B signals.

The EPR spectrum of Hyd-1 R509K shows peaks that differ greatly from the published values for the states of Hyd-1.⁴⁵ These investigations found that after 6 days of purging the enzyme with H₂, peaks that could represent inactive states like Ni-A or Ni-B did decrease (g_{xyz} = 2.330, 2.226, 2.002).⁴⁵ Other peaks with g values (g_{xyz} = 2.240, 2.103, 2.045), indeed the dominant peaks of the spectrum in the as-isolated enzyme, showed no change over the course of reduction with H₂ (Figure 7.4).⁴⁵ Interestingly, these peaks correspond very well to the Ni-X species seen in the EPR spectrum of reduced native Hyd-2 in H₂ oxidation turnover but had not been previously reported for native Hyd-1.^{44,45}

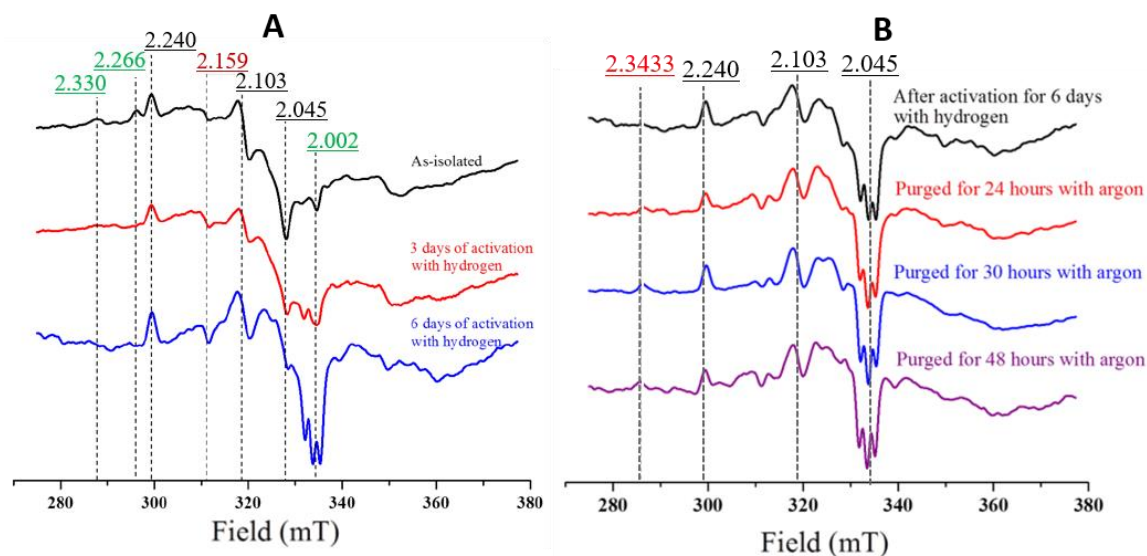


Figure 7.4: EPR spectra at 70 K of Hyd-1 R509K in the (A) as-isolated and H₂ activation state as well as (B) after subsequent treatment with Ar for varying lengths of time. Adapted from reference.⁴⁵

After being purged with Ar for 48 h, the only change in the observed EPR signal of Hyd-1 R509K is the growth of a peak at $g=2.343$. The species with $g_{xyz} = 2.240, 2.103, 2.045$ remained unchanged after purging with Ar and the addition of sodium dithionite, though it disappeared after the addition of potassium ferricyanide.⁴⁵ Interestingly, the peak at $g=2.343$ also remained after the addition of sodium dithionite but disappeared after the addition of potassium ferricyanide, suggesting that the peak is likely associated with a reduced state with a higher g -value like Ni-L. Finally, after 8 hours of treatment with potassium ferricyanide, a peak corresponding to $g=2.295$ grew and disappeared with further H₂ purge.⁴⁵ This species is most likely associated with an inactive, oxidized species.

These results show that while the spectrum for as-isolated Hyd-1 R509K contains an oxidized species, it also contains a reduced species that can only be oxidized with harsh chemically oxidizing conditions. As Hyd-1 spends over 20 hours in air between cell lysis and being quench frozen, reduced enzyme in the as-isolated spectrum is

unexpected. Moreover, all of the species seen for Hyd-1 R509K also appeared at g values that are not seen in native Hyd-1.

7.1.5 Proteins and mimics bind O₂ reversibly

Proteins that reversibly bind O₂ have long been of great interest due to their relevance in biological systems.^{46,47} They have also served as model systems of metalloproteins that have spurred the development of bioinorganic chemistry.^{47,48} A detailed review of the intricacy of these systems is beyond the scope of this thesis, but a basic description of the debate over how O₂ binds to haem is useful for a comparison to the current diatomic ligand found at the active site of Hyd-2 R479K-OP.

Two well-studied O₂ binding proteins found in humans are myoglobin, responsible for diffusing O₂ from the blood to tissue, and haemoglobin, responsible for transporting blood from the lungs throughout the body. Both myoglobin and haemoglobin contain at least one Fe atom coordinated by a tetradentate porphyrin as well as the N3 of a histidine (termed the proximal histidine). Another histidine is located above the Fe positioned with the N3 of the histidine (His_d) within 5 Å of the Fe (Figure 7.5). Such an arrangement is reminiscent of the pendant arginine above the [NiFe]-cofactor of [NiFe]-hydrogenase.

Even though the structures at a high resolution for unoccupied (deoxy), oxygenated (oxy), and carbon monoxide-bound (carboxy) haemoglobin elucidated the structural details of the binding modes of this important O₂-transporting protein, the nature of the Fe-O₂ bonding is still disputed. Molecular oxygen coordinates end-on to the Fe of the haem in a bent fashion (Fe-O-O 121°), and the distal histidine shifts towards the bound O₂ to facilitate hydrogen bonding to the bound O₂.⁴⁹ Haemoglobin is also strongly inhibited by CO as it binds orders of magnitude stronger with CO binding in a

colinear fashion (Fe-C-O 180°), and one of the roles of the distal histidine is to sterically hinder the colinear binding of CO to haem and thus increase the likelihood for O₂ to outcompete CO.⁵⁰

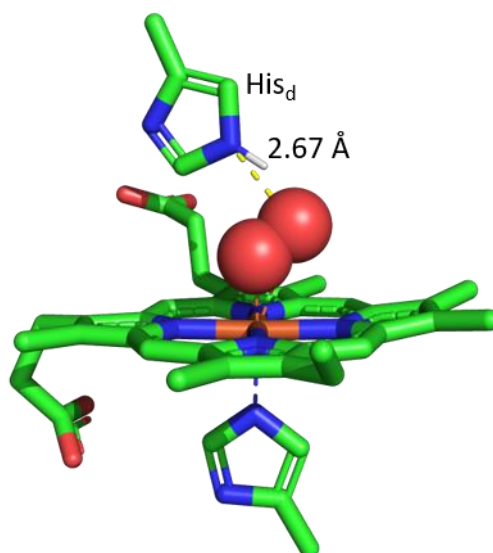


Figure 7.5: Structure of oxy haem from myoglobin (PDB 1A6M) showing the conformational change of haem of the distal histidine that helps coordinate the O₂ molecule in the haem.

The details of how O₂ binds to the Fe is still debated and recent evidence shows that there may be a difference in how O₂ is bound to haem in solution and in crystallized form.⁵¹ The two most prominent models for how O₂ binds to Fe differ on the redox state of the O₂ molecule and the Fe atom. The Pauling model describes the Fe as Fe(II) bonded to singlet O₂^{52,53} whereas the Weiss model contends that it is an Fe(III) bound to superoxide (O₂⁻).⁵⁴ Whether Fe donates an electron to O₂ determines other important characteristics of the binding mode of O₂ and has therefore been the subject of intense investigation. One way to determine the redox state of O₂ is to look at the bond length of the O₂ molecule because O₂⁻ has a longer bond length than O₂, and many earlier investigations were focused on measuring the length of the O₂ bond when O₂ binds Co or Fe heme mimics.⁵⁵ Superoxide (O₂⁻) typically has a bond length of 1.28 Å, whereas O₂

typically has a bond length of 1.21 Å. In recent structures of haemoglobin, the O₂ bond length has been measured at 1.24 Å which does not rule out either O₂ or O₂⁻ as the ligand. Recent XANES investigation that used synthetic haem mimics to compare to measurements taken on haemoglobin have proposed a hybrid of those models, showing that the O₂ is bound with superoxide characteristics to an inter-spin Fe (II).⁵⁶ This finding only further proved that the reality of the bonding situation in oxy haemoglobin is subtle, and standard binding models do not describe the Fe-O₂ adduct perfectly.

Other mimics have focused more generally on transition metals that can bind O₂ as possible catalysts.⁵⁷⁻⁶⁰ Recent efforts have been successful in synthesizing and characterizing a limited number of Ni dioxygen adducts.⁵⁹ The resulting end-on Ni-superoxo complexes have a O₂ bond length of 1.301 Å and a Ni-O-O bonding angle of 119.3°, and these complexes have been well characterized by Raman Resonance. Although these adducts require highly acidic CF₃SO₃ and high concentrations of O₂ or H₂O₂ for synthesis and therefore do not represent highly reversible O₂ binding at a transition metal, they could serve as important benchmarks for characterizing future Ni-O₂ adducts.

7.1.5 Aims of this chapter

This chapter postulates an identity for the diatomic molecule that was found at the active site of as-isolated Hyd-2 R479K-OP. The implications for the mechanism of aerobic inactivation for [NiFe]-hydrogenases as well as the larger implications of the ability for Ni to reversibly coordinate O₂ are discussed.

7.2 Results

As mentioned in the previous chapter, the structure of as-isolated Hyd-2 R479K-OP revealed unexpected electron density at the active site that is at 80% occupancy in the as-isolated enzyme. This electron density is located off the Ni atom and in a similar position as the CO molecule in the structure of *D. vulgaris* MF.²¹ Reduction of the enzyme by H₂ after exposure to CO converts this diatomic molecule to an additional conserved water molecule at the active site (Figure 7.6). That water molecule is likely removed upon further electrochemical reduction (see below) which allows for an accurate characterization of the enzymatic activity of Hyd-2 R479K-OP (see Chapter 6).

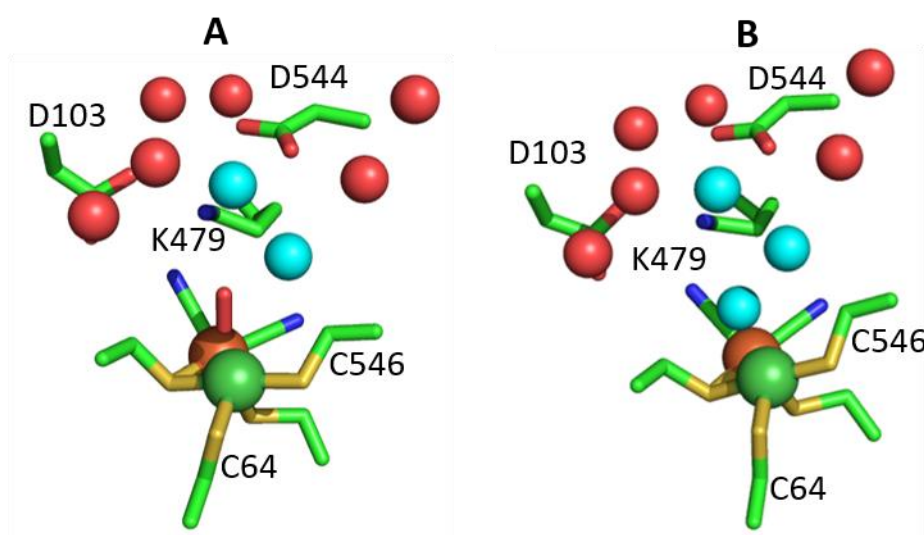


Figure 7.6: Structure of the active site of Hyd-2 R479K-OP including conserved water molecules. The water molecules that are found in R479K-OP but not in native Hyd-2 are coloured blue. The electron density coordinating the Ni is interpreted as an **(A)** O₂ molecule in as-isolated Hyd-2 R479K-OP and a **(B)** conserved water molecule in reduced Hyd-2 R479K-OP. See Figure 7.8 for detailed electron density of the ligands at the active site.

The distribution of the electron density suggested a homodiatomic ligand as there was equal electron density on both sides of the molecule. The enzyme had been prepared in air which limits the number of likely homodiatomics to two: N₂ and O₂. Moreover,

because of the strength with which CO displaces O₂ and its presence in the atmosphere, CO was also considered as the possible identity of the diatomic.

The average bond length in the five oxidized Hyd-2 R479K-OP structures with a resolution better than 2.0 Å between the two centres of electron density is 1.20 +/- 0.02 Å and the distance from the Ni to the closest centre of electron density is 1.77 +/- 0.08 Å. The bonding angle between the Ni and the diatomic is 168.4 +/- 3.3°. The bond length is in good agreement with O₂ as the ligand, yet the bond angle is more linear than typical metal O₂ complexes. The distance to the N of K479 is 2.7 Å which is appropriate for a strong hydrogen bond.

7.2.1 Crystallographic characterization

7.2.1.1 Hyd-2 R479K-OP

As crystallography first revealed the diatomic ligand positioned at the active site, the diatomic was further investigated by X-ray crystallography. Subsequent crystal structures of as-isolated Hyd-2 R479K-OP confirmed the presence of the diatomic at the active site. In an attempt to reduce the enzyme to clear out the diatomic and examine structures intentionally inhibited by diatomic gases, enzymes were treated with H₂ and then CO or O₂ (Figure 7.7) Aliquots of the enzyme were taken at every step for crystallization. Initial results revealed that simply flowing H₂ through the enzyme for 9 h only cleared the enzyme of half the occupancy of the diatomic. Trials were set up with different lengths of time for H₂ exposure to see if the level of diatomic cleared depended on the length of time reducing with H₂ (Figure 7.7).

Initial results revealed that the diatomic was not increasingly cleared with more time exposed to H₂ under pressure and at 4°C i.e. the protein that had been purged for

12 hours (3RED_12H) had a diatomic at equal occupancy as the protein purged for 6 days (3RED_6D) (structure not shown but refinement statistics are presented in Appendix B). After H₂ purging for 6 days, the protein was exposed to ³⁶O₂ with the intention of using the resulting crystals for X-ray diffraction and Raman resonance. Detecting the isotopically labelled ³⁶O₂ by Raman resonance and attributing the signal to the active site would be conclusive evidence of the Ni-O₂ adduct. Unfortunately, the structure of 3OX18 was identical to 3RED_6D suggesting that perhaps the protein breaks down over prolonged periods making it less reactive to gasses. For total replacement of the diatomic with ³⁶O₂, Hyd-2 R479K-OP had to be fully reduced, which prompted other attempts to fully clear the active site of the diatomic.

The structure of Hyd-2 R479K-OP labelled 2REDL, which was purged with H₂, CO, and then H₂ suggested that by treating the protein with CO before reduction may help fully remove the diatomic before treating it with other gases (Figure 7.7). However, the 2REDL crystal diffracted at a poor resolution 2.0 Å, which meant that the interpretation of the electron density was less certain. In two attempts to crystalize Hyd-2 R479K-OP under sequential treatments of CO and H₂, no Hyd-2 R479K-OP was ever crystallized (Figure 7.7). A treatment with ³⁶O₂ after H₂ reduction and CO exposure crystalized, but the resulting crystals diffracted to a poor resolution. Fortunately, reducing the protein while stirring it under an H₂ purge for 20 h (4RED) and a slightly higher temperature 8-10°C, rather than reducing it under pressure without agitation in 4°C, led to a structure of Hyd-2 R479K-OP with a cleared active site (see the H₂ reduced structure in Chapter 6). None of the fully cleared Hyd-2 R479K-OP (4REDL) that underwent subsequent gas treatments crystallized (Figure 7.7).

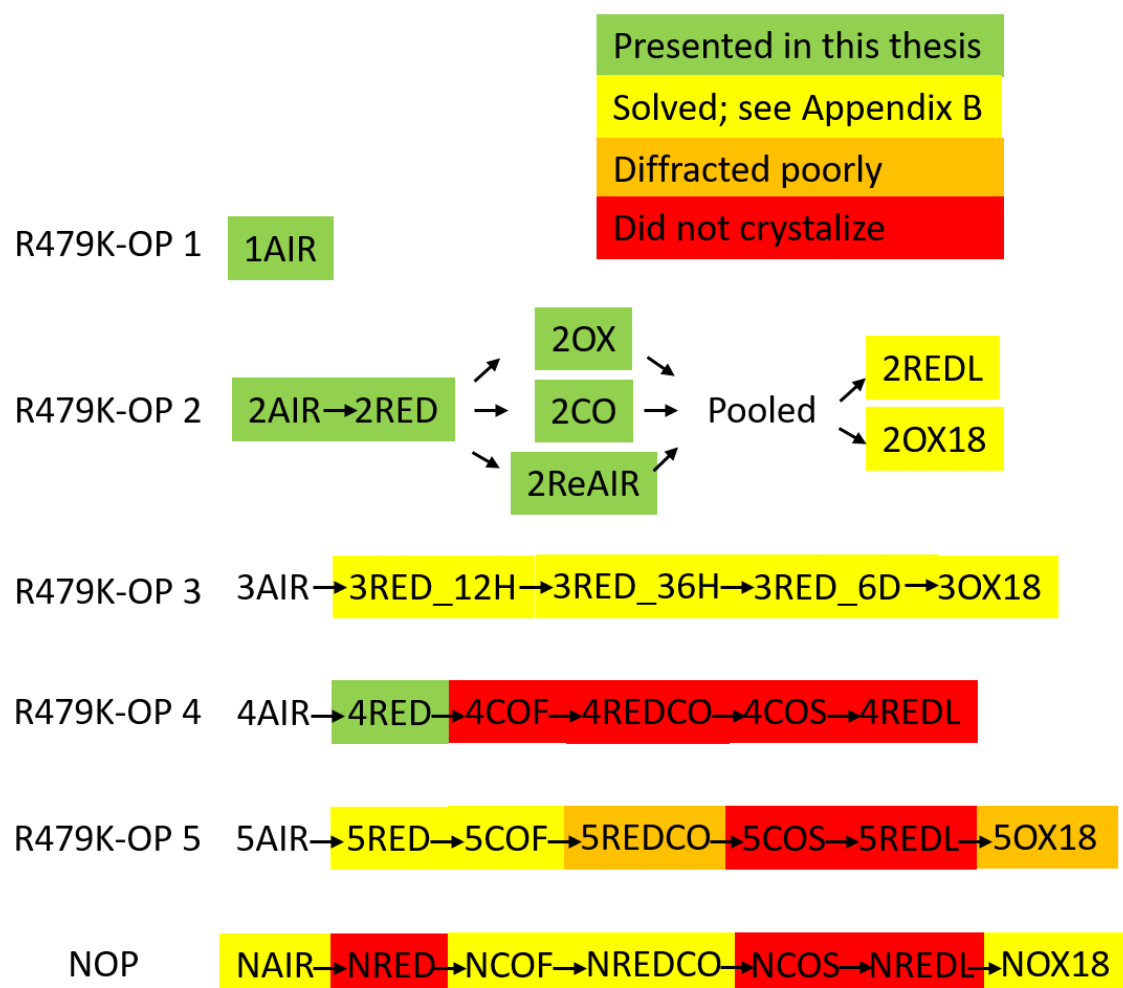


Figure 7.7: Schematic for gas treatment of Hyd-2 R479K-OP and Hyd-2 NOP used to characterize the diatomic found in as-isolated Hyd-2 R479K-OP. All coloured protein treatments were set up to crystallize. The structure of the protein in **green** are presented in this thesis. Protein in **yellow** crystallized and the structure was solved and generally conformed to the structure of protein already solved and presented in this thesis. Data from the structures of the protein in yellow are used for averages of distances (Appendix A for refinement parameters of those data sets) but are not explicitly presented in this thesis. Protein in **orange** crystallized, but the crystal diffracted to a poor resolution, and the structure was not solved. Protein in **red** did not crystallize. Legend: RED=reduced by H₂, suffix L=last, REDCO=treated purge of CO and H₂ simultaneously, 12H=12 h, 36H=36 h, 6D=6 days; CO=treated by CO, suffix F=first, S=second; AIR=exposed to air, prefix Re=exposed to air after reduction; OX=purged with O₂, prefix 18=purged with ¹⁸O₂. See Appendix B for all refinement parameters and detailed description of purge conditions.

Analogous structures of Hyd-2 NOP were also solved to compare the variant and native protein after gas treatments. In contrast to Hyd-2 R479K-OP, Hyd-2 NOP crystallized after a simultaneous purge of the protein with CO and H₂. Protein that experienced further treatment with CO and H₂ reduction did not crystallize, but protein

that had been exposed to ¹⁸O₂ after the extended treatment with CO and H₂ did crystallize, and those crystals diffracted well enough for the structure to be solved.

While the failures to crystallize Hyd-2 R479K-OP consistently over a variety of gas treatments limited the ability to characterize the diatomic found at the active site of Hyd-2 R479K-OP, the crystals that were solved under different gas treatments provided valuable insight into the nature of the diatomic (Table 7.1).

Table 7.1: X-ray data collection and refinement statistics for Hyd-2 R479K-OP treated with different gases.

Sample	2AIR	2RED	2OX	2CO	2ReAIR
Sample treatment	As-Isolated	H ₂ reduced (9h flow)	O ₂ treatment after H ₂	CO treatment after H ₂	Air exposure after H ₂
Data Collection					
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit cell dimensions (Å)	a=100.79, b=101.24, c=171.10	a=100.47, b=101.18, c=170.80	a=100.71, b=101.35, c=170.69	a=100.65, b=101.00, c=170.83	a=100.48, b=100.97, c=170.78
Resolution (Å)	86.8 – 1.3 (1.32-1.3)	86.6- 1.4 (1.42-1.40)	87.1-1.3 (1.32-1.30)	86.9-1.3 (1.32-1.3)	86.9-1.5 (1.53-1.5)
Total reflections	3,434,112 (156,725)	2,854,687 (140,000)	1,574,700 (76,634)	3,653,850 (176,526)	2,411,672 (107,477)
Unique reflections	425,274 (19,281)	329,447 (15,922)	390,605 (19,730)	424,259 (20,649)	271,524 (13,124)
Completeness (%)	99.4 (91.7)	97.0 (95.3)	92.1 (94.5)	99.9 (98.9)	98.4 (97.0)
Multiplicity	8.1 (8.1)	8.7 (8.8)	4.0 (3.9)	8.6 (8.5)	8.9 (8.2)
<I/σ>	9.8 (1.4)	9.1 (1.2)	6.0 (1.2)	6.6 (1.0)	8.6 (2.0)
R _{merge} (%)	10.5 (190)	12.1 (154)	14.4 (89.8)	11.8 (204)	13.4 (104)
R _{pim} (%)	4.2 (70.7)	4.5 (56.8)	8.9 (58.3)	4.6 (77.5)	5.0 (40.5)
CC _{1/2}	0.998 (0.686)	0.997 (0.572)	0.986 (0.49)	0.982 (0.49)	0.996 (0.764)

Refinement					
R _{work} /R _{free} (%)	11.9/14.8	15.5/17.7	11.9/14.8	13.6/16.5	11.1 (15.4)
No. of atoms	14,623	14,343	14,539	13,881	14,560
Macromolecule	12,834	12,797	12,812	12,794	12,848
Solvent	1789	1546	1,727	1,087	1712
Average B-factors					
Macromolecule	15.7	16.5	13.4	16.9	16.3
Solvent	30.9	28.8	29.2	25.9	29.9
RMSD bond lengths (Å)	0.02	0.019	0.02	0.022	0.022
RMSD angles (°)	1.74	1.78	1.76	1.86	1.84
Ramachandran plot (%) favoured / outliers	96.3 / 2	96.3/ 2	96.0/ 2	96.1/ 2	96.0/ 2
Clashscore	0.75	0.67	0.75	0.63	0.75

Values in parenthesis refer to data in the highest resolution shell. See Section 2.8 for more information about R_{merge}, R_{meas}, R_{pim}, R_{work}, R_{free}, and CC_{1/2}.

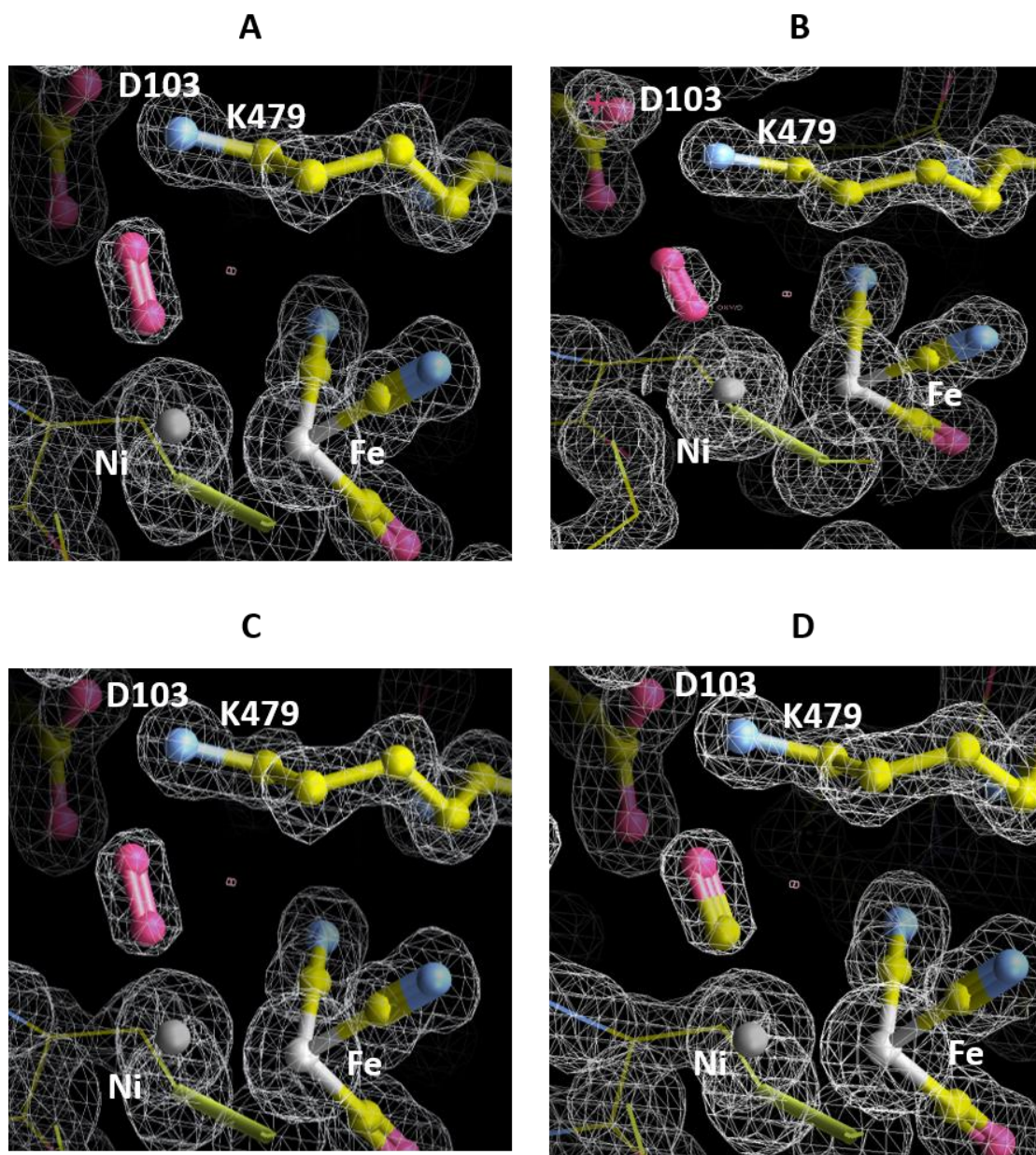


Figure 7.8: Structure of Hyd-2 R479K-OP under different gas treatments with the electron density modeled as well as the interpreted structure. **(A)** As-isolated Hyd-2 R479K-OP (2AIR) was **(B)** activated under 100% H₂ for 9 h (2RED). The reduced enzyme was then **(C)** exposed to pure O₂ for 20 min (2OX) or **(D)** exposed to pure CO for 20 min before crystallization (2CO). The resulting electron density at the active site is shown.

The crystal structure of 2RED revealed that purging the as-isolated enzyme with H₂ for 9 hours only decreased the occupancy of the homodiatomic ligand from 80% to 40% (Figure 7.8). Reduced enzyme that was purged with CO (2CO) resulted in increased occupancy of a diatomic back to 80%, but the electron density is changed. The electron

density observed at the active site of 2CO is now indicative of a heterodiatomic ligand. Greater electron density at one end of the molecule, modelled as an O atom, than the other, modelled as a C atom, makes the structure look different than as-isolated Hyd-2 R479K-OP. The C-O bond length is 1.22 Å, and the Ni-C distance is 1.85 Å while the Ni-C-O bonding angle is 169.1°. Enzyme purged with O₂ (2OX) successfully restores 80% of the homodiatomic and looks identical to the structure of the as-isolated enzyme.

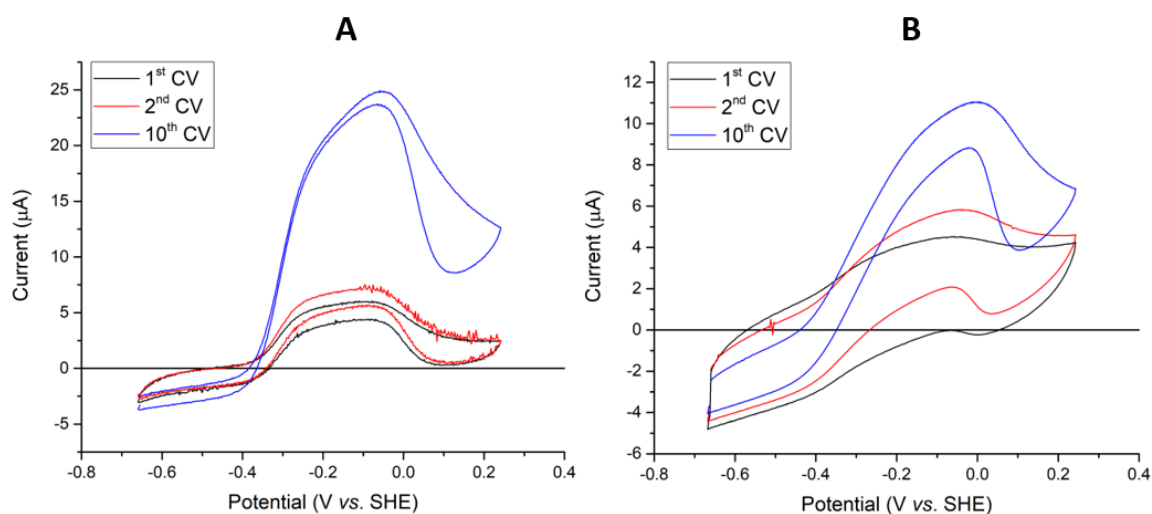


Figure 7.9: Activation profile of Hyd-2 R479K-OP during the first two CVs for **(A)** as-isolated Hyd-2 R479K-OP and **(B)** Hyd-2 R479K-OP that had been activated under a H₂ flow for 9 hours. Other conditions: pH 6.0, 30 mV/sec, 100% H₂, and 1000 RPM.

Interestingly, enzyme that has been purged with H₂ also requires an activation process similar to that needed for as-isolated enzyme, but the activation process results in initial CVs that are distinct from the activation profile of as-isolated Hyd-2 R479K-OP. The rise of current after the thermodynamic H₂/H⁺ equilibrium is less sharp in the enzyme that has been purged with H₂ (Figure 7.9). Moreover, this sharp rise is typically followed by a plateau of current that is common among early CVs during the activation protocol of Hyd-2 R479K-OP. The shape of the initial CVs of pre-purged enzyme more closely resemble CVs of Hyd-2 R479K-OP after the electrochemical activation protocol, but

activation is still required on the pre-purged enzyme as there is still significant growth in current from the first CV.

The structures of Hyd-2 NOP purged with CO are very different than R479K-OP purged with CO. (Appendix B) The NCOF (native enzyme final CO exposure, see Appendix B for detailed description) structure exposed to CO after only a short H₂ purge (10 min) revealed that the enzyme was mainly in the Ni-B state. This finding is consistent with previous experiments that have shown that [NiFe]-hydrogenase in the Ni-B state is unreactive to CO. The structure of Hyd-2 NOP solved after prolonged simultaneous purging with H₂ and CO reveals that the Ni-Fe distance is short (2.6 Å) with electron density bridging the [NiFe] cofactor that resembles a hydride. These characteristics are consistent with enzyme that is in a mixture of Ni-R and Ni-C states. These two Hyd-2 NOP structures differ from the CO-bound structures previously reported for *D. vulgaris* in that the structures of Hyd-2 NOP were purged with CO before crystallization, whereas crystals of *D. vulgaris* were purged with CO shortly before being frozen. Thus, in the 48 h between treating the protein with CO and freezing the crystals, the inhibitor (CO) left the enzyme and Hyd-2 NOP returned to an active state. This reactivation of the native enzyme is in stark contrast to Hyd-2 R479K-OP that traps the inhibitor, which further confirms the importance of K479 in stabilizing diatomic molecules bound to the Ni atom.

7.2.2 Spectroscopic characterization

7.2.2.1 EPR of gas-treated native Hyd-2 NOP

In order to more accurately compare Hyd-2 R479K-OP and native Hyd-2, EPR was used to investigate the resulting states of Hyd-2 NOP after gas treatments similar to those used in the crystallographic investigations of Hyd-2 R479K-OP. Although Hyd-2 had been

previously investigated by EPR, previous characterization relied on chemical reduction or oxidation.

As expected, reducing Hyd-2 NOP with H₂ gives strong Ni-C, Ni-L, and Ni-X signals (Figure 7.10). For Hyd-2 NOP, purging the reduced enzyme with Ar gives a spectrum absent of Ni-X. Warming the enzyme that has been purged with H₂ then purging with Ar gives a spectrum with just Ni-C. As a reversible enzyme, Hyd-2 NOP under an Ar purge likely produces small amounts of H₂, but without an electron donor, H₂ production activity is limited to electrons stored in the reduced Fe-S clusters. Given that both the enzyme under H₂ and Ar conditions were prepared in light, the lack of Ni-X at all temperatures and Ni-L at 70K after the purge with Ar gives insight into the energetics of the states that can only be sustained by fully reduced enzyme.

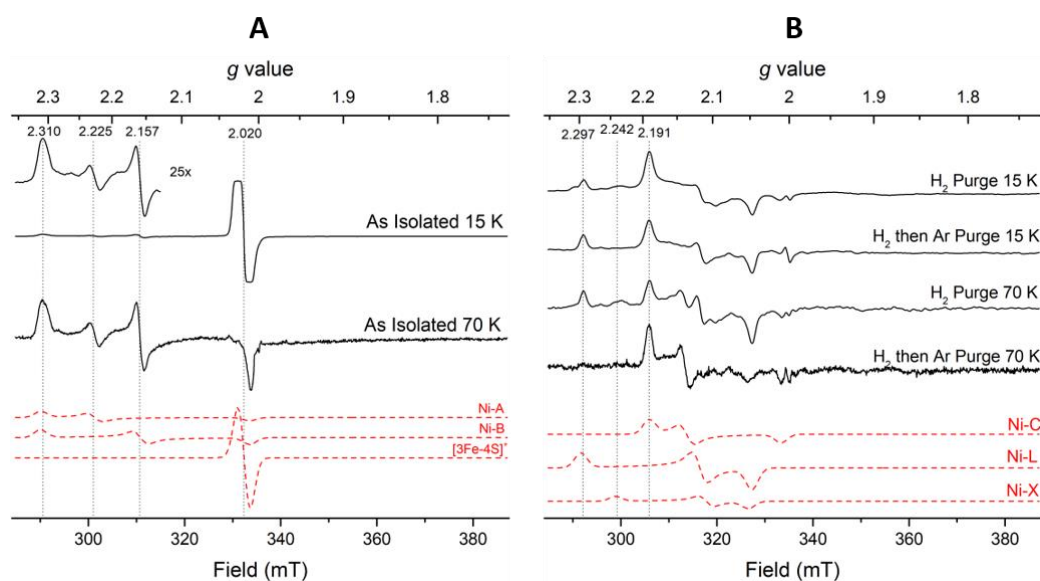


Figure 7.10: EPR of Hyd-2 NOP in the (A) as-isolated state at 15 K and 70 K. Hyd-2 NOP was then reduced with H₂ and finally purged with Ar, and EPR measurements were taken after every step (B). Red lines are simulated EPR signals. Microwave power 2.00 mW; modulation amplitude 1.0 mT. Protein concentration was ~25 μ M.

7.2.2.2 Spectroscopy of Hyd-2 R479K-OP

Attempts to characterize the enzyme by IR or Raman resonance were met with limited success. The crystals of protein that had undergone different gas treatments appeared to give different signals in different areas of the crystal examined (data not shown). This could be a result of either sample preparation or the fact that the intensity of light used to get a discernible signal is changing the state of the enzyme. The only successful spectroscopic characterization was the EPR done on as-isolated Hyd-2 R479K-OP.

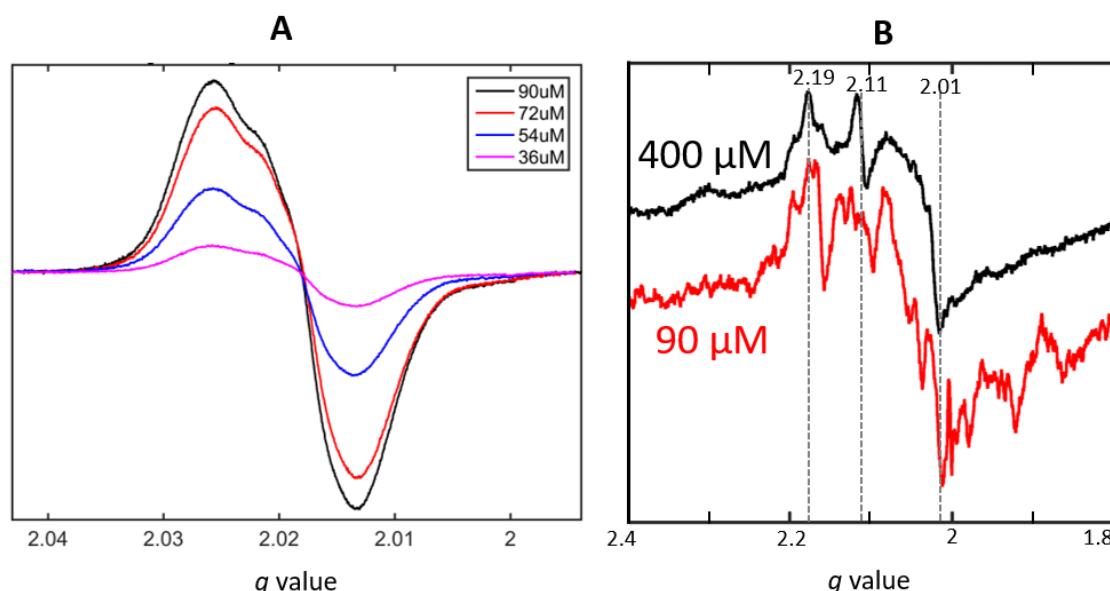


Figure 7.11: EPR for as-isolated Hyd-2 R479K-OP at **(A)** 15 K and **(B)** 70 K. Microwave power 2.00 mW; modulation amplitude 1.0 mT. For EPR spectrum of protein at 400 μ M, 1.6mm high precision tube was used and for all others a 4mm tube was used. Dr. Will Myers (CASER EPR Facility, University of Oxford) performed all EPR measurements on Hyd-2 R479K-OP.

Expectedly, at low temperatures (15K) the EPR spectra of as-isolated Hyd-2 R479K-OP is dominated by the [3Fe-4S]⁺ signal (Figure 7.11). The intensity of the EPR signal is consistent with an enzyme that is almost entirely oxidized. While such an EPR signal at low temperatures in native Hyd-2 would be paired with a high temperature EPR

signal that contains strong Ni-A and Ni-B EPR signals, Hyd-2 R479K-OP appears to give a weak EPR signal that best resembles a state found in reduced Hyd-2.

The strongest EPR signal for as-isolated Hyd-2 R479K-OP at 70 K is $g_x=2.19$, $g_y=2.11$, $g_z=2.01$ closely resembling Ni-C ($g_x=2.193$, $g_y=2.137$, $g_z=2.01$). Moreover, the EPR signal from Hyd-2 R479K-OP at 70 K is so weak that rather than using concentrations similar to native Hyd-2 (25 μ M averaged for <30 s), Hyd-2 R479K-OP requires significantly higher concentrations of enzyme (400 μ M) as well as averaging a signal for >12 h to give a discernible EPR signal. The weakness of the signal likely signifies that the vast majority of the enzyme's active site is in a diamagnetic state. The only other discernible peak is around 2.3, and it is difficult to associate with another peak, which makes it impossible to interpret.

7.3 Discussion

7.3.1 Activation of Hyd-2 R479K-OP

The data show that activation by purging with H₂ alone is insufficient to fully activate the enzyme. Instead, purging Hyd-2 with H₂ leads to the replacement of the diatomic inhibitor with a smaller molecule which is interpreted as a conserved water molecule in the electron density maps. The CVs of Hyd-2 R479K-OP after it has been purged with H₂ reveals that it is in a different state than the as-isolated enzyme, but it still requires electrochemical activation to be fully active. The activation that occurs after Hyd-2 R479K-OP is on an electrode likely reflects the need to clear the active site of the additional water molecule formed from the reduction of the diatomic inhibitor in order for the catalytic cycle to proceed (see Section 6.3.4 for discussion of the state of reduced Hyd-2 R479K-OP in solution).

7.3.2 Identity of the diatomic

The R479K mutation changed the environment around the [NiFe] cofactor in such a way that it trapped a diatomic molecule at the active site. While the active site of [NiFe]-hydrogenase has been solved before with a CO bound, the enzyme in that case was intentionally inhibited after crystallization. The as-isolated structure of Hyd-2 R479K-OP revealed that the diatomic became trapped under standard conditions, which means that the molecule at the active site is likely found in the atmosphere.

One appealing explanation for the trapped diatomic is that the arginine to lysine mutation enhanced the stability of one of the intermediates formed by O₂ attack. In the DFT calculations, the arginine was suggested to contribute approximately 11.0 kcal/mol to the stabilization of the transient Ni-O₂ adduct, yet the distance between the arginine and Ni atom (4.2 Å) in the native enzyme likely limits the stability of the bulky diatomic. In Hyd-2 R479K-OP, the lysine is further removed from the Ni (5.2 Å) and that could further increase the stability of the Ni-O₂ adduct by allowing an appropriate O-O (1.20 Å) distance and permit a strong hydrogen bonding distance between the lysine and the bound O₂ (2.7 Å). Such an arrangement might trap an otherwise transient Ni-O₂ adduct. The correlation between the appearances of the Ni-O₂ adduct in as-isolated R479K-OP and a water molecule after reduction of R479K-OP (Chapter 6) supports this explanation.

Interestingly, the homologous R509K variant in Hyd-1 did not experience any similar phenomenon in the as-isolated enzyme, which suggests that the trapped molecule might be an extension of a large difference that already exists between Hyd-1 and Hyd-2: sensitivity to O₂ or CO. Perhaps the same mechanisms that make Hyd-1 tolerant to O₂, such as the increased supply of electrons to the active site, decreased access to the active site, or the majority resting state, prevents such a diatomic from

inhibiting the active site in the R509K variant. Moreover, the R509K variant was always in a configuration in which the lysine was within 4.4 Å of the Ni atom which would have acted more like the native enzyme in the stabilization of O₂ intermediates.

While inconclusive, the structural and spectroscopic results support the conclusion that the homodiatom at the active site of as-isolated Hyd-2 R479K-OP is O₂. The bond length of the bound diatomic, 1.20 Å, is consistent with an O₂ molecule. The bonding angle is straighter than what is typically observed for O₂, yet the coordination of the lysine may sterically force the O₂ into a configuration that is atypical for bound O₂. Similar to carboxy myoglobin in which the CO is forced into a more bent configuration than typical, the O₂ in Hyd-2 R479K-OP may be forced into an atypical bonding angle in order to be stabilized by the pendant lysine. This conclusion is supported by Hyd-2 R479K-OP that was intentionally inhibited by CO that shows that CO adopts almost the same bonding angle as O₂ rather than the more bent configuration that CO prefers at the active site of [NiFe]-hydrogenases or the straight configuration that typically characterizes the coordination of CO to transition metals. This would suggest that the sterics of the active site force a specific orientation that both diatomics must take up regardless of the ideal binding angle.

The crystal structure of Hyd-2 R479K-OP under different gas treatments further supports the conclusion that the bound diatomic in the as-isolated enzyme is O₂. The obviously heterodiatom nature of CO in the electron density maps of CO inhibited-Hyd-2 R479K-OP suggests that CO is not the molecule in the as-isolated enzyme. Given that the enzyme was crystallized in an anaerobic box of N₂, the decreased occupancy of the diatomic after purging with H₂ makes it unlikely that N₂ is the molecule at the active site.

One key question is how photoreduction of the enzyme that took place during X-ray diffraction affected the structure of Hyd-2 R479K-OP under different gas treatments. Like all the other structures reported in this thesis, linear scans were used to reduce the effect of photoreduction. However, it is likely that at least minor photoreduction occurred that would have decreased the occupancy of the diatomic in almost all structures solved.

7.3.3 Possible binding mode of O₂ and state of hydrogenase

Like Hyd-1 R509K, the EPR spectrum of Hyd-2 R479K-OP contains a species that best resembles a reduced species (Ni-C). The heterogeneous nature of as-isolated Hyd-2 R479K-OP is in stark contrast to the clear spectrum of as-isolated Hyd-2 NOP that contains only two paramagnetic states: Ni-A and Ni-B. The presence of a reduced state could signify that in addition to the slower rate of H₂ production and oxidation, Hyd-2 R479K-OP could also have a lower rate of formation of oxidized inactive states. Alternatively, the EPR signal could indicate that the enzyme in the paramagnetic state observed is less susceptible to O₂ attack.

The most important conclusion from the EPR of Hyd-2 R479K-OP is that the majority of the enzyme is diamagnetic as the strength of the signal suggests that less than 1% of the enzyme is EPR active. This finding indicates that the Hyd-2-O₂ adduct is in a diamagnetic Ni(II) state. Along these lines, a HS Ni(II) is also unlikely as it would have two unpaired spins and would therefore be paramagnetic. These results validate the suggestions of the DFT calculations in the literature that show that a LS Ni atom is preferred in the calculation of O₂ attack on both Ni-C and Ni-SI_a.

Assuming that the O₂ inhibition seen in Hyd-2 R479K-OP follows the same initial steps as aerobic attacks on Hyd-2 NOP postulated by the DFT calculations above,³⁵ the characteristics of the diatomic found in Hyd-2 R479K-OP confirms and clarifies three key characteristics of O₂ attack. First, the Ni-O₂ adduct is diamagnetic which likely means that in Hyd-2 R479K-OP either the inhibited enzyme is formed from attacking Ni-Sl_a, or the Ni-O₂ adduct is reduced shortly after the initial binding to Ni-C to form Ni(II). Second, if the Ni-O₂ adduct is formed from O₂ attack on Ni-Sl_a, the lysine likely plays an important role in stabilizing the end-on O₂ arrangement over the theoretically more favored side-on O₂ binding. In the multiple structures solved with O₂ bound (either by intentionally exposing the enzyme to O₂ or as-isolated), the diatomic was always in an end-on arrangement. Third, the bond distance of the diatomic supports O₂ over O₂⁻ as the redox state of the bound O₂, though the sterics of the active site may play a role in compressing the molecule. Moreover, the best resolution of a structure of oxidized Hyd-2 R479K-OP is 1.30 Å, and a structure with higher resolution would be helpful to definitively confirm the bond length of the O₂.

7.4 Conclusion and Perspective

The unexpected finding of a diatomic at the active site of as-isolated Hyd-2 R479K-OP has important implications both within and outside the hydrogenase field. These initial experiments heavily suggest that the diatomic at the active site is O₂. There are currently ongoing efforts to create a homogeneous sample of Hyd-2 R479K-OP that is labeled with ¹⁸O₂ and to spectroscopically characterize the enzyme (Raman Resonance and infrared). If the preliminary results are confirmed, then R479K-OP would be the first example of a Ni reversibly binding O₂ under standard conditions. Moreover,

this would be the first trapped intermediate of O₂ attack in [NiFe]-hydrogenases and would allow further study of the mechanism of aerobic attack. Together, these results could pave the way for a better understanding of how to design Ni compounds capable of only certain interactions with O₂.

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Chapter 8

Exploring a proton route in/out of the
active site of Hyd-2

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8.1 Introduction

While the hydrogenase field draws great interest due to its application to renewable energy and links to bacterial pathogenicity,^{1,2} hydrogenases are also a good platform for studying the fundamental science of proton and electron transfer in redox proteins.^{3,4} Molecular hydrogen (H_2) is the simplest of molecules and is made up of only four quantum particles, i.e. two protons and two electrons. Hydrogen activation occurs at the active site buried deep in the protein that shields it from bulk solution that could interfere with the delicate reaction. This sequestration necessitates long range proton and electron transfer to/from the protein surface and the active site ($> 30 \text{ \AA}$).

For Hyd-2 (section 4.2.5), and indeed all [NiFe]-hydrogenases,^{1,5} long range electron transfer is performed by employing Fe-S clusters between which electrons can rapidly tunnel to the active site, provided the redox centres are within electron tunnelling distance of one another:^{6,7} electrons can tunnel up to $14 \text{ \AA}$, so only three Fe-S clusters are typically needed to span the distance between the surface of [NiFe]-hydrogenases and the active site (see section 1.3.1 and chapter 4 for hydrogenase structure and Fe-S cluster naming convention).⁸ However, these redox-active units can conformationally rearrange upon exposure to *highly* oxidizing potentials. While degradation of the cluster is oftentimes a sign of oxidative damage,⁹ some [NiFe]-hydrogenases have evolved mechanisms to decrease the impact of oxidative cluster breakdown.^{10,11} In the case of standard [NiFe]-hydrogenases, rearrangement of the proximal cluster prevents loss of one of the irons from the [4Fe-4S] cluster which could be advantageous for surviving aerobic conditions (see section 4.2.5).

For O₂-tolerant [NiFe]-hydrogenases, the ability for the special proximal [4Fe-3S]6cys cluster to perform two 1-electron transfers,^{12,13} the second by a large conformational rearrangement, and its relevance to O₂ tolerance is described in depth elsewhere in this thesis (Section 4.1.3). Importantly, this superoxidized state necessitates a structural rearrangement that is unique to O₂-tolerant [NiFe]-hydrogenases.^{10,11,14}

8.1.1 Proton transfer

In contrast to Fe-S clusters which can be seen in even low-resolution X-ray diffraction crystal structures, protons cannot be seen by X-ray crystallography as they lack electron density. Compared to electrons, protons only tunnel much shorter distances (~45 times shorter), and therefore depend on a proton hopping mechanism in which protons hop from one ionisable group to another.^{6,7} Generally, protons are modelled tunnelling distances up to 1-2 Å,⁷ yet depending on the flexibility of the transfer molecules, protons are typically modelled as traveling 4-5 Å between each step.⁴ The limited mobility of protons complicates the transfer pathway and requires significantly more residues to transfer protons into and out of the enzyme.

Theoretical studies of the proton transfer pathways through [NiFe]-hydrogenase have predicted that proton transfer likely depends on a number of possible pathways of glutamates, histidines, and water molecules as these are the prominent residues able to protonate/deprotonate at near neutral pH.^{4,15} Aspartates and cysteines in the [NiFe]-hydrogenase could also be part of the pathway, but they are active in proton transfer conditions outside neutral pH.⁴ However, it is difficult to model the interplay between

proton and electron transfer as studies have shown that the redox state of the Fe-S clusters can affect the pK_a of local residues.¹⁶

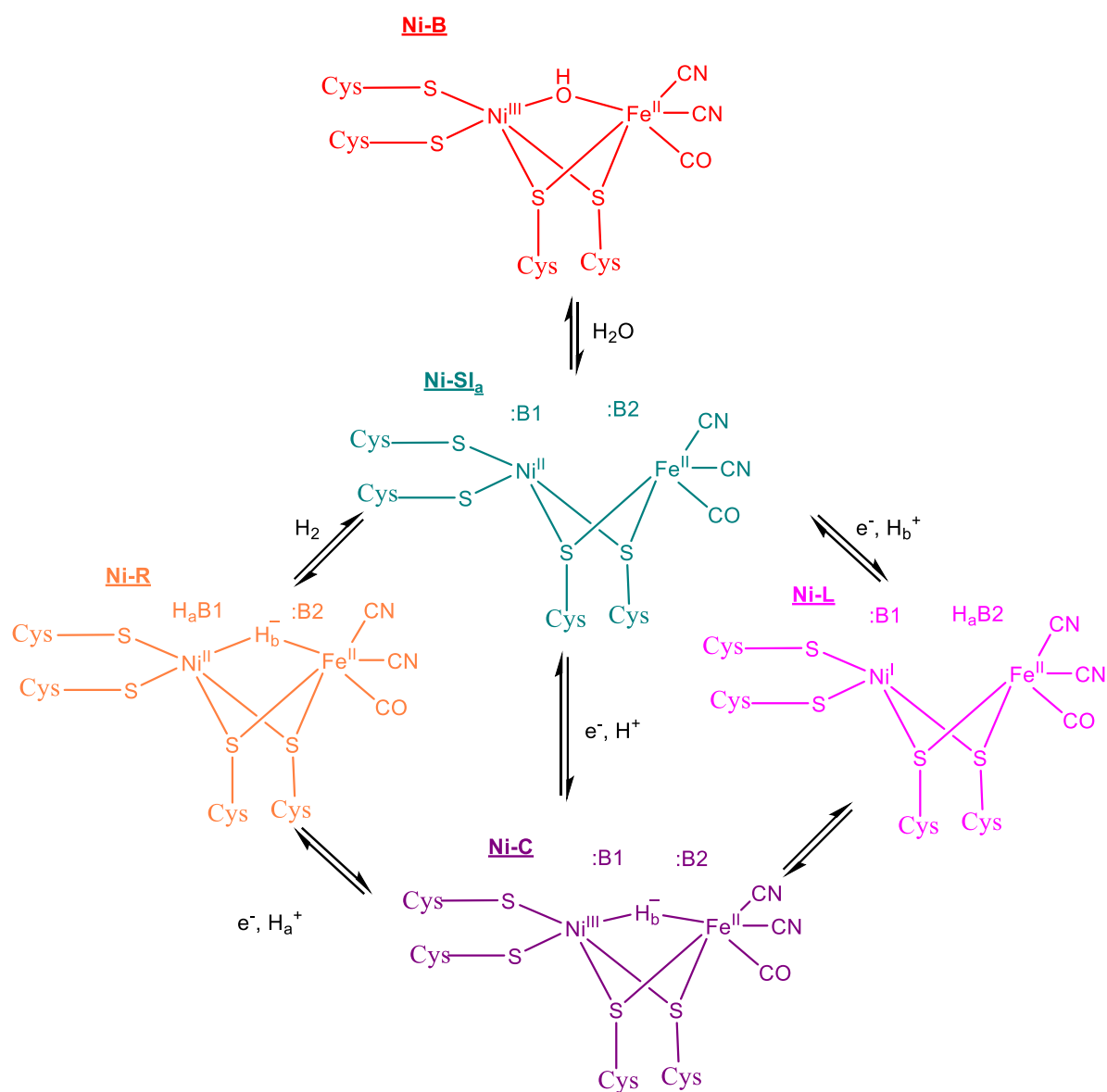


Figure 8.1: Schematic of active site states showing the two protons, labelled H_a^+ and H_b^+ , transported away from the active site during H_2 oxidation by ionisable groups, labelled B1 and B2 (see text). The two protons are modelled leaving by different pathways though they could take the same pathway or both pathways out of the active site. Ni-L is also modelled as a possible intermediate though it has only been shown to be a true intermediate for some [NiFe]-hydrogenases (section 1.3.2).

Complicating the matter further, there is no reason that the two protons released during H_2 activation need follow the same pathway out of the active site

(Figure 8.1). For simplicity sake, in this chapter the proton abstracted from molecular H_2 is denoted as H_a^+ , differentiating it from the remaining hydride that is likely transported away from the active site as a proton, denoted H_b^+ .^{17,18} The two protons are theorized to start in different places in the protein with H_a^+ starting by being abstracted by a base (both R479 and C546 have been postulated)^{1,19,20} and H_b^+ as a bridging ligand between Ni and Fe in the Ni-R and Ni-C states (see Section 1.3.2).^{21,22} Spectroscopic techniques that are sensitive to small changes in the distribution of electrons at the active site of [NiFe]-hydrogenases, like IR spectroscopy, have registered multiple Ni-R and Ni-L states.²³ Not all [NiFe]-hydrogenases exhibit the spectroscopic signature of all Ni-R and Ni-L states, but a review of the investigations of [NiFe]-hydrogenase by IR spectroscopy suggests that there are two Ni-R signals and three Ni-L signals.¹ There are also trends concerning which states are present in different [NiFe]-hydrogenase that depend on the type (i.e. O_2 -tolerant or standard) of [NiFe]-hydrogenase.²³ The physical interpretation of the difference between the two Ni-R and three Ni-L states is unknown, though it is theorized that the states differ by distance or location of the leaving proton as both Ni-R and Ni-L are states in which H_a^+ and H_b^+ , respectively, are migrating away from the active site.²³

Typically, IR spectroscopy follows the ν_{CO} of the carbonyl ligand on the Fe atom, and the multiple states indicate that there is more than one group in the proton pathway that is close enough to the [NiFe] cofactor to affect the electrostatics of the carbonyl.^{24,25} However, mapping important residues in the proton transfer pathway by site-directed mutagenesis is challenging as water molecules can shift in response to the mutation.²⁶ Together, these elements have limited insight on proton transfer pathways,

yet there have been notable exceptions that have elucidated residues likely involved in proton transfer (see below).

8.1.2 E14Q variant

The important work by Dementin et al showed that mutating a glutamate to glutamine (E14Q using Hyd-2 numbering) in the standard [NiFe]-hydrogenase from *Desulfovibrio fructosovorans* renders the enzyme nearly totally inactive (<99.9% activity) at pH 6-11.³ However, the work showed that the inactive variant was still capable of para/ortho H₂ interconversion³ demonstrating that the active site of the E14Q variant can still cleave H₂, but the protons cannot leave the active site. This work inspired various homologous variants in different [NiFe]-hydrogenases resulting in different levels of inactivity, though most variants lose the majority of the activity of the native enzyme.^{18,27} Recently, it was reported that replacing E28 (equivalent to E14) in *E. coli* Hyd-1 with a glutamine led to an enzyme that was almost entirely inactive.²⁸ Together these studies strongly suggest that E14 is necessary for proton transport out of the active site, yet they are indeterminate as to which of the two protons (possibly both) require E14 as a residue in the proton pathway.

8.1.3 Implication of E14 on H₂ activation mechanism

The proximity of E14 to terminal cysteine C546 has been used as evidence to support theories that H_a⁺ is abstracted by the sulphur of C546 and rapidly transported away from the active site by E14.^{18,20,29,30} While the importance of E14 does not preclude the possibility that the pendant arginine acts as a base to abstract H_a⁺, protons abstracted by R479 would likely take a different pathway out of the active site than E14

(Chapter 6). Regardless of the base that abstracts H_a^+ , H_b^+ could still leave the active site via E14. Indeed, there is significant evidence that E14 acts as a proton relay for H_b^+ (Figure 8.1).¹⁸

8.1.4 A second look at the activity of Hyd-1 E28Q

While it has been recently reported that the Hyd-1 E28Q variant is totally inactive as measured by PFE experiments at pH 6.0 and for potentials between -0.60 V and 0.200 V,²⁸ Hyd-1 E28Q on electrodes modified with carbon nanotubes to increase surface area (see section 2.10.4) show that there is low activity of E28Q at pH 6.0.³¹ That activity is also concealed by the long activation procedures required to fully reactivate as-isolated Hyd-1 E28Q.³¹ While native Hyd-1 typically takes hours to fully reactivate, Hyd-1 E28Q required days of an electrochemical reactivation protocol to reveal its full activity.³¹ This finding cast doubt on earlier studies showing total inactivity for the glutamate variant, as these studies performed only the standard reactivation protocol on the glutamate variant and could have therefore been measuring the activity of inactive enzyme.³

Moreover, a unique current profile is revealed by increasing the potential range of the experiment: E28Q activity increases in two different potential regimes, denoted as section I and section II in Figure 8.2. The current between the onset potential and 0.05 V (section I at pH 6) for Hyd-1 E28Q increases with pH up to pH 10. Whereas native Hyd-1 has maximal hydrogen oxidation activity at pH 8.0, electrochemical measurements of Hyd-1 E28Q show increasing current with increasing pH beyond pH 8 (Figure 8.2). By contrast, native Hyd-1 has experienced nearly a ten-fold decrease in activity at pH 10 when compared to pH 6 (Figure 8.2). Steady-state solution assays

comparing activity of native Hyd-1 and Hyd-1 E28Q (using methylene blue, a mediator with a potential of 11 mV)³² agreed well with data from electrochemical experiments showing a strong correlation between pH and enzyme activity for the variant.³¹

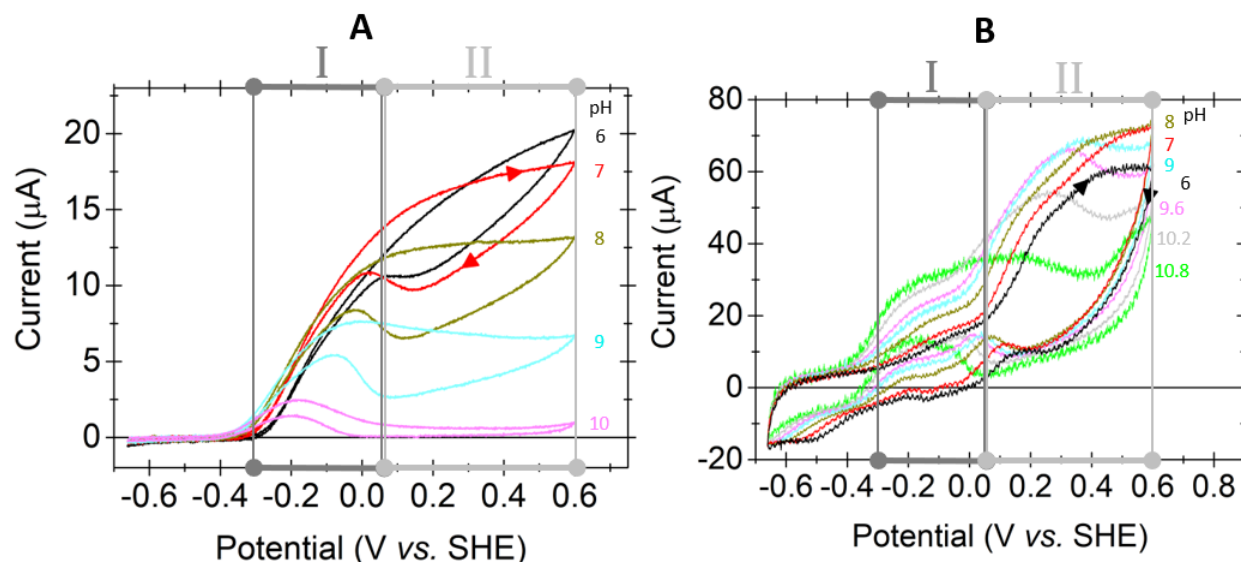


Figure 8.2: Comparison of the pH dependence of CVs of (A) native Hyd-1 and (B) Hyd-1 E28Q. (A) On a standard PGE electrode, the potential was scanned at 5 mV/s (for clarity, the scan direction is indicated with arrows on the pH 7 scan, red). The film was exposed to the mixed buffer system at each pH in the order 6, 4 (not shown), 5 (not shown), 7, 8, 9, 10. For E28Q (B) using a modified electrode (see section 2.10.4), the potential was scanned at between -0.6 to +0.6 V in 100% H₂ (for clarity, the scan direction is indicated with arrows on the pH 6 scan, black). The film was exposed to a potassium phosphate (100 mM), NaCl (100 mM) buffer at each pH in the order 6, 7, 8, 8.5 (not shown), 9, 9.6, 10.2, 10.8. For both native Hyd-1 and E28Q the buffer was returned to pH 6 in between each pH to account for film loss. Each buffer was pre-equilibrated with 100% H₂ and 2 scans were taken at each pH to ensure complete gas and temperature equilibration. Other conditions: 100% H₂, 37 °C, total gas flow rate maintained at 1000 scc/min, $w = 2500$ rpm. All protein production and electrochemistry for Hyd-1 enzymes performed by Dr. Rhiannon Evans (University of Oxford). The sections are based on the CVs at pH 6 based on the potential at which the increase in current is seen for Hyd-1 E28Q, which is approximately 50 mV. Figure adapted from reference.³¹

Interestingly, the cyclic voltammograms of Hyd-1 E28Q (Figure 8.2) revealed that at pH 4 through pH 9 there is a large increase in the current at potentials higher than 0.05 V (section II) compared to the current at 0 V. Scans of native Hyd-1 at potentials greater than 0.05 V show only slight increases in activity as Ni-B formation (section 1.3.2) is more stable at higher potentials. By contrast, Hyd-1 E28Q shows rapidly increasing current at 0.05 V due to a lack of formation of Ni-B (see below). Further

electrochemical and solution-based experiments confirmed that the second potential regime is not an electrochemical artefact as the current is H₂-dependent.

8.1.5 PFIRE from Hyd-1 E28Q

Protein film infrared electrochemistry (PFIRE) is a powerful tool for revealing the distribution of hydrogenase states during turnover conditions. In short, it includes all the elements of traditional PFE (section 2.10) while also measuring the IR spectrum of the adsorbed hydrogenase *in situ*. The IR measurements typically report the vibrational frequency of the CO ligand bound to the active site Fe as it is a reliable indicator of the electronic distribution at the active site. The states of native Hyd-1 as measured by PFIRE are well documented.^{17,23}

At an oxidizing potential (0.356 V) in an inert gas, native Hyd-1 is almost exclusively in the Ni-B state. Under turnover conditions with H₂ at that same potential, native Hyd-1 resides mainly in three Ni-L states and three Ni-R states (Section 8.1.1) though there are also significant populations of Ni-C and Ni-SI_a (Figure 8.1).^{17,23} Lowering the potential to -0.074 V while maintaining an H₂ flow does not significantly change the distribution of active states, though it does decrease the population of Ni-B. However, holding Hyd-1 at -0.074 V under Ar forces Hyd-1 into predominantly Ni-SI_a with some populations of Ni-B, Ni-C, and Ni-L.¹⁷

Infrared measurements of Hyd-1 E28Q under hydrogen oxidation conditions showed that Ni-R dominates the IR spectrum with additional formation of Ni-C. In contrast to native Hyd-1 (which has a Ni-L dominance over Ni-C at ~2:1),³³ almost no Ni-L is present in E28Q under turnover conditions except for at very high potentials. Even at these potentials, the quantity of Ni-L is negligible.³¹ These results suggest that the

proton transfer pathway for H_a^+ is less affected by the mutation than H_b^+ , though only one of the two Ni-R states was observed in Hyd-1 E28Q which could be an indicator that the second Ni-R state relies on E14 to transport H_a^+ .³¹

The PFIRE experiments also revealed that even at very high potentials as the current from Hyd-1 E28Q sharply decreases, it is not accompanied by a sharp increase in the rate of Ni-B formation typically present under turnover conditions.³¹ Under an H_2 atmosphere at oxidizing potentials (0.356 V), Hyd-1 E28Q exhibits no Ni-B formation in contrast to native Hyd-1.^{17,31} The lack of Ni-B in Hyd-1 E28Q at high potentials is surprising given that the reverse scan of the CV for Hyd-1 E28Q (Figure 8.2) shows a current decrease of approximately nearly 80% that would at first glance traditionally be ascribed to anaerobic inactivation.³¹ However, PFIRE reveals that at high potentials, all the states (Ni-R and Ni-C) that are typically present under turnover conditions continue to exist as if the variant hydrogenase at high potential stalls in the reduced active states Ni-R and Ni-C.³¹

8.1.6 Structural insight from Hyd-1 E28Q

To understand the cause of the two different regions of activity, the structure of Hyd-1 E28Q was solved under different pH and oxidation conditions. The H_2 reduced protein was crystallized at pH 6 and pH 8, and some crystals grown at pH 8 were soaked in pH 10 cryoprotectant solution before being flash frozen in $N_2(l)$. Comparing the structures of the reduced variant at pH 10, pH 8, and pH 6 revealed that at higher pH a H_2O/OH^- molecule appears close to Q28 which could be capable of transferring protons (Figure 8.3). Much like the water molecule that fills the role of D103 in Hyd-2 D103A (see structure of Hyd-2 D103A-OP in section 6.2.1) or various examples throughout the

literature,²⁶ a $\text{H}_2\text{O}/\text{OH}^-$ could fill the role of E28 transferring protons along the pathway and restoring activity. This trend would explain the increasing activity with increasing pH as high pH coincides with a $\text{H}_2\text{O}/\text{OH}^-$ in the position needed for restoration of the proton transfer pathway. This explanation applies in section I of the CV (Figure 8.2) and explains the increase in activity with increasing pH above 7.0. The increasing activity in section II of the CV (Figure 8.2) can be explained by another key component of the Hyd-1 structures: the presence of the superoxidized proximal cluster.

Comparing the reduced and as-isolated structures of Hyd-1 E28Q (both at pH 6.0) shows that the structural rearrangement of the proximal Fe-S cluster as it forms the superoxidized state in the as-isolated enzyme coincides with a $\text{H}_2\text{O}/\text{OH}^-$ molecule in similar proximity to Q28 as seen at basic pH conditions. The electron density of the $\text{H}_2\text{O}/\text{OH}^-$ indicated that it was only there at a partial occupancy and the electron density of the molecule became increasingly intense with pH. This observation could explain why at potentials high enough to cause the reorganization of the $[\text{4Fe-3S}]6\text{cys}$ proximal cluster there is a large increase in activity. As the cluster reorganizes, the electron density ascribed to the $\text{H}_2\text{O}/\text{OH}^-$ molecule close to Q28, and thus the ability of that variant to transport protons out of the active site, increases. The question was asked as to whether this phenomenon is linked to the superoxidation of the proximal cluster unique to Hyd-1 and other O_2 -tolerant $[\text{NiFe}]$ -hydrogenases which contain the special $[\text{4Fe-3S}]6\text{cys}$ proximal cluster.^{5,14,34} The proximal cluster in Hyd-2 undergoes a slight, but reversible, reorganization upon air oxidation (see section 4.2.5). If the equivalent Hyd-2 variant (E14Q) is sufficiently active for steady-state measurements, subsequent steady-state PFIRE measurements would give further insight on the proton pathways of standard O_2 -sensitive $[\text{NiFe}]$ -hydrogenases.

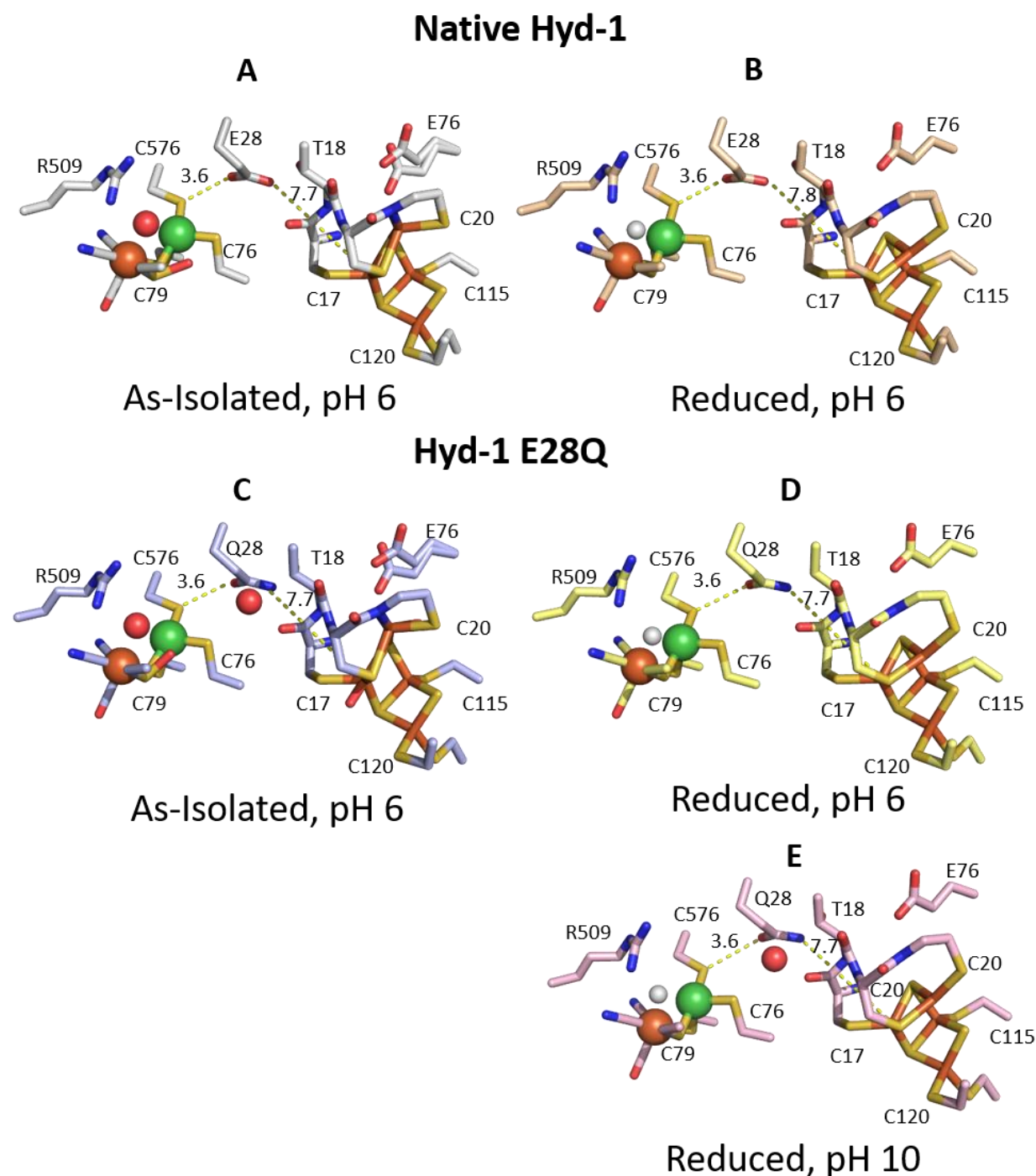


Figure 8.3: Structural comparison of native Hyd-1 and Hyd-1 E28Q under different conditions. In all structures, OH^- is modelled as a red sphere (both near Q28 and in the bridging position) and H^- is modelled as a grey sphere. **(A)** Native Hyd-1 (PDB 6FPO) in the as-isolated state at pH 6 shows the rearrangement of the proximal cluster (section 8.1.1) in the superoxidized state as well as the bridging hydroxide of the $[\text{NiFe}]$ cofactor. **(B)** Native Hyd-1 after reduction at pH 6 (PDB 6FPW) shows the proximal cluster in its reduced state and a bridging hydride at the $[\text{NiFe}]$ cofactor. **(C)** Hyd-1 E28Q at pH 6 in its as-isolated state (PDB 5LMM) shows the superoxidized proximal cluster rearrangement, as seen previously for native Hyd-1, as well as the $\text{H}_2\text{O}/\text{OH}^-$ molecule in close proximity to Q28. **(D)** Hyd-1 E28Q at pH 6 in the reduced state (PDB 6FPI) lacks the $\text{H}_2\text{O}/\text{OH}^-$ near Q28. **(E)** Reduced Hyd-1 E28Q crystallized at pH 8 and soaked at pH 10. Note that at pH 10 the electron density of the $\text{H}_2\text{O}/\text{OH}^-$ close to Q28 is at a lower occupancy than the as-

*isolated structure but increases with pH. Both reduced native Hyd-1 and E28Q are in a mixture of Ni-R and Ni-C. All distances measured in Å. Figure adapted from reference.*³¹

8.1.7 Hyd-1 C19G/C120G/E28Q

Because the crystal structure of as isolated Hyd-1 E28Q implicated the [4Fe-3S]6cys proximal cluster in the high potential activity of Hyd-1 E28Q, a Hyd-1 E28Q variant was made in which the [4Fe-3S]6cys cluster was exchanged for a [4Fe-4S] cluster by mutating the supernumerary cysteines to glycine (C19G/C120G) (see Section 4.1.3). The effect of mutating the supernumerary cysteines has been studied previously in *E. coli* Hyd-1 and *R. eutropha* MBH and the replacement of the [4Fe-3S]6cys cluster with the standard cubane [4Fe-4S] cluster found in O₂-sensitive [NiFe]-hydrogenase has been confirmed by EPR^{35,36} and structural characterization of *E. coli* Hyd-1 C19G.³⁷ The C19G variant is incapable of forming the superoxidized state, and the need for the cysteines in the superoxidized state is discussed elsewhere in this thesis (Section 4.1.3).

The triple E28Q/C19G/C120G variant exhibits a similar pH dependent current increase in section I of the CV compared to the E28Q variant (Figure 8.4), but it does not exhibit the increase in activity at high potential (section II).³¹ While this finding supports the theory that the reorganization of the special proximal cluster is responsible for the second high-potential region increase in activity in E28Q, the triple variant is not ideal for understanding the [4Fe-4S] cluster as it is not native to the enzyme. There is also no evidence that the [4Fe-4S] cluster in an O₂-tolerant enzyme undergoes the same rearrangement seen in standard [NiFe]-hydrogenases.³⁷

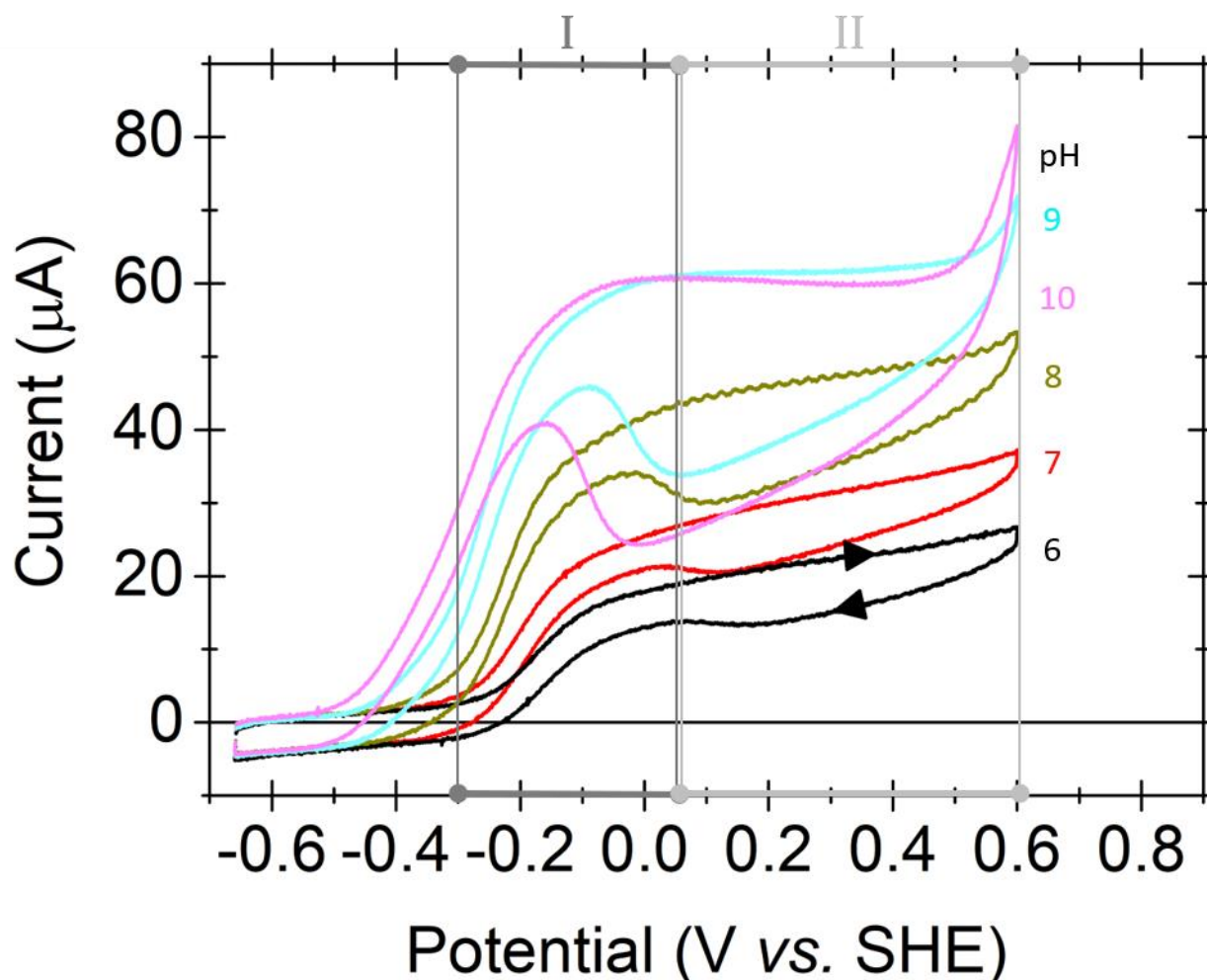


Figure 8.4: The pH dependence of Hyd-1 C19G/C120G/E28Q in the mixed buffer system pH 6-10. The potential was scanned at 5 mV/s and after each pH, the film was returned to pH 7 to correct for film loss. Each buffer was pre-equilibrated with 100% H₂ and two scans at each pH were taken in order to ensure complete equilibration. Arrows are shown on pH 7 scan (black) for clarity of scan direction. Other conditions: 37 °C, total gas flow rate maintained at 1000 scc/min, $\omega = 1000\text{-}2500$ rpm, and 100% H₂. The sections are based on the CVs at pH 6 based on the potential at which the increase in current is seen for Hyd-1 E28Q, which is approximately 50 mV. Figure adapted from reference.³¹

By contrast, Hyd-2 would make a better study for how the standard cubane affects the activity of the glutamate variant at high potential. Hyd-2 is stable over a smaller pH range (see below), and while only capable of limited pH studies, Hyd-2 would offer insight into the effect of the standard [4Fe-4S] cluster in the E14Q variant. Similar to the reasoning for comparative Hyd-2 canopy variants (Chapter 6), the similar periplasmic environment of Hyd-1 and Hyd-2 makes the [NiFe]-hydrogenases powerful

tools for comparing the mechanism of proton transfer in O₂-tolerant and standard hydrogenases.

8.1.8 The effect of changing pH on Hyd-2

The catalytic activity of Hyd-2 at different pH values was characterized in some of the earliest studies of natively expressed Hyd-2.³⁸ These studies relied on solution assays with redox active dyes of Hyd-2 purified by cleaving the transmembrane (TM) domain of HybO.³⁸ The systematic characterization of the two [NiFe]-hydrogenases in *E. coli* also examined the effect of changing pH on both enzymes.³⁹ Although this approach purified Hyd-2 with an intact TM domain and relied on electrochemical characterizations of Hyd-2, the results were in good agreement with the previously published characterization.³⁹ These studies found that Hyd-2 is not stable outside near neutral pH, and detailed electrochemical characterizations were limited to pH 5–pH 8.³⁹ One key difference between Hyd-1 and Hyd-2 is the minimal overpotential requirement of Hyd-2 (see section 9.1.1).⁴⁰ With increasing pH, Hyd-2 displays a Nernstian shift in the H₂/H⁺ thermodynamic equilibrium (i.e. -59 mV/pH). Thus, a direct comparison of the activity at different pH, particularly when comparing activities at potentials close to the H₂/H⁺ thermodynamic equilibrium, is challenging. Such a comparison must take into consideration the relative driving force at each potential. However, a general comparison of the activity of Hyd-2 NOP ranks pH 7 as the most active with pH 6 and pH 8 approximately equivalent to each other (see below). Electrochemical characterization also showed that while there is no obvious difference in the rate of inactivation at different pH (at 30 mV/sec) the extent of inactivation increases at basic pH values, and

this effect can be seen at slower scan rates.³⁹ This result is unsurprising given that higher pH correlates to a higher concentration of OH⁻.

8.1.9 Aims of this chapter

In this chapter, the structural and enzymatic characterization of Hyd-2 E14Q-OP is described and compared to Hyd-1 E28Q in order to draw conclusions about the effect of the proximal cluster on possible proton transfer pathways out of the active site during H₂ oxidation.

8.2 Results

8.2.1 Production of Hyd-2 E14Q-OP

The E14Q mutation was introduced into the HJ001-hyp strain (see section 2.4 for pMAK, and tables 2.5, 2.6, and 2.7 for a detailed description of the strain, plasmid, and primers used to create the strain) to enable the overproduction of Hyd-2 E14Q-OP. The enzyme variant expresses at similar levels as Hyd-2 NOP (approximately 0.15 mg/L). Data from an SDS-PAGE as well as size exclusion chromatography shows that Hyd-2 E14Q-OP purifies as the HybOC dimer as expected (data not shown).

8.2.2 The effect of changing pH on Hyd-2 NOP and Hyd-2 E14Q-OP

The activity of Hyd-2 NOP was measured electrochemically at pH 6, 7 and 8, and the film was returned to pH 6 between every scan (Figure 8.5). While the decrease in current from exposing the film to pH 7 is expected for a normal rate of film loss (section 2.10.3.2), the dramatic drop in current following exposure to pH 8 conditions was elevated above this normal rate indicating enzyme/film instability at pH 8. The relative

rates of H₂ oxidation at different pH are in good agreement with previously published data for natively expressed Hyd-2 (Chapter 3).^{38,39}

Characterization of Hyd-2 E14Q-OP at different pH values is challenging as Hyd-2 E14Q-OP is almost totally inactive. Even when using concentrated enzyme and modified electrodes to increase surface area and covalently link the enzyme to the electrode surface (section 2.10.4), electrochemical measurements were difficult. Activation was done for 2-4 hours, but Hyd-2 is more prone to film loss than Hyd-1 prohibiting the days-long activation protocol used for the E28Q variants.³¹ Thus, the electrochemical measurements of Hyd-2 E14Q-OP likely represent the activity of enzyme that is not fully active. Moreover, the increased capacitance resulting from modifying the electrode with multi-walled carbon nanotubes (section 2.10.4) results in poor signal-to-noise measurements requiring slow scan rates (1 mV/sec) to reduce the effect of capacitance on the scans of Hyd-2 E14Q-OP.

Electrochemical measurements over the limited pH range at which Hyd-2 is stable show that there is a slight increase in current that corresponds to increasing pH for the potential range between the onset potential and -0.1 V (Figure 8.5). For Hyd-2 E14Q-OP, scanned at 1 mV/sec, the effect of anaerobic oxidative inactivation is greater at higher pH values causing a decrease in current at high potentials. While this inactivation would typically be attributed to Ni-B formation, the tendency for the homologous Hyd-1 variant to stall in reduced active states of the catalytic cycle as measured by PFIRE (see Section 8.1.5) prohibits such a conclusion for Hyd-2 E14Q-OP. However, the inactivation at high potential clearly shows that the catalytic activity of Hyd-2 E14Q-OP differs greatly in section II of the CV from Hyd-1 E28Q (Figure 8.2) and is incapable of a second region of greatly increased activity.

The combination of the limited pH range at which Hyd-2 is stable, plus the low level of activity of Hyd-2 E14Q-OP makes the slight increase in activity in section I of the CV difficult to distinguish from the general increase with pH that is seen in Hyd-2 NOP (compare Figure 8.5A and B). Electrochemical characterization of Hyd-2 is particularly sensitive because even small changes in the folding, affinity, and orientation induced by pH shifts could cause a decrease in the electroactive population of enzyme. Early solution assays of Hyd-2 were able to sample the activity of Hyd-2 over a much wider pH range, likely due to the difference in the timescale of the two experiments: a 1 mv/sec scan requires approximately 30 min while solution assays require less than 5 min.

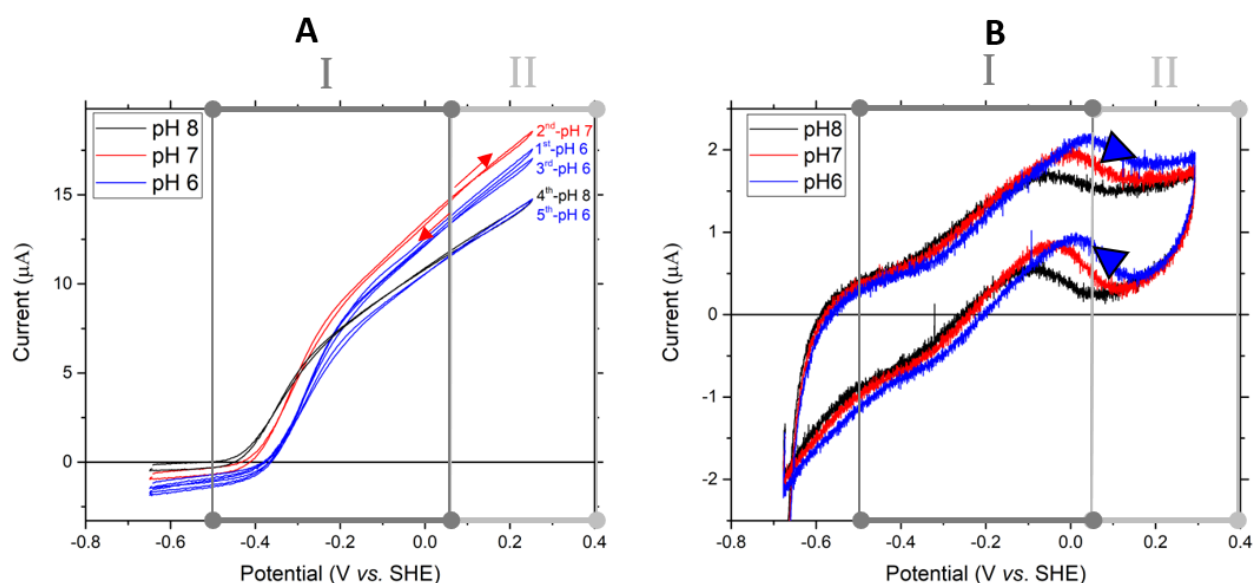


Figure 8.5: The pH dependence of **(A)** Hyd-2 NOP and **(B)** Hyd-2 E14Q-OP at pH 6–pH 8. **(A)** For Hyd-2 NOP on an unmodified electrode, the potential was scanned at 30 mV/s returning to pH 6 between each scan. The order of the scans is labelled, and the direction of the scans is labelled at pH 7 (red) for clarity. **(B)** Using covalently attached enzyme on modified electrodes, Hyd-2 E14Q-OP was assessed using highly concentrated enzyme ($>8 \text{ mg mL}^{-1}$) at 1 mV/s. The films were not corrected for film loss, but activity was measured in pH descending order (pH 8, pH 7, pH 6). For clarity, arrows are shown on the pH 6 scan. Other conditions: 30 °C, total gas flow rate maintained at 1000 scc/min, $\omega = 1000 \text{ rpm}$, and 100% H_2 . The sections are based on the CVs at pH 6 based on the potential at which the increase in current is seen for Hyd-1 E28Q, which is approximately 50 mV.

8.2.3 Steady-state solution assays of Hyd-2 E14Q-OP

Steady-state solution assays were performed with Hyd-2 E14Q-OP to determine if the activity of the variant could be better ascertained in solution. The same assays were done for Hyd-2 NOP, and the relative rates of Hyd-2 NOP at varying pH (i.e. H_2 oxidation rates by Hyd-2 measured with benzyl viologen is best at pH 8.0) agree well with previous measurements reported in the literature (section 3.2.7).³⁸ For example, the turnover rate measurements for Hyd-2 NOP reported in Chapter 3 is $218 \pm 17 \text{ s}^{-1}$, which is higher than the $133.18 \pm 33.3 \text{ s}^{-1}$ reported here, but they are under two different conditions. In order to maximize reactivation time for Hyd-2 E14Q-OP, both Hyd-2 NOP and Hyd-2 E14Q-OP were both reactivated under an H_2 purge for 18-24 hours, opposed to the 8-12 hours that Hyd-2 NOP underwent previously. The prolonged reactivation time would likely result in similar levels of time dependent degradation for the two enzymes but would allow Hyd-2 E14Q-OP to be more fully active.

Steady-state solution assays show that while Hyd-2 NOP reaches maximum activity at pH 8.0, Hyd-2 E14Q-OP continues increasing activity at pH 9.0 (Figure 8.6, Table 8.1). The decrease in activity at pH 9.0 of Hyd-2 NOP is likely an effect of the breakdown of the protein and agrees well with previous experiments that have shown that Hyd-2 is less active at high pH.^{38,41} The increase in activity of Hyd-2 E14Q-OP therefore occurs in spite of a similar level of protein breakdown as the E14Q mutation is unlikely to instill an increased global protein stability. It is important to note that the potential of benzyl viologen is slightly more oxidizing (-358 mV at pH 7)³² than the H_2/H^+ thermodynamic equilibrium (-414 mV at pH 7) and solution assays therefore reflect activity of Hyd-2 E14Q-OP within section I of the cyclic voltammogram.

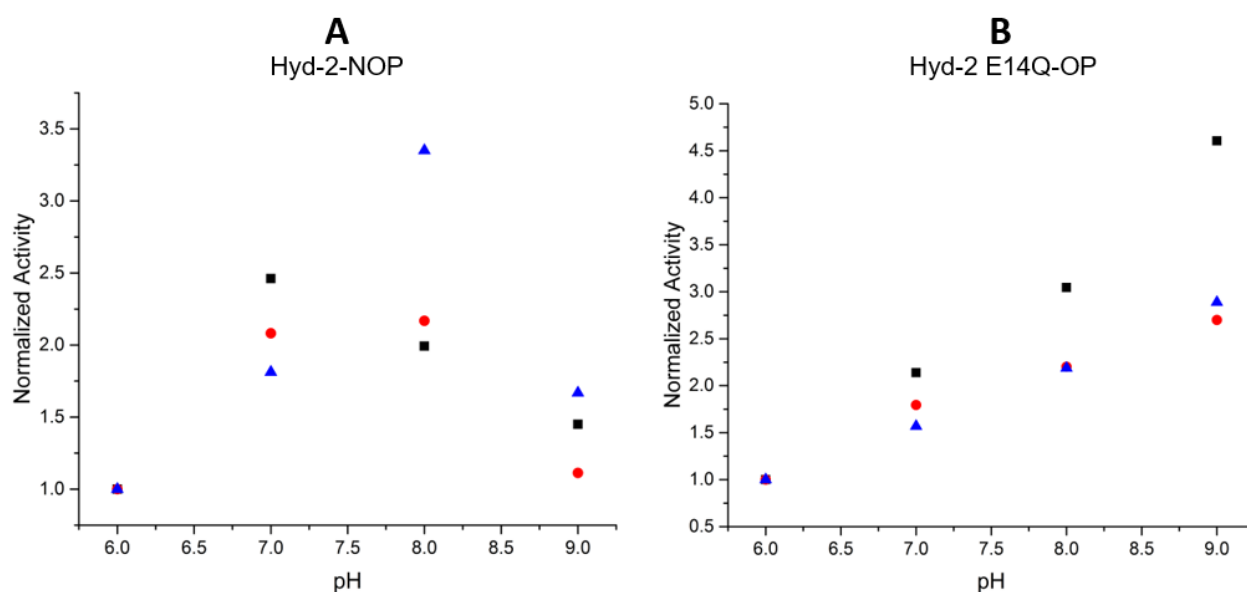


Figure 8.6: Steady-state solution assays of **(A)** Hyd-2-NOP and **(B)** Hyd-2 E14Q-OP. Assays performed in a H_2 -saturated solution (i.e. 1 mL, 2 mM benzyl viologen, in 0.10 M potassium phosphate, 0.10 M NaCl, pH 6-9, titrated at room temperature). At each pH the solution assay was repeated at least 3 times, denoted by each symbol, see also Table 8.1. Figure adapted from reference.³¹

Not only are these results in good agreement with the PFE results but they also show a key difference between Hyd-2 NOP and Hyd-2 E14Q-OP in that at pH 9.0, the activity of Hyd-2 E14Q-OP continues to increase.

Table 8.1: Steady-state solution assays for H_2 oxidation for Hyd-2 and E14Q enzymes at various pH. Table represents values presented in Figure 8.7 using benzyl viologen as electron acceptor. Relative rates are given to represent the steady-state rate at each pH as a proportion of activity at pH 6. Errors are given in parentheses and represent the standard deviation from the mean of at least three repeats. All enzymes were activated under a constant flow of H_2 for 18-24 h. Figure adapted from reference.³¹

	Solution assay rate at pH 6		pH dependence of average rate relative to pH 6			
	Unit	pH 6	6 (%)	7 (%)	8 (%)	9 (%)
Hyd-2 NOP	$[\mu\text{mol of } H_2 \text{ min}^{-1} \text{ (mg of enzyme)}^{-1}]$	85.53 (21.4)	100 (0)	212 (27)	250 (60)	141 (23)
	(s^{-1})	133.18 (33.3)				
E14Q-OP	$[\mu\text{mol of } H_2 \text{ min}^{-1} \text{ (mg of enzyme)}^{-1}]$	0.13 (0.1)	100 (0)	179 (29)	247 (40)	339 (86)
	(s^{-1})	0.20 (0.1)				

This characteristic of Hyd-2 E14Q-OP is masked in PFE likely because breakdown of the protein's periphery can destroy the electroactive connection between the electrode and the enzymes. In solution assays, the enzyme is immersed in the electroactive redox dye and slight breakdown of the protein has a smaller impact on the activity of the enzyme.

8.2.4 Structural characterization of Hyd-2 E14Q-OP

Structural characterization of Hyd-2 E14Q-OP was necessary to ensure that the protein did not experience any other structural change as a result of the E to Q mutation and probe the possible presence of a $\text{H}_2\text{O}/\text{OH}^-$ near Q14. In order to reduce the enzyme in a time period that would limit degradation of the protein, the enzyme was reduced in a pressure vessel (Tynclave Steel, Büchiglasuster) at 4°C and at 4 bar H_2 for 12 h. This strategy was similar to the reduction used for different preparations of Hyd-2 R479K-OP, which showed that such a treatment was not detrimental to the protein (see Chapter 6). The protein successfully crystallized in the conditions originally optimized for Hyd-2 NOP (section 4.2.1), and structures of both reduced and as-isolated Hyd-2 E14Q-OP were solved to a high resolution (Table 8.2).

Table 8.2: X-ray data collection and refinement statistics for Hyd-2 E14Q-OP. Dr. Steve Carr solved all structures.

Sample	Hyd-2 E14Q-OP As-isolated	Hyd-2 E14Q-OP H_2 reduced
Data collection		
pH during crystallization	5.9	5.9
PDB ID	6GAM	6GAN
Space group	$P2_12_12_1$	$P2_12_12_1$

Unit cell dimensions (Å)	a=99.48, b=100.47, c=168.66	a=99.84, b=100.39, c=168.77
Resolution (Å)	168–1.4 (1.42–1.4)	168–1.60 (1.63–1.6)
Total reflections	2,373,441 (115,915)	1,360,314 (67,265)
Unique reflections	330,431 (16,196)	222,822 (10,935)
Completeness (%)	100 (100)	100 (99.7)
Multiplicity	7.2 (7.2)	6.1 (6.2)
$\langle I/\sigma \rangle$	9.4 (1.5)	5.1 (1.9)
R _{merge} (%)	10.4 (117)	13.5 (135)
R _{pim} (%)	4.5 (50.4)	6.5 (64.1)
CC _{1/2}	0.998 (0.637)	0.995 (0.511)
Refinement		
R _{work} /R _{free} (%)	15.0/17.0	16.2/19.0
No. of atoms	13,897	13,865
Macromolecule	12,829	12,807
Solvent	1,068	1,058
Average B-factors		
Macromolecule	16.0	19.7
Solvent	22.7	25.8
RMSD bond lengths (Å)	0.016	0.016
RMSD angles (°)	1.74	1.72
Ramachandran plot (%) favored / outliers	96.5/0.2	96.2/0.2
Clashscore	1.27	1.35

Values in parenthesis refer to data in the highest resolution shell. See section 2.8 for more information about R_{merge}, R_{meas}, R_{pim}, R_{work}, R_{free}, and CC_{1/2}. Both E14Q datasets were refined with isotropic B-factors.

Structural characterization of reduced and as-isolated Hyd-2 E14Q-OP at pH 6 revealed that the as-isolated enzyme lacks the H₂O/OH[−] (Figure 8.7) observed in as-

isolated Hyd-1 E28Q (Figure 8.3). The lack of an ionisable group near Q14 would likely severely inhibit proton transfer out of the active site.

As-isolated Hyd-2 E14Q-OP also lacked some of the signature features of as-isolated Hyd-2 NOP (section 4.2.4 and 4.2.5): while the hydroxide bridging the active site Ni and Fe atoms was present as expected, there was no observed alternate conformation of the proximal cluster nor was there oxidative damage on the active site cysteine C546 (or any other active site cysteine; see Figure 8.7).

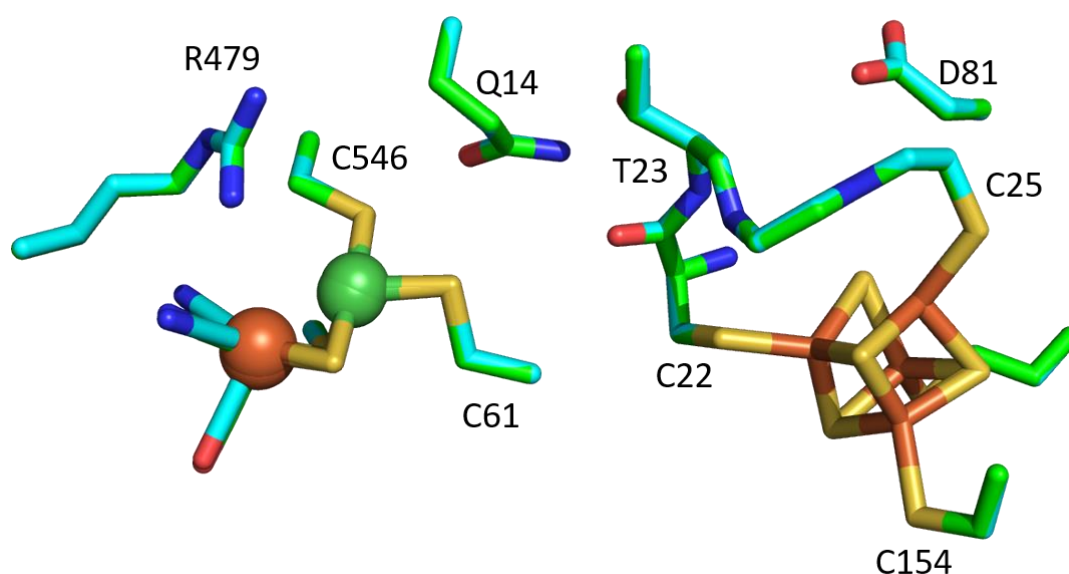


Figure 8.7: Overlay of H_2 reduced (green) and as-isolated (cyan) Hyd-2 E14Q-OP at pH 6 showing the lack of electron density near the Q14 amide (which was seen in oxidized Hyd-1 E28Q, see Figure 8.3). Electron density of OH^- bridging the [NiFe] cofactor in the as-isolated structure omitted for clarity. There was also no evidence of proximal cluster breakdown or oxidation at C546.

8.3 Discussion

8.3.1 Activity at high pH

The increasing activity at high pH (pH 8 and 9) in Hyd-2 E14Q-OP complements the trend seen in the Hyd-1 E28Q variants. The proton transfer pathway depends on a group that becomes increasingly ionized at high pH. Structural evidence from Hyd-1

E28Q links this activity to the $\text{H}_2\text{O}/\text{OH}^-$ found at only partial occupancy (10%) near the glutamine. Due to the increasing concentration of OH^- at higher pH, this group is likely to be an amide-hydroxide pairing that could help shuttle protons in the proton pathway in the role fulfilled by glutamate in the native enzyme.

For Hyd-2 E14Q-OP, the presence of the $\text{H}_2\text{O}/\text{OH}^-$ near Q14 is never observed in structural characterization of the enzyme. At high pH, the $\text{H}_2\text{O}/\text{OH}^-$ molecule is possibly present in very small amounts to facilitate increasing proton transfer with increasing pH, but the effect is still marginal as the rate of H_2 oxidation for the E14Q variant never exceeds 0.5% of the activity of the native enzyme. The almost total lack of activity in Hyd-2 E14Q-OP is a reflection of the fact that the limited proton transport impairs full reactivation of the as-isolated variant. In Hyd-1 E28Q, the as-isolated enzyme is in the superoxidized state allowing the OH^- near the Q28, and thus the as-isolated E28Q has a somewhat viable proton pathway. This relationship between aerobic exposure that occurs upon isolation and increased proton transfer in Hyd-1 E28Q allows the variant to be more easily activated than it would otherwise be without a repaired proton transfer pathway. Even so, Hyd-1 E28Q takes days to reactivate. In Hyd-2 E14Q-OP, the as-isolated enzyme does not form a superoxidized state that facilitates proton transport. It would therefore be expected that Hyd-2 E14Q-OP might take weeks to fully reactivate, yet the instability over time prevents that possibility. Thus, the measured activity is too low for significant characterization by PFE or PFIRE.

8.3.2 Activity at high potential

Comparing the activity of the two Hyd-1 variants and Hyd-2 E14Q-OP is further evidence that the reorganization of the special proximal cluster in Hyd-1 E28Q allows a

second region of increase in activity that could coincide with $\text{H}_2\text{O}/\text{OH}^-$ near the glutamine. There is no increase in the activity of Hyd-2 E14Q-OP at high potentials, and the increase of current for Hyd-1 E28Q is at very oxidizing potentials where the proximal cluster would undergo rearrangement.¹²

Moreover, aerobic conditions exert a powerful oxidizing force that has been shown to cause the proximal cluster to rearrange in Hyd-1, and structures of Hyd-1 E28Q have confirmed the presence of the $\text{H}_2\text{O}/\text{OH}^-$ near Q14 that could facilitate proton transfer. Comparing the structures of reduced and as-isolated Hyd-2 E14Q-OP confirms that as-isolated Hyd-2 at pH 6 shows no evidence of a hydroxide-amide pairing that could be the cause for the increased activity.

The lack of other features characteristic of as-isolated Hyd-2 in the structure of Hyd-2 E14Q-OP (i.e. oxidative damage at C546, movement of D81, and change in Fe binding at the proximal cluster) might be caused by the near total lack of activity in Hyd-2 E14Q-OP. Typically, Hyd-2 NOP resides in the cytosol, and it is likely activated before purification and exposure to O_2 . The oxidative damage could be a product of oxygen attack on highly reduced sites like the active site and Fe-S clusters. However, Hyd-2 E14Q-OP is almost totally inactive, and thus it is unlikely that cofactors in Hyd-2 E14Q-OP would be in highly reduced states upon purification. Another explanation is that proton transfer to and from the active site are required for the oxidative damage seen in as-isolated Hyd-2 NOP. As the active site and proximal cluster are insulated from protons without E14, the structure of Hyd-2 E14Q-OP does not display the same oxidative damage seen in Hyd-2 NOP.

8.4 Conclusion

The Hyd-2 studies presented here confirm observations made in Hyd-1 E28Q concerning the role of the thoroughly investigated E14Q (or equivalent) variant. While this variant has largely been described as nearly totally inactive,^{3,28} the results presented herein show that the glutamine variant becomes increasingly active under two conditions. First, activity increases with increasing pH which is true for both [NiFe]-hydrogenase variants tested, and this likely is due to a hydroxide-amine couple replacing the glutamate's role. Second, the activity of the variant increases at high potential for Hyd-1 and possibly other O₂-tolerant [NiFe]-hydrogenases because of the rearrangement of the special [4Fe-3S]6cys cluster that coincides with the appearance of a hydroxide-amide pair even at near-neutral pH.

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Chapter 9

Design and exploitation of an
orientation-specific binding strategy for
Hyd-2

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9.1 Introduction

Covalently attaching redox-active enzymes to a wider range of electrodes or semiconductors is of great interest as it enables their use in a wide range of applications from sensing to chemical synthesis. Specifically, Hyd-2 is already used in a variety of biotechnological applications.^{1,2} There is also interest in using Hyd-2 and other hydrogenases in model light-driven systems for H_2 production (Figure 9.1).^{3,4} These nascent technologies, however, would benefit from more robust methods to couple enzymes in an electroactive configuration to electrodes. Experimentally, covalent attachment would also stabilize the enzyme on electrodes for better characterization. While Hyd-1 and Hyd-2 are stably adsorbed to PGE electrode for hours to days, other redox enzymes of great interest like nitrogenase have proved more difficult to couple to an electrode in an electroactive manner for full electrochemical characterization.

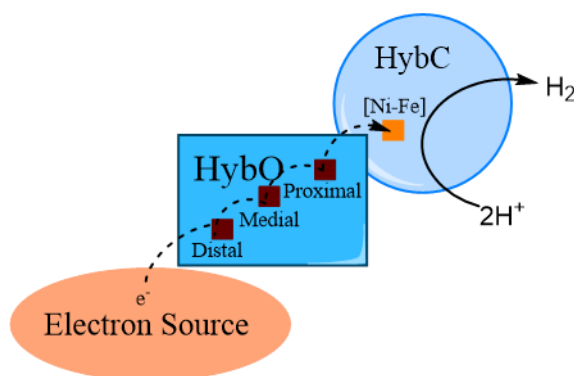


Figure 9.1: Schematic of Hyd-2 NOP on an electrode for H_2 production. The brown squares of HybO are Fe-S clusters that are used for electron transport to and from the [NiFe] active site represented by the orange square of HybC. For H_2 oxidation, the electron flow would be reversed, and the unit that is labelled electron source would accept electrons rather than donate them

9.1.1 Distal cluster controls bias

An important component of schemes to couple Hyd-2 NOP to an electrode in an electroactive configuration is maintaining an orientation that limits the distance between the distal Fe-S cluster and electrode to a maximum of 14 Å to allow for rapid electron tunneling.⁵ This has led to efforts focused on mutating the environment around the distal cluster to incorporate elements into the enzyme that can bind to an electrode surface,⁶ yet mutations in the protein environment around the distal cluster can also affect the apparent bias of the enzyme.^{7,8} An enzyme can have no bias besides that dictated by thermodynamics.⁹ Nevertheless, an enzyme is said to have an apparent bias when the enzyme has greater activity in either direction of the redox reaction, i.e. greater H₂ oxidation rate than H₂ production rate. This apparent bias arises from a variety of factors including proton transfer, electron transfer, sterics at the active site, and other enzymatic characteristics.^{10,11} An extreme apparent bias is reflected by a significant overpotential in which the enzyme requires additional driving force to catalyse a reaction.

The overpotential requirement of Hyd-1 for H₂ oxidation has long been theorized to be related to the redox potential of the distal cluster.^{12,13} It was proposed that if the potential of the distal cluster is higher than the potential of the H₂/H⁺ couple, an overpotential will be required to drive the H₂ oxidation reaction.¹³ The overpotential requirement decreases with pH, and Hyd-1 becomes nearly a reversible hydrogenase at pH 3.¹² This change in apparent bias is due to the fact that the potential of the Fe-S clusters is less affected by shifts in pH than the H₂/H⁺ thermodynamic equilibrium. The thermodynamic equilibrium shifts more positive by 60 mV per pH unit. Hence, the potential of the distal cluster of Hyd-1 was theorized to be significantly higher than the H₂/H⁺ thermodynamic equilibrium at pH 6.

Recent experiments have confirmed the distal cluster potential in Hyd-1 to be -123 ± 31 mV, approximately 180 mV higher than the potential of the H_2/H^+ couple at pH 6.⁸ In the same study that reported the distal cluster potential for Hyd-1, Adamson et al reported four Hyd-1 variants with mutations to individual amino acids in the primary coordination sphere of the distal cluster. Two of those mutants were designed based on the differences between the conserved amino acids in O_2 -tolerant and standard hydrogenases, but neither Hyd-1 K189N or Hyd-1 Y191E have a distinct distal cluster potential.⁸ The study focused on R193 (R198 in Hyd-2) that is within 3.5 Å of H187 that coordinates one of the irons. The proximity of the arginine and positive charge at biologically relevant pH suggests that it is capable of electrostatic interaction with the histidine.⁸ A variant replacing the positively charged arginine with a negatively charged glutamate, R193E, did not express, but the R193L variant expressed at normal levels. The R193L variant showed measurable H_2 production at pH 6 and a significantly lowered distal cluster potential.⁸

These findings have shown which amino acids near the distal cluster of [NiFe]-hydrogenases are important for controlling the catalytic activity or viability of the enzyme. While the study aimed to tune the catalytic bias of Hyd-1, the results should also inform future attempts to mutate the environment around the distal cluster for attachment to an electrode.

9.1.2 Electrode-protein conjugation in biohybrid systems

While incorporating enzymes into artificial light-driven systems is likely unfeasible for large-scale hydrogen production, these systems serve as important efforts to inspire and benchmark future catalyst designs. Typically, photosynthetic hydrogen production

assemblies comprise a photosensitizer that upon light stimulation emits an electron, a semiconductor that can help direct and transmit the electrons and holes, and hydrogenase to catalyse the hydrogen production reaction. While highly efficient cheap H_2 catalysts are still being designed, biohybrid systems that incorporate hydrogenases are important tools to explore and optimize different portions of these model assemblies. However, these systems represent only half of the redox reaction and therefore typically require a sacrificial electron donor that can repopulate the electrons of the oxidized photosensitizer.

Early work by Reisner et al showed that a [NiFeSe]-hydrogenase coupled to TiO_2 nanoparticles with a ruthenium dye as a photosensitizer produced H_2 at a turnover rate of $50\ s^{-1}$ with a sacrificial electron donor present.³ A comprehensive review of semiconductors is outside the scope of this thesis, yet it is important to mention that TiO_2 is an n-type semiconductor that biases attached enzymes towards reduction. It is commonly used in photocatalytic systems,¹⁴ yet Hyd-2 does not adsorb in an electroactive configuration to TiO_2 and thus gave only marginal turnover rates.³ The inability for the Hyd-2 to adsorb in an electroactive configuration on TiO_2 is in stark contrast to its ability to adsorb more generally. Reisner et al measured the amount of Hyd-2 left in solution after addition of colloidal TiO_2 and found that approximately 75% of the enzyme adsorbed to TiO_2 .³ Other efforts to incorporate hydrogenases into a hybrid system have involved [FeFe]-hydrogenases adsorbed on CdTe nanocrystals and CdS nanorods capped with 3-mercaptopropionic acid.^{4,15} The latter system showed an impressive H_2 production turnover rate of $380-900\ s^{-1}$ with a sacrificial electron donor.¹⁵ While the turnover rate is expected to be higher with the [FeFe]-hydrogenase, this

enzyme is significantly more O₂ sensitive than both the [NiFeSe]-hydrogenase used in Reisner's work and Hyd-2.

9.1.3 Covalent connections

While there are diverse ways to link enzymes to a surface, it is challenging to create a stable electroactive connection. As mentioned previously, an electroactive linker would have to orient the enzyme such that the distal cluster is within 14 Å of the electrode surface to facilitate rapid electron transfer between the enzyme and the electrode surface.⁵ There are currently various techniques that rely on different common surface residues. The modification by Krishnan et al (used in this thesis in Chapter 8) relies on π - π stacking interactions between multiwalled carbon nanotubes (MWNT) and 1-pyrene butyric acid (Py).¹⁶ The carboxylic acids of the MWCNT/Py assembly is then functionalized with EDC/NHSS to facilitate covalent attachment to the amino of surface lysine residues. Fortunately, there are sufficient surface lysine residues for Hyd-1 and Hyd-2 to create an electroactive connection, yet it is likely that only a portion of the attached enzymes are coupled in an electroactive configuration as the binding of surface lysine residues does not specify an orientation ensuring distal cluster/electrode interface. This is partially ameliorated by the 3D morphology of the MWCNT and the corresponding increase in surface area, yet an easy orientation-specific technique linking method would still be valuable.

Orientation-specific linking mechanisms are even more challenging and often require engineering the protein of interest to create a unique surface interface to bind to the linker group. Specifically, for the [NiFe]-hydrogenase from *E. coli* previous efforts were focused on mutating Y231 (numbered Y227 in Hyd-1) to a non-natural amino acid

to facilitate orientation specific linkage, yet these efforts were abandoned due to the cost and difficulty associated with producing sufficient quantities of enzyme with non-natural amino acids.⁶

9.1.4 AgNC connection

Recent efforts have focused on using Ag nanoclusters (here denoted AgNCs) whose light absorbing properties and affinity for sulphur atoms create the possibility of a molecule that serve as both photosensitizer and covalent linking group. Work by Zhang et al has recently shown that these nanoclusters, templated in poly(methacrylic acid) (PMAA), demonstrated successful linkage of carbon monoxide dehydrogenase (CODH) to a TiO₂ electrode.¹⁷ The connection is created by the AgNCs that are capable of forming a covalent bond with the sulphur atom from a surface cysteine and the free carboxylate groups of PMAA that are suited for binding to the surface of TiO₂.^{18,19}

Moreover, suspended TiO₂ nanoparticles with CODH I/AgNC-PMAA can successfully convert CO₂ to CO with a turnover frequency two orders of magnitude larger than what has been reported for other colloidal systems.³ Interestingly, a mixture of CODH I/AgNC-PMAA without TiO₂ nanoparticles had a turnover rate an order of magnitude slower suggesting that efficient catalysis depends on TiO₂. This poor catalytic activity of CODH I/AgNC-PMAA could be due to, among other reasons, lack of surface cysteines, distance of surface cysteines to active site, or need for TiO₂ to manage the charge transfer. Unfortunately, the structure of CODH I is not solved making it difficult to understand the reason for the poor catalytic activity of the dimeric assembly. With knowledge of the structure and an easy platform for genetic manipulation, the small sub-

unit of Hyd-2 makes an ideal candidate for studying the mechanism of AgNC interaction with the protein.

9.1.5 Aims of this chapter

This chapter will explore efforts to engineer the small subunit of Hyd-2 for applications in biohybrid systems. Specifically, Hyd-2 variants designed for direct covalent attachments by AgNCs were created and characterized.

9.1.6 Contributions

Dr. Liyun Zhang did all initial research for AgNCs and their attachment to proteins including the foundational work on CODH/AgNCs-PMAA/TiO₂ assemblies.¹⁷ We collaboratively designed the variants, primers, and molecular biology strategy for mutating Hyd-2 NOP to alter the surface cysteines. Dr. Liyun Zhang performed all the molecular biology and purification under my direction. I advised Dr. Liyun Zhang on technical portions of the experiment relating to overexpressed Hyd-2, such as the activation procedure required before assembling the photosynthetic H₂ production systems and helped interpret results. Dr. Liyun Zhang carried out all assembly of AgNCs-PMAA/TiO₂, electrochemical and gas chromatography measurements.

9.2 Results

9.2.1 Need for distal cluster variants

It has formerly been unclear why Hyd-2 is less likely to form an electroactive connection with TiO₂ (a qualitative characteristic of enzymes previously termed ‘titaniaphilicity’)³ when compared with other hydrogenases, yet the structure of Hyd-2

gives insight into the lack of affinity. Colloidal TiO_2 has an isoelectronic point of 5.3.²⁰ Experiments with TiO_2 and Hyd-2 have typically been done at near neutral pH which means, in these experiments, TiO_2 is negatively charged. The structure of Hyd-2 NOP reported in this thesis (see Chapter 4 for detailed structure of Hyd-2 NOP) shows that the surface of Hyd-2 close to the distal cluster is neutral with negatively charged regions at the interface between the two small subunits (Figure 9.2). Thus, the ideal location for an electroactive connection between TiO_2 and the enzyme is unfavourable due to charge repulsion. The positively charged regions of the surface of Hyd-2 are in cavities limiting the surface area for connection to TiO_2 , and the most positive regions are at a prohibitive distance ($>16 \text{ \AA}$) for rapid electron tunneling.⁵ These isolated regions of positively charged surface prohibitively far from the Fe-S clusters also explain why the majority of Hyd-2 in solution adsorb to colloidal TiO_2 yet do not provide the expected current response upon applying a potential to a TiO_2 -Hyd-2 electrode.³ Upon analysis of the structure of Hyd-2 NOP, it was clear that investigations incorporating Hyd-2 into biohybrid systems with TiO_2 must find ways to covalently couple the enzyme in an electroactive configuration with TiO_2 .

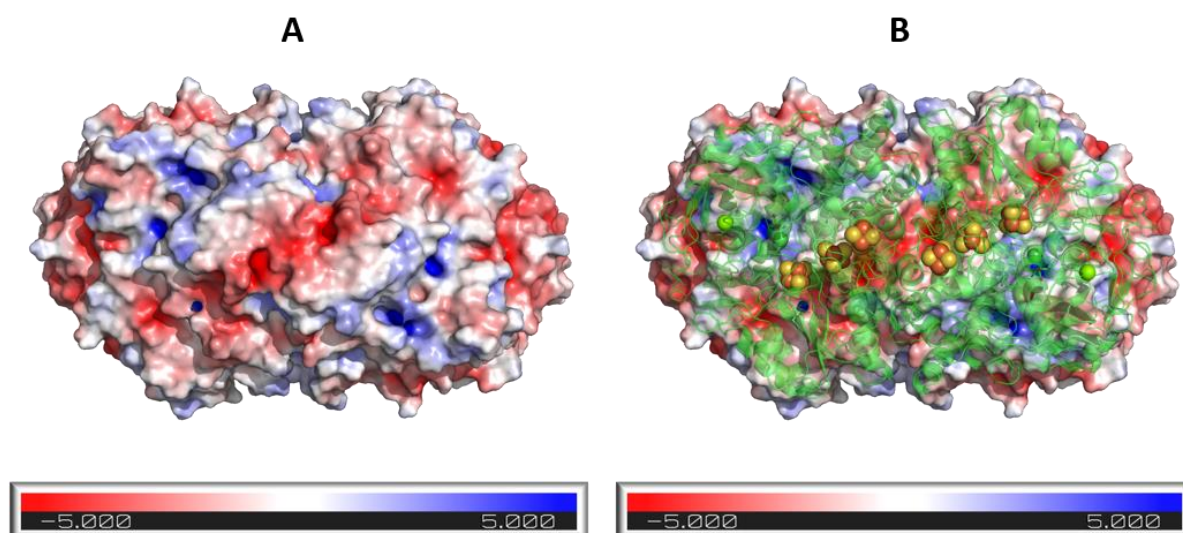


Figure 9.2: Electrostatic Surface of Hyd-2. **(A)** View of the electrostatic surface (APBS) of reduced Hyd-2 (PDB 6EN9) viewed from the 'underside' where the HybO makes contact with its natural redox partner, HybA. **(B)** Overlay of the structure of Hyd-2 NOP with the surface electrostatics (APBS) of Hyd-2 NOP highlighting the negative charge of the region around the Fe-S clusters.

9.2.2 Design of distal cluster variants

The structure of Hyd-2 NOP also revealed that there are no cysteines on the surface of Hyd-2 NOP that are within 14 Å of the electron transfer pathway or the active site (Figure 9.3). One of the amino acids close to the distal cluster was therefore mutated to create an orientation specific binding site for AgNCs. When choosing which amino acid to mutate, it was planned that the mutation would not change the potential of the distal cluster or the bias of the enzyme. The understanding that Hyd-2 NOP is typically arranged in a heterodimeric configuration (HybOC)₂ also guided the mutation to the distal cluster away from the interface between the two dimers. If the mutation were at the interface between the two protein subunits, it would make simultaneous coordination to both sites difficult.

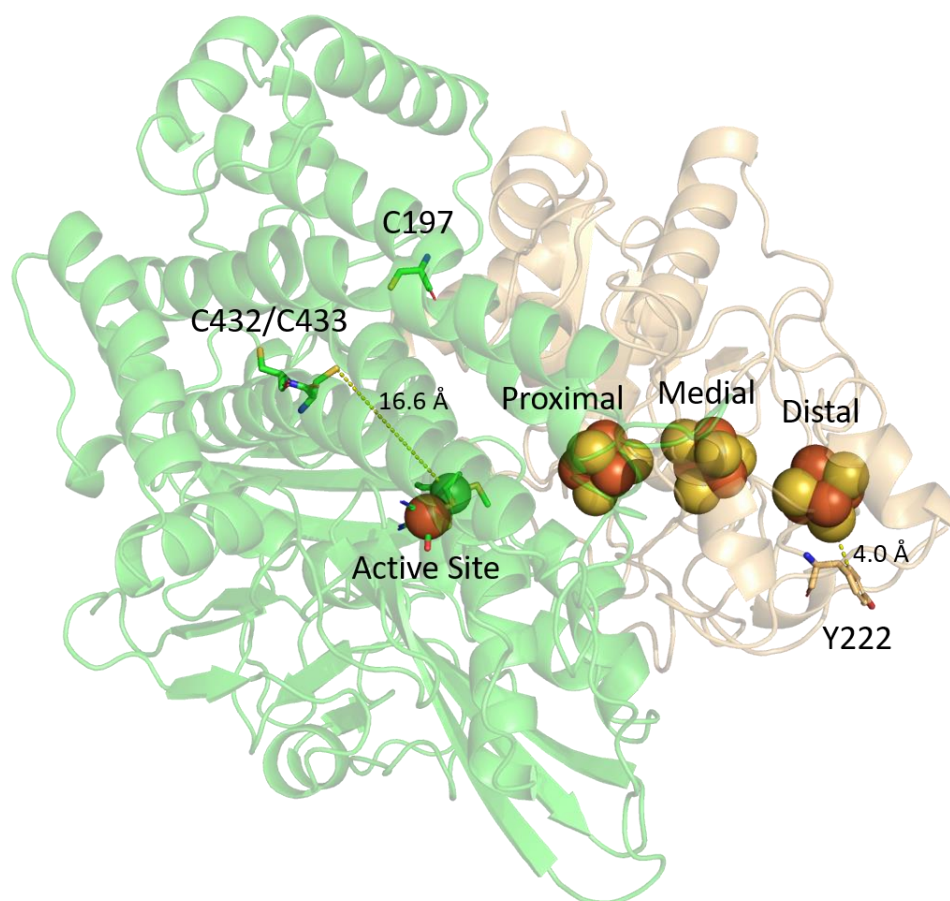


Figure 9.3: Schematic of surface cysteines on a single dimer of Hyd-2 NOP and the potential electron relay channels when viewed from the protein interface between the two dimers. HybC is shown in green, and HybO is shown in pale yellow. Measurement of the closest cysteine to the active site of the Fe-S clusters shows that any possible surface cysteines are at a distance that is prohibitively far from the cofactors of Hyd-2 for electron transfer. By contrast, Y222 would be the ideal candidate to mutate for direct injection of electrons into the distal cluster.

The Y'222C mutation (to distinguish mutations between the two subunits, mutations in HybO all contain an apostrophe) was chosen as it fits these requirements, and it is also in a less occupied region of the structure, likely signifying that it will be surface exposed with room to coordinate AgNCs (Figure 9.3). Y222 is neutral and 4 Å away from amino acids coordinating Fe or S atoms of the distal cluster. Moreover, Y222 is located within 5 Å of one distal cluster and more than 18 Å away from the distal cluster on the opposing small subunit, thus ensuring access of a colloidal system to both dimers.

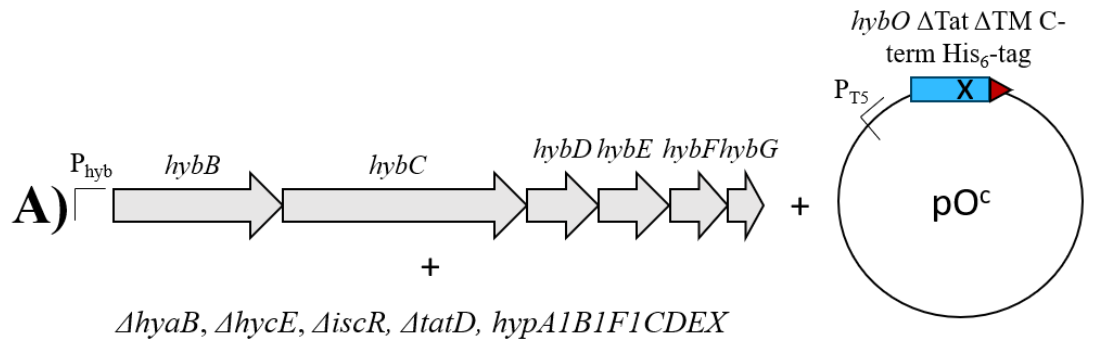
This arrangement also mimics the theorized stoichiometric ratio of the natural redox partner of HybO which should optimize the rate of H₂ turnover (Section 4.3.4).

To further probe the effect of the sulphur close to the distal cluster, all possible sulphurs that could be targeted by AgNCs on the surface of Hyd-2 NOP were removed. Thus, the three surface exposed cysteines in HybC (C197, C432, and C433) were mutated to serine. This mutation was chosen as cysteine to serine is the most conservative mutation, which should minimize the structural impact of the mutation.

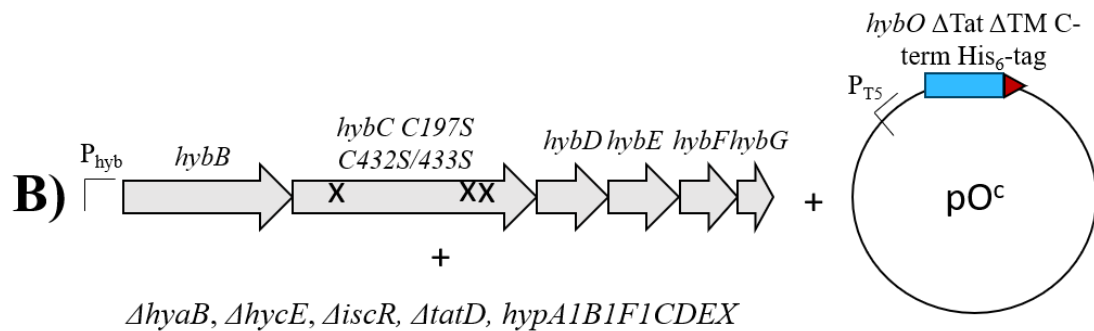
9.2.3 Creation of mutants

The three cysteine to serine mutations were incorporated into the pMAK-hybC plasmid by a three-part Gibson assembly (C432S and C433S were engineered in the same primer). The triple mutant pMAK-hybC was created by a similar strategy as the three-part Gibson assembly for pOC (see Appendix A). The mutations were then incorporated into HJ001-hyp via the pMak cycle (Section 2.4). The Y'222C mutation was created by mutating the plasmid pO^C, and it was transformed into HJ001-hyp as well as the triple-mutant strain in which all surface cysteines of HybC were mutated to serine (Figure 9.4).

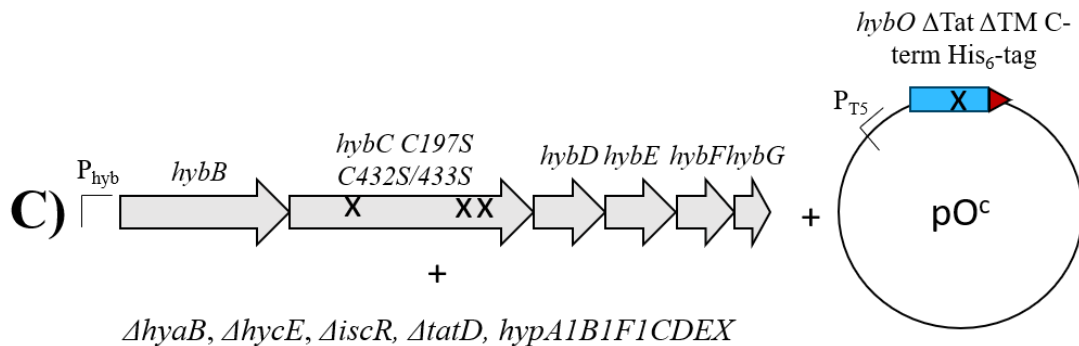
Thus, three Hyd-2 variants were produced to investigate the distal cluster variants. One that contained the three surface exposed cysteines of the native protein and an additional surface-exposed cysteine near the distal cluster (Y'222C-OP), a second that contained a sole surface exposed sulphur near the distal cluster (3S-Y'222C-OP), and a third that contained no surface exposed cysteines (3S-OP) (Figure 9.4).



HJ001-hyp + pO^c Y'222C(Hyd-2 Y'222C-OP)



HJ001-hyp-3S + pO^c (Hyd-2 3S-OP)



HJ001-hyp-3S + pO^c Y'222C(Hyd-2 3SY'222C-OP)

Figure 9.4: A representation of the *hyb* operon and pO^c for the three surface exposed cysteine variants.

9.2.4 Production of variants

All variants expressed at levels comparable to Hyd-2 NOP. Visualization by SDS-PAGE revealed that the HybO and HybC subunits purified at a similar ratio with no additional subunits (Figure 9.5).

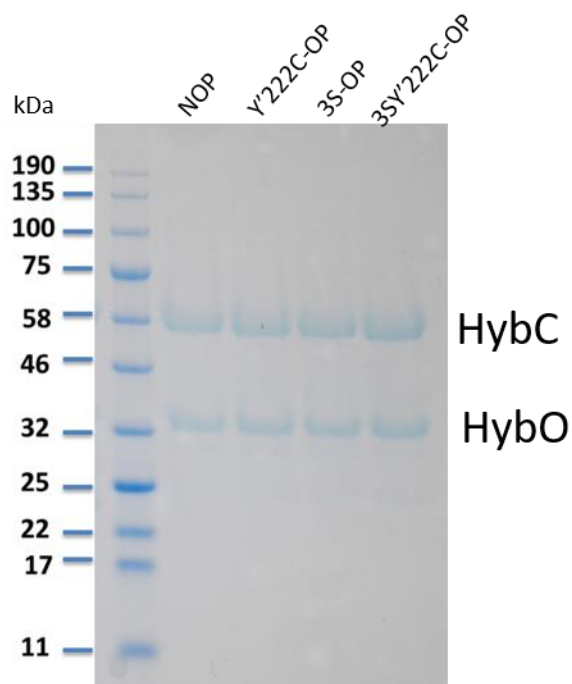


Figure 9.5: SDS-PAGE denaturing gel of Hyd-2 NOP, Hyd-2 Y'222C-OP, Hyd-2 3S-OP, and Hyd-2 3SY'222C-OP. A sample (10 μ L) of enzyme is mixed with loading dye, boiled, and loaded. SDS-PAGE gel run at 200 V for approx. 55 min. HybC (61 kDa) and HybO (37 kDa) are present in an approximately equivalent ratio. Cysteine variants purified by and SDS-PAGE was run by Dr. Liyun Zhang (University of Oxford). Figure adapted from reference.²¹

9.2.5 Hyd-2 Y'222C-OP

Electrochemical investigations on PGE of the distal cluster variants showed that all three of the variants had nearly identical electrochemical profiles as Hyd-2 NOP (Figure 9.6). All three variants are active for both H_2 oxidation and H_2 production, and as expected, with decreasing partial pressures of H_2 , all Hyd-2 variants become increasingly active in the direction of H_2 production. In agreement with the native enzyme, the ratio of max H_2 production to max H_2 oxidation is approximately 1:3. The distal cluster variants also show no significant overpotential requirement in either direction.

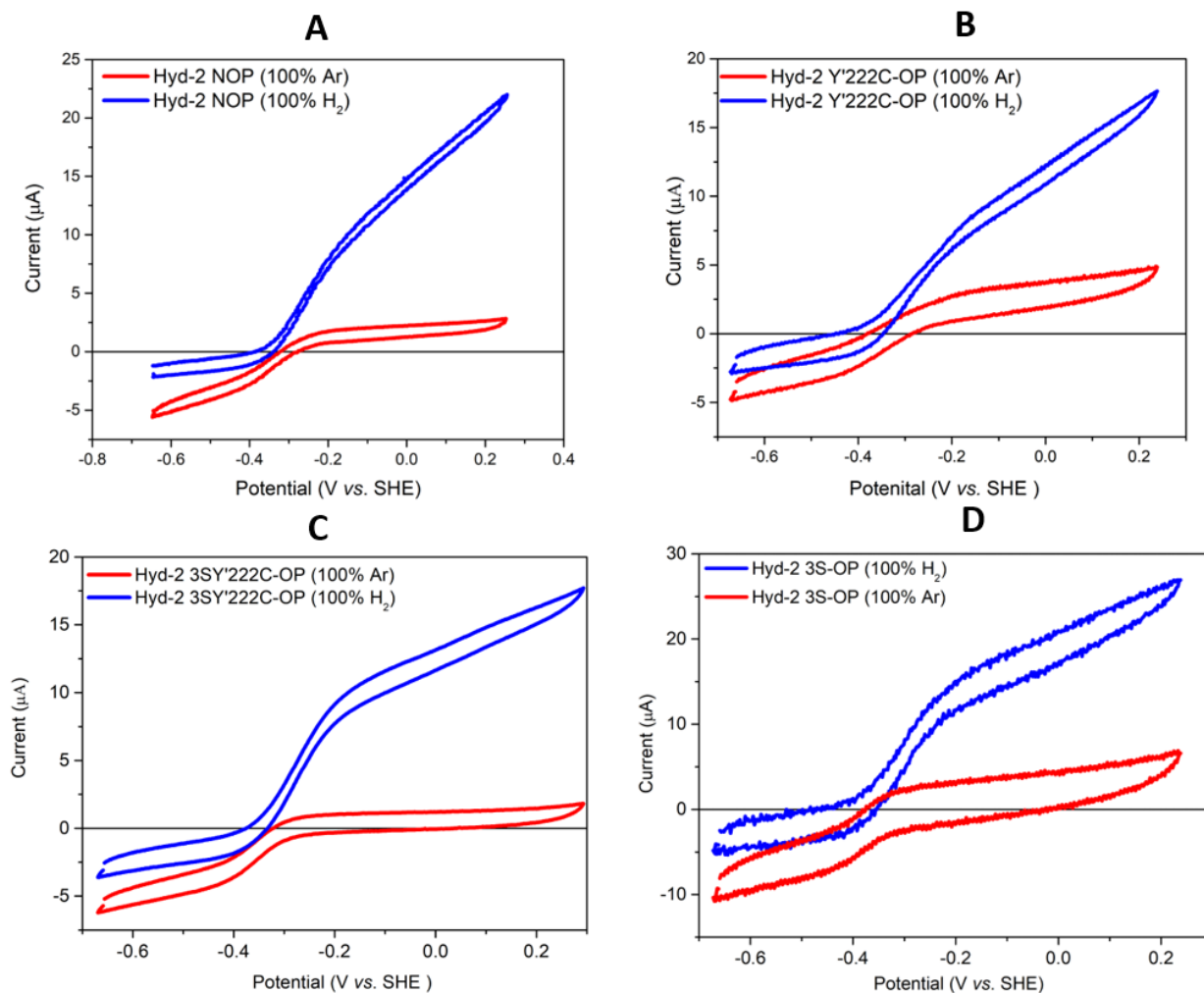


Figure 9.6: PFE comparison of Hyd-2 cysteine variants on PGE demonstrating that all variants are active in both the H_2 production and H_2 oxidation direction with similar CV profiles when scanned at 30 mV/sec. All electrochemical measurements on cysteine variants performed by Dr. Liyun Zhang (University of Oxford). (A) Hyd-2 NOP on PGE. (B) Y'222C-OP on PGE. (C) 3SY'222C-OP on PGE. (D) 3S-OP on PGE. Other conditions: pH 6, 30 °C, total gas flow rate maintained at 1000 scc/min, $\omega = 1000$ rpm. Figure adapted from reference.²¹

On TiO_2 , Hyd-2 NOP/AgNC-PMAA was electroactive and allowed electrochemical analysis of Hyd-2 with TiO_2 as the electrode. Interestingly, all three cysteine variants, when applied to a TiO_2 electrode with AgNC-PMAA, were electrochemically active including 3S-OP that was engineered to be free of any surface cysteines (data of variants on TiO_2 electrode not shown).

For photosynthetic hydrogen production, the hydrogenase/AgNC-PMAA/TiO₂ system was active for all cysteine variants. However, the rate for the hydrogenase variant lacking surface cysteines, Hyd-2 3S-OP/AgNC-PMAA/TiO₂, was approximately four-fold lower than the rest of the Hyd-2 variants (Figure 9.7). By contrast, the rate of H₂ production by the variants where the AgNC-PMAA linker can directly inject electrons into the chain of Fe-S clusters, Hyd-2 Y'222C-OP and Hyd-2 3SY'222C-OP, had the highest rates of H₂ production ($193.3 \pm 5.1 \text{ s}^{-1}$).

Assemblies without TiO₂ that directly receive electrons from AgNC-PMAA reveal that only the Y'222C-OP variants (with or without native surface cysteines) are active. However, the activity from this two-component set up is approximately 10% of the assembly with TiO₂, demonstrating the importance of TiO₂ in the assembly.

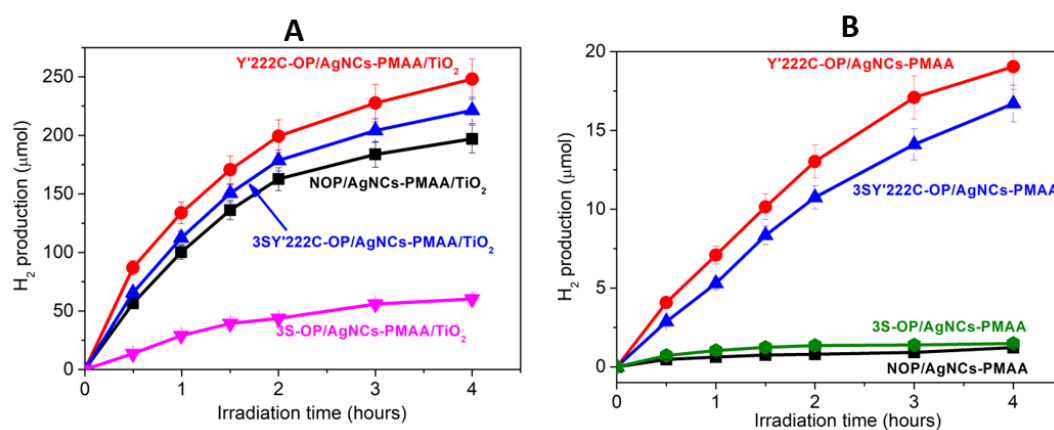


Figure 9.7: Comparison of Hyd-2 cysteine variants in photosynthetic systems with **(A)** AgNCs-PMAA/TiO₂ or only **(B)** AgNCs-PMAA. H₂ produced by systems under visible-light irradiation in 5 mL 0.1 M TEOA buffer contained 5 mg of AgNCs-PMAA/TiO₂ and 0.25 nmol enzyme at pH 7.0, 25 °C under visible light irradiation, followed using GC. All photosynthetic H₂ production experiments performed by Dr. Liyun Zhang (University of Oxford). Figure adapted from reference.²¹

9.3 Discussion

The use of AgNCs-PMAA is the first ever successful electroactive linkage between Hyd-2 and TiO₂. The importance of TiO₂ in biotechnological applications makes this

advance fundamental for incorporating Hyd-2 into biohybrid systems. The electroactivity of Hyd-2 NOP on AgNCs-PMAA/TiO₂ is an extension of previous work with CODH as the electroactivity relies on non-orientation specific covalent bonds between the AgNCs which are suspended in the PMAA that is covalently attached to the TiO₂ electrode. Due to the prohibitive distance between the surface cysteines in Hyd-2 NOP and the Fe-S clusters, the AgNCs that are covalently bound to the enzyme are not capable of electron transfer. Instead, other AgNCs that are suspended in the PMAA likely act as mediators to shuttle electrons from the TiO₂ electrode to the bound enzyme.

However, the electroactivity of Hyd-2 3S-OP/AgNCs-PMAA/TiO₂ elucidates that AgNC-PMAA can also act as electron mediators without having surface cysteines on Hyd-2 that can mediate covalent attachment. As previous investigations have shown that native Hyd-2 adsorbs well onto TiO₂ in non-electroactive configurations, the results of the Hyd-2 3S-OP/AgNCs-PMAA/TiO₂ assembly demonstrate that the adsorbed enzyme can be activated by simply providing metal centres capable of shuttling electrons from the electrode surface to the enzyme. Unsurprisingly, this connection also works for colloidal TiO₂ onto which Hyd-2 adsorbs and AgNCs-PMAA act as traditional mediators shuttling electrons between the electrode and the hydrogenase. In the colloidal assembly, the AgNCs-PMAA also act as a photosensitizer to give electrons to TiO₂. The decreased rate from the assembly with Hyd-2 3S-OP confirms that the probability of obtaining an electroactive connection is lower for enzymes without surface cysteines, and this connection is also more likely to be limited by electron transport.

Work with Y'222C variants present an advance over the previously published study on CODH by designing Hyd-2 to bind AgNCs in an orientation-specific manner. For the two cysteine variants with the Y'222C mutation, the same AgNC that covalently links

to C222 can also shuttle electrons from TiO_2 to the hydrogenase as the AgNC bound to C222 is within 14 Å of the distal cluster. Indeed, the two Hyd-2 variants with the Y'222C mutation have the highest rates which likely indicates that this method of electron transfer allows for more efficient electron transfer and thus a higher rate of H_2 turnover. Moreover, the photosynthetic assembly without TiO_2 confirms that the AgNCs-PMAA do not need a semiconductor for H_2 production with Hyd-2 and can instead directly inject photoexcited electrons into the enzyme. The large decrease in rate without TiO_2 is revealing. Without TiO_2 in the assembly, the rate of H_2 production decreases tenfold. The obvious mechanistic role for TiO_2 in assemblies with hydrogenases and Y'222C-OP present is mediating electron transfer from the sacrificial electron donor (triethanolamine) and delivering them to the AgNCs. Finally, the measured rates of H_2 production by the Hyd-2 Y'222C-OP/AgNCs-PMAA/ TiO_2 assembly is nearly 40-fold higher than the rate of Hyd-2 NOP measured by traditional solution assays with reduced methyl viologen as the electron donor. This finding suggests that enzymes like Hyd-2 that are reported to have low rates of H_2 production may be much better H_2 -producers once their electron transfer pathways are optimized.

9.4 Conclusion

Mutations around the distal cluster of Hyd-2 offer opportunities for further optimization and study of the properties of [NiFe]-hydrogenase. The first ever orientation-specific binding of Hyd-2 allows rapid covalent attachment to TiO_2 . This advance allowed the incorporation of Hyd-2 into a photosynthetic H_2 producing system with AgNC-PMAA and colloidal TiO_2 . Experiments reveal that while the TiO_2 is not necessary for H_2 production with AgNC-PMAA, it increases the rate of H_2 production by

nearly an order of magnitude. These findings and the creation of the Hyd-2 variants with specific surface cysteines have therefore opened the door to more specific characterization of TiO_2 and possibly other semi-conductors in biohybrid systems.

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Chapter 10

Conclusions and Implications

10.1 General conclusions and implications

The hydrogenases of *E. coli* have been important model systems in the investigation of the mechanism of hydrogen activation in nature. The higher native expression levels of Hyd-1 have made detailed characterization of the O₂-tolerant [NiFe]-hydrogenase easier.¹ Consequently, mutagenic studies have focused on Hyd-1 due to its known structure and robust expression levels.^{2–4} Increasingly complex studies, though, require comparison across different enzymes with contrasting properties. The shared native environment and contrasting properties of membrane-bound Hyd-1 and Hyd-2 make them ideal for comparison, yet the variable, low native yield of Hyd-2 had previously prevented structural characterization. Prior efforts to increase the yield of [NiFe]-hydrogenases have had limited success likely due to the complex metal cofactor synthesis required for protein maturation.

The results presented in Chapter 3 detailed an innovative overexpression system that resulted in approximately an order of magnitude increase in the expression of Hyd-2. This approach was based on expressing *hybO* without its transmembrane domain or TAT signal on a plasmid in a strain optimized for [NiFe] hydrogenase maturation. It is interesting to note that expressing *hybC* and *hybD* on a plasmid with *hybO* even with *hybC* present in the chromosome resulted in significantly decreased Hyd-2 expression. This result highlights the challenges of overexpression [NiFe]-hydrogenases as *hybC* likely depends so heavily on maturation proteins encoded in the *hyb* operon that collocated translation is necessary for proper protein-protein targeting. Nonetheless, expression of even one of the subunits from a plasmid greatly simplifies mutagenic work on the small subunit and allowed greater ease of expressing variants with different large subunits.

The higher level of expression also was shown to have a negligible effect on the function of the redox protein and enabled the structure to be solved. Chapter 4 presented the structure of HybOC. While HybOC strongly resembles other membrane-bound hydrogenases, the structure revealed four key components of Hyd-2 that enabled a range of subsequent investigations.

First, the hydrophobic patches on the surface of HybO give hints as to where the ferredoxin associated with HybOC, HybA, could interact. This allowed a more accurate prediction of the quaternary structure of Hyd-2. As Hyd-2 is an important enzyme in the colonization and infection by pathogens, this quaternary structure will provide insight into different approaches targeting Hyd-2. Most immediately, it guided investigations into the mechanism and role of intermembrane proteases that had been shown to be necessary for infection and that interact with the transmembrane domain of HybA. In collaboration with Guyang Liu, the role of the rhomboid GlpG in *Shigella sonnei* was investigated and presented in Chapter 5. Although it was originally theorized that GlpG could be necessary for Hyd-2 maturation, that theory was proved incorrect. Further investigations ruled out a role in mediating the aerobic/anaerobic interface, and further investigation is necessary to determine the exact role of cleavage of HybA by GlpG.

Second, the structure revealed that as expected, the ‘canopy’ of the active site was highly conserved between Hyd-1 and Hyd-2. More importantly, the ability to solve the structure suggested that the structure of future canopy variants could be solved. This encouraged and guided the creation of Hyd-2 canopy variants. Two canopy variants that had been shown to cause the largest change in hydrogenase activity in Hyd-1 were D118A and R509K. Thus, the homologous mutations were made in Hyd-2 and

incorporated into the overproduction system. The D103A variant differed greatly from Hyd-1 D118A and this difference might be attributed to conserved water molecules in the active site of D103A that replaced the functionality of the carboxyl. Hyd-2 R479K-OP exhibits a 50-fold lower H_2 oxidation activity as would be expected from a pendant arginine mutation. Importantly, the 150-fold decrease in H_2 production in Hyd-2 R479K-OP extends the observation of the importance of the pendant arginine in H_2 oxidation to H_2 reduction. Further, Hyd-2 R479K-OP has a decrease in activation enthalpy which could indicate that the arginine plays a role in formation of the rate limiting step. Solving the structure of Hyd-2 R479K-OP revealed that the arginine mutations also traps a homodiatomic, likely O_2 , at the active site of the as-isolated enzyme. Moreover, the reduced enzyme traps a water molecule in the active site in a state resembling the proposed structure of an inactive state that has only been previously characterized by IR-spectroscopy. Further spectroscopic characterization of these enzymatic states is needed to validate the initial characterization of what are likely trapped intermediates of inactivation pathways. If confirmed, these structures could provide valuable insight into the mechanism of aerobic and anaerobic inactivation in [NiFe]-hydrogenases.

Third, solving the structure of Hyd-2 NOP in both its reduced and oxidized states suggested the importance of the well-studied glutamate residue, E14, that has been shown to be important in proton transfer. This result combined with mutagenic studies of the homologous residue in Hyd-1 that reported increased activities for Hyd-1 28Q at high potentials prompted characterization of Hyd-2 E14Q-OP. The E14Q-OP variant however does not have the same high-potential activity observed in Hyd-1 and comparing the reduced and oxidized structures of Hyd-2 E14Q-OP with native Hyd-1

and Hyd-1 E28Q have elucidated the role of the special [4Fe3S]6cys proximal cluster in the high-potential activity of the variant.

Fourth, the structure helped explain the inability for Hyd-2 to establish an electroactive connection with TiO₂ and prompted investigations on how to link Hyd-2 to semi-conductors in a covalent, electroactive, orientation-specific manner. Work in collaboration with Liyun Zhang have shown that AgNCs can be used for simple orientation-specific linkage with simple modifications. Characterizing these variants also provided further evidence that TiO₂ is useful in managing electron transfer in catalytic applications.

In conclusion, the work in this thesis has developed Hyd-2 as a model enzyme in understanding the hydrogenase mechanism and its application in pathogenicity and biotechnology. By increasing the expression level of Hyd-2 and solving the structure, this study has enabled detailed work on Hyd-2 both in its own right and in comparison to Hyd-1. The ability to compare the effects of a mutation on both Hyd-1 and Hyd-2 has already given mechanistic insight in the importance of the secondary coordination sphere on hydrogen activation and will likely continue to be used for future mutagenic studies in [NiFe]-hydrogenases. Moreover, the use of overexpressed Hyd-2 and variants into a light-driven H₂ production system shows that the use of Hyd-2 in biotechnological applications can provide important insight into the mechanism of these systems. As humankind continues to draw inspiration from natural mechanisms of energy production and storage, the work in this thesis has developed Hyd-2 as a platform for further insight.

10.2 References

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Appendix A

A1.1 Design, production, and initial characterization of Hyd-2 D'81N

To probe the effect of the negatively charged D81 on coordinating the Fe lost under aerobic conditions (see section 4.2.5 and 4.3.1), the aspartate was mutated to asparagine. The D to N mutation is the most conservative mutation in this position and preserves the size of the residue. Asparagine is also polar, so it still might be able to facilitate the coordination of the Fe of the proximal cluster after breakdown under aerobic conditions. A mutation that conserved the size of the residue was chosen to prevent introducing inherent instability in the Fe-S cluster unrelated to O₂ attack.

Hyd-2 D'81N-OP was produced at levels similar to Hyd-2 NOP. Visualization on SDS-PAGE gel showed that the protein purified as overproduced HybO and native HybC in approximately equal proportion with no other subunits (data not shown). Hyd-2 D'81N-OP is electrochemically active, and it has a similar electrochemical profile as Hyd-2 NOP.¹ Initial characterization of Hyd-2 D'81N-OP showed that the approximate shape of the CV is similar though Hyd-2 D'81N-OP appears to be slightly more biased towards H₂ oxidation with little H₂ production at reducing potentials. Hydrogen production was confirmed by poisoning the electrode at low potentials and cycling the gas between 100% H₂ and 100% Ar and measuring the current response (data not shown). This experiment confirmed that Hyd-2 D'81N-OP is inhibited by H₂ and is capable of H₂ production. The measured E_{SWITCH} for Hyd-2 D'81N-OP is within the range of what has been measured for Hyd-2 NOP (Section 3.2.6).

The importance of D81 in the reversible conformational change of the proximal cluster upon oxidation was tested by measuring the response of Hyd-2 D'81N-OP to transient O₂ exposure. As measured by PFE, there was no drastic change in the reversibility of inactivation by transient O₂ exposure (Figure A1.1).

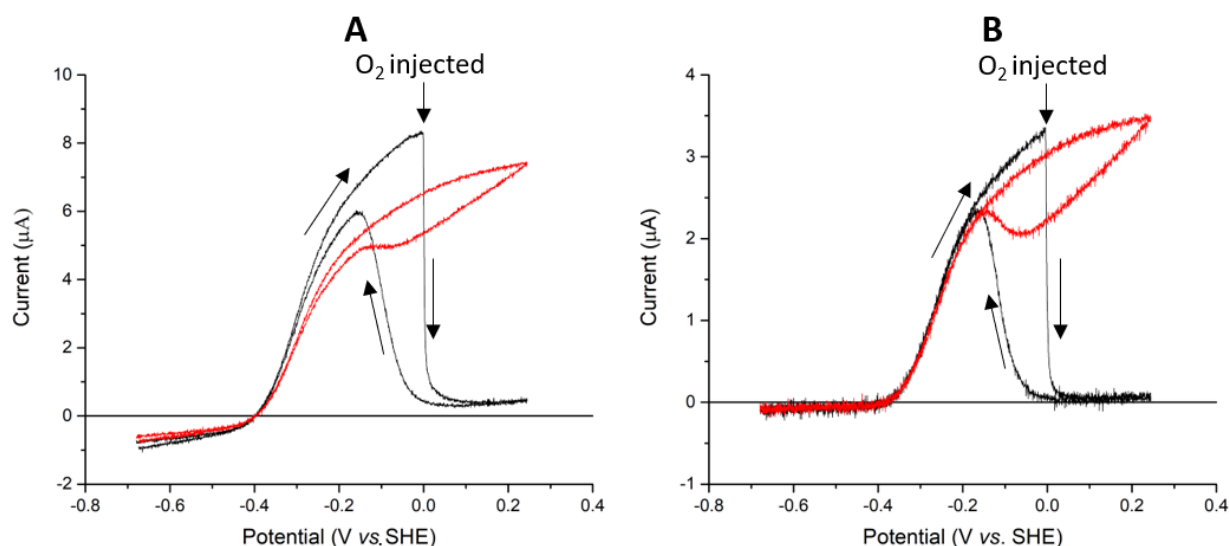


Figure A.1: O_2 injection of **(A)** Hyd-2 NOP and **(B)** Hyd-2 D'81N-OP in a 100% H_2 atmosphere. Scan during the O_2 injection is in black while the subsequent scan is in red. Both enzymes were injected with O_2 -saturated buffer to give a final concentration of 16 μM . Higher activity for this particular film of Hyd-2 NOP allows for greater film loss between scans. Thus, although the scans of Hyd-2 NOP appear to have greater film loss in absolute current between the two scans, the decrease in current is similar between Hyd-2 NOP and Hyd-2 D'81N-OP. The low H_2 production activity of Hyd-2 D'81N-OP is also seen in the low negative current in the reverse scan. Other conditions: 30°C, ω =1000 rpm, 100% H_2 , scan rate 0.5 mV/sec, total gas flow rate of 1000 scc min⁻¹.

The D'81N-OP variant reactivates to the same extent as the native enzyme does after transient exposure to O_2 (Figure A1.1). There are a range of possibilities to explain this result. Hyd-2 D'81N-OP is aerobically purified and relies on an activation procedure to reduce the enzyme into an active state. This activation procedure likely reduces the active site and reverses the oxidative damage of Fe-S clusters. It is possible that Hyd-2 D'81N-OP is not capable of coordinating the Fe lost from the proximal cluster upon prolonged aerobic exposure, and the proximal cluster forms a [3Fe-4S] cluster upon activation. Such a large change in the Fe-S cluster would be expected to alter the potential of the Fe-S clusters. The mutations that replaced the [4Fe-4S] medial cluster in [NiFeSe]-hydrogenases with a [3Fe-4S] resulted in a 400 mV increase in the redox potential of the cluster,² and a shift on that magnitude would be expected. Such a drastic increase in the potential could explain the shift in the apparent bias seen in D'81N-OP

towards H₂ oxidation. In the case where the majority of the D'81N-OP has a [3Fe-4S] cluster at the proximal position, transient exposure to O₂ would not further change the proximal cluster.

If instead, the [4Fe-4S] cluster is properly formed in the enzyme on the electrode, there are additional explanations for the fact that Hyd-2 D'81N-OP is equally reversibly inactivated. Either transient O₂ exposure is insufficient for cluster deformation which would prevent the need for D81 to coordinate a Fe atom lost from the proximal Fe-S cluster, or the Fe atom only needs a polar residue for coordination in the different conformation. It is also possible that, like in Hyd-1 E28Q,³ the asparagine facilitates a H₂O/OH molecule close to the amide which helps coordinate the Fe lost in oxidative damage to the proximal cluster. Further investigations for Hyd-2 D'81N-OP should include solving the as-isolated and reduced structure to see if the proximal cluster breakdown occurs reversibly in the variant. Alternative mutations like D81A could make the Fe-S cluster inherently less stable and would certainly affect the electrostatic environment of the Fe-S cluster, possibly altering the potential, but testing additional variants could also elucidate the importance of D81. Structural characterization of the proximal cluster variant would be needed in order to better understand the effect of the mutation on the enzyme.

A1.2 Design, production, and initial characterization of Hyd-2 G'24C/G'125C

In an attempt to engineer the [4Fe-3S]6cys cluster that is present in O₂-tolerant [NiFe]-hydrogenases like Hyd-1 into Hyd-2, mutations around the proximal cluster in Hyd-2 were made. The same proximal cluster mutations attempted in natively expressed Hyd-

²¹ (G'24C/G'125C) were made in the small subunit of overexpressed Hyd-2 (for clarity mutations made in the small subunit of Hyd-2 are designated with an apostrophe in this thesis). The reason why the G'24C/G'125C variant protein was not produced from the chromosomally-expressed variant strain is unknown. Regardless of whether overexpression of the concomitant variant was successful in incorporating the [4Fe-3S] cluster into Hyd-2, the differences in the expression system could give insight into the obstacles to expression. This variant also takes advantage of the simplified approach required for mutating the small subunit given that it is expressed from a plasmid rather than the chromosome. The mutations to *hybO* were made to the pO^C plasmid and expressed in native HJ001-hyp (see section 3.2.3).

In order to make the double mutation on pO^C, primers were designed to take advantage of the ability of Gibson Assembly to join together multiple fragments (Figure A1.2). While this possibility is inherent to Gibson Assembly, the high GC content of *hybO* and *hybC* has limited the success of previous mutagenic efforts in Hyd-2 (personal communication, Prof. Frank Sargent, University of Dundee). A similar approach was used as detailed for pMAK *hybC* (section 2.3.2).

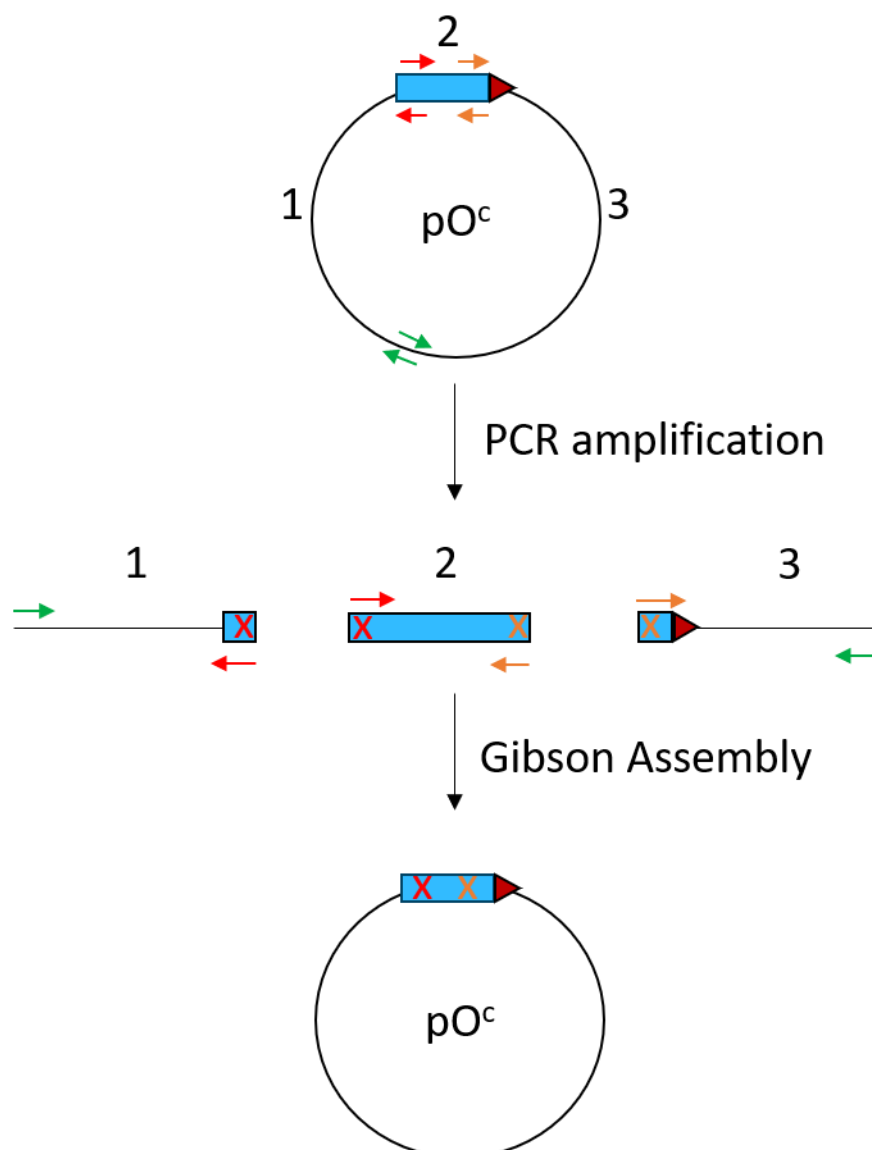


Figure A.2: Diagram of approach for site-directed mutagenesis using Gibson Assembly of pO^c to incorporate double mutants into the Hyd-2 overexpression system. Mutagenic primers are coloured red and orange. The primers that amplify in pO^c are coloured green, and the mutations left in the *hybO* gene are marked as an X. Mutant pO^c G'24C/G'125C is expressed in HJ001-hyp (see Tables 2.5-2.7 for a list of the strains, plasmids, and primers, respectively).

Attempts to express Hyd-2 G'24C/G'125C-OP followed the conditions that were previously optimized for expression of Hyd-2 NOP (section 3.2.4). However, no protein was produced for the Hyd-2 G'24C/G'125C-OP strain. There was no evidence of the mutated *HybO* gene expression on the FPLC chromatogram during Ni-affinity purification and the subsequent SDS-PAGE gel revealed no protein at the size corresponding to native

HybC or overproduced HybO (data not shown). The chromatographic fractions corresponding to the imidazole concentration where Hyd-2 NOP typically elutes were nonetheless pooled and concentrated and applied to an electrode. There was no signal apparent under any condition attempted (e.g. activation protocol at low and high potential, cyclic voltammograms, etc. data not shown).

The failed attempt to insert the [4Fe-3S]6cys cluster in the proximal position of Hyd-2 NOP elucidates the challenges of engineering the special proximal cluster into standard [NiFe]-hydrogenases. Part of the challenge could be the highly conserved region close to the proximal cluster in O₂-tolerant hydrogenase that differs greatly from the homologous region of standard hydrogenases. Figure A1.3 shows two alignments: (1) an alignment of the residues around the proximal cluster of O₂-tolerant [NiFe]-hydrogenases and (2) the alignment of that same region in O₂-tolerant and standard [NiFe]-hydrogenases. These alignments show that for O₂-tolerant [NiFe]-hydrogenases, this region is highly conserved, yet it is very dissimilar to the homologous region in standard [NiFe]-hydrogenases. Glycine is also capable of a range of orientations due to its lack of steric bulk in the functional group and replacing glycine with another residue that has more limited possibilities (like what was done in the C19G/C120G Hyd-1 variant) is more likely to result in unstable protein than the inverse mutations done to the O₂-tolerant hydrogenases.

O₂ tolerant membrane-bound [NiFe]-hydrogenase

E. coli Hyd-1 (1d) PVVWIHGLECTCCTESFIRSAHPLAKDVILSLISLDYDDTLMAAAGTQAE
S. typhimurium Hyd5 (1d) PVIWLHGLECTCCTESFIRSAHPLAKDAILSLISLDYDDTImAAAGQQAE
R. eutropha MBH (1d) PVLWLHGLECTCCSESFIRSAHPLAKDVVLSMISLDYDDTLMAAAGHQAE
A. aeolicus (1d) PVLWIHGLECTCCESEFSIRSATPLASDVVLSMISLEYDDTLsAAAGEAVE
-**-***** ** *.-*-**--*****- *****

Standard membrane bound [NiFe]-hydrogenases

G24

E. coli Hyd2 (1c) PVIWIGAQECTGCTESLLRATHPTVENLVLETISLEYHEVLSAAFGHQVE
D. gigas (1b) SVVYLHNAECTGCSESLLRTVDPYVDELILDVISMDYHETLMAGAGHAVE
* : : * * * * * : : * * * * * : : * * * * *

Figure A.3: Sequence alignment of O₂-tolerant and standard [NiFe]-hydrogenases in the region around G24 of Hyd-2. The sequence alignment shows the highly conserved region in O₂-tolerant [NiFe]-hydrogenases around the proximal cluster. The first row of symbols shown is for the alignment among O₂-tolerant hydrogenases followed by an alignment of hydrogenases from both groups. Asterisks represent amino acids that are strictly conserved, a colon represents amino acids that are highly conserved, and a period represents amino acids that are weakly conserved.

The inability for the Hyd-2 overproduction system to produce the Hyd-2 G'24C/G'125C-OP shows certain possible biochemical limitations of the original native expression system are not responsible for inhibiting incorporation of [4Fe-3S]6cys clusters into Hyd-2. For example, the HJ001-hyp strain contains the maturation proteins from *R. eutropha* which are active in *E. coli*.⁴ The MBH from *R. eutropha* contains the [4Fe-3S]6cys proximal cluster,⁵ and it was postulated that the maturation proteins from *R. eutropha* may help properly assemble the Hyd-2 G'24C/G'125C-OP variant. Thus, as the double cysteine variant was not produced in the overexpression system, the inability to insert the special [4Fe-3S]6cys cluster into standard hydrogenases is less likely to be related to the lack of maturation proteins. HybO produced by the overproduction system is not processed by the Tat system, and thus the failure to express the double cysteine variant in the overexpression system also shows that the failure is unrelated to the Tat system that checks HybO for proper insertion of the metal cofactors. Further investigations involving mutations of larger sections of the region around the proximal cluster in standard [NiFe]-hydrogenases will be required in an attempt to insert the special [4Fe-3S]6cys cluster into standard hydrogenases.

The results above confirm that the glycine residues play an important structural role in the coordination of the proximal cluster of Hyd-2. Mutating those glycine residues to cysteine produces a protein that is either fundamentally unstable in the cell and/or does not meet the requirements of the maturation proteins that check for properly folded and functional protein. In the case of overproduced protein, there are two sets of maturation proteins potentially capable of inserting the [4Fe-3S]6cys cluster, including those native to *E. coli* (encoded in the *hya* operon) and the proteins from *R. eutropha* (encoded by the synthetic *hyp* operon). Nevertheless, more work must be done either on the maturation proteins encoded in *E. coli* or *hybO* in an attempt to produce a standard [NiFe]-hydrogenase with the special [4Fe-3S]6cys cluster.

A1.3 References

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Appendix B

B1.1 Gas treatments to characterize the diatomic found at Hyd-2 R479K-OP

In an attempt to characterize the diatomic that was found near the Ni atom of as-isolated Hyd-2 R479K-OP (see Chapter 7), different protein preparations were treated with different gases under different conditions. Particularly because the activity of Hyd-2 R479K-OP was found to be highly dependent on temperature, a detailed description of how the enzyme was treated is important to interpret the resulting electron density at the active site. There is also an important distinction between how the temperature is reported for protein treatments. Protein preparations treated with a flow of gases were cooled in a water-jacketed glass cell, and the reported temperature reflects the temperature of the water bath. Although the temperature of the protein was never measured directly, other experiments using buffers cooled with similar glass cells have shown that the contents in the cell are typically 2°C-4°C warmer than the 4°C reported for the water bath. By contrast, protein treated in a pressure vessel kept in the fridge is consistently within 0.5°C of 4°C.

The first preparation of as-isolated Hyd-2 R479K-OP (termed 1AIR in Figure 7.7) was purified and then stored overnight in anaerobic conditions at room temperature (20°C). Protein was then set up in crystal trays in anaerobic conditions and were sealed. The trays were then placed in aerobic conditions and crystals were allowed to grow in conditions that became increasingly aerobic with time. Crystals were then picked in aerobic conditions and snap frozen in N₂(l).

The second preparation of Hyd-2 R479K-OP (termed 2AIR) was purified and reduced overnight at 4°C and stirred in a flow of 100% H₂. Four aliquots of the reduced enzyme were taken. One aliquot was crystallized in the reduced form (2RED) while the other three aliquots underwent either treatment with O₂ (2OX), CO (2CO), or air (2ReAir).

The protein aliquots that were treated with pure gases (2OX and 2CO) were exposed to the air for 20 min at 4°C before being sparged with Ar. The protein exposed to air after H₂-reduction was at room temperature (20°C) in air for 1.5 h before being taken into an anaerobic environment for crystallization. For this round of protein crystallization and all subsequent crystallization the protein was set up for crystallization in fully anaerobic conditions, the crystals grew at room temperature (20°C), and the crystals were picked and snap frozen in N₂(l) under anaerobic conditions. There was protein remaining from each aliquot and the remaining protein was pooled regardless of the previous gas treatment and was placed in a pressure vessel (Tynclave Steel, Büchiglasuster) of 100% H₂ at 4 bar at 4°C. The protein was then divided into two samples: one sample then underwent exposure to 100% ³⁶O₂ for 20 min at 2 bar (218OX) and the other was simply set up for crystallization (2REDL).

The third purification of Hyd-2 R479K-OP was placed in a pressure vessel (Tynclave Steel, Büchiglasuster) of 100% H₂ at 4 bar at 4°C, and aliquots were taken at 12 h (3RED_12H), 36 h (3RED_36H), 6 days (3RED_6D). A portion of the 3RED_6D protein was then exposed to ³⁶O₂ (3OX18).

A fourth purification of Hyd-2 R479K-OP was exposed to a flow of H₂ while being stirred at 4°C for 23 h. An aliquot of the reduced enzyme was then taken and set aside and stored in an N₂ environment at 4°C (4RED). The remaining protein was then exposed to a sequential flow of gases under the same conditions (i.e. stirred, 4°C) after which an aliquot was taken for each step and placed in an N₂ environment at 4°C: 100% CO for 20 min (4COF), 50% H₂ and 50% CO for 3.5 h (4REDCO), 100% CO for 20 min (4COS), and finally 100% H₂ for 16 h (4REDL).

The final purification of Hyd-2 R479K-OP was exposed to a flow of H₂ while being stirred at 4°C. An aliquot of the reduced enzyme was taken and set aside and stored in an N₂ environment at 4°C (5RED). The remaining protein was then exposed to a sequential flow of gases under the same conditions (i.e. stirred, 4°C) after which an aliquot was taken for each step and placed in an N₂ environment at 4°C: 100% CO for 10 min (5COF), 50% H₂ and 50% CO for 20 min (5REDCO), 100% CO for 10 min (5COS), 100% H₂ for 16 h (5REDL), and finally ³⁶O₂ for 45 min (5OX18).

Hyd-2 NOP was then purified and underwent identical treatments as the fifth treatment of Hyd-2 R479K-OP.

B1.2 Table of refinement statistics

Tables B.1, B.2, B.3, and B.4 report that refinement statistics of structures of Hyd-2 R479K-OP (Table B.1, B.2, and B.3) and Hyd-2 NOP (Table B.4).

Table B.1. X-ray data collection and refinement statistics for Hyd-2 R479K-OP after various treatments with different gasses. Dr. Steve Carr solved all structures.

Sample treatment	2REDL*	2OX18*
Data collection		
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit cell dimensions (Å)	a=99.17, b=99.97, c=169.1	a=99.84, b=100.41, c=169.05
Resolution (Å)	70.5 – 1.45 (1.47-1.45)	86.4- 1.7 (1.74-1.7)
Total reflections	1,359,775 (63,729)	1,176,165 (54,034)
Unique reflections	291,846 (13,698)	183,286 (8,637)

Completeness (%)	98.5 (94.1)	99.8 (95.4)
Multiplicity	4.7 (4.7)	6.4 (6.3)
$\langle I/\sigma \rangle$	7.0 (1.3)	3.4 (1.1)
R _{merge} (%)	8.6 (64)	14.9 (117)
R _{pim} (%)	5.1 (37.5)	5.9 (46.6)
CC _{1/2}	0.992 (0.708)	0.999 (0.5)
Refinement		
R _{work} /R _{free} (%)	18.5/20.6	15.2/17.3
No. of atoms	14,721	13,193
Macromolecule	12,715	12831
Solvent	2006	362
Average B-factors		
Macromolecule	29.5	25.8
Solvent	38.9	25.9
RMSD bond lengths (Å)	0.02	0.015
RMSD angles (°)	2.0	1.73
Ramachandran plot (%) favoured / outliers	96.5 / 2	96.0 / 2
Clashscore	2.4	0.88

Values in parentheses refer to data in the highest resolution shell. Sample treatments marked with an asterisk represent sample with only partial refinements that were refined until the occupancy at the active site became clear. See section 2.8 for more information about R_{merge}, R_{meas}, R_{pim}, R_{work}, R_{free}, and CC_{1/2}.

Table B.2. X-ray data collection and refinement statistics for Hyd-2 R479K-OP after various treatments with different gasses. Dr. Steve Carr solved all structures.

Sample treatment	3RED_12H	3RED_36H	3RED_6D	3OX18

Data collection				
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions (Å)	a=99.37, b=100.19, c=169.12	a=99.53, b=100.16, c=169.19	a=99.50, b=99.99, c=168.91	a=99.52 b=100.01, c=169.04
Resolution (Å)	49.5-1.2 (1.22-1.20)	86.2-1.14 (1.16-1.14)	49.75-1.26 (1.28-1.26)	86.08-1.19 (1.21-1.19)
Total reflections	2,588,723 (125,991)	4,247,308 (169,424)	3,291,666 (159,417)	3,754,148 (178,421)
Unique reflections	359,726 (17,835)	609,090 (30,194)	450,641 (22,352)	534,762 (26,534)
Completeness (%)	100 (100)	100 (99.6)	100 (99.9)	100 (100)
Multiplicity	8.8 (1.0)	7.0 (5.6)	7.3 (7.1)	7.0 (6.7)
$\langle I/\sigma \rangle$	7.2 (7.1)	6.6 (1.0)	12.3 (1.2)	9.3 (1.2)
R _{merge} (%)	14.4 (89.8)	9.1 (142)	9.2 (175)	6.0 (184)
R _{pim} (%)	5.4 (33.5)	4.6 (77.5)	3.4 (65.6)	2.3 (71)
CC _{1/2}	0.999 (0.500)	0.999 (0.5)	0.999 (0.500)	0.999 (0.600)
Refinement				
R _{work} /R _{free} (%)	12.8/14.9	14.5/17.2	11.8 (14.5)	12.7/14.9
No. of atoms	14,077	14,072	14,073	13,570
Macromolecule	12,858	12,857	12,858	12,836
Solvent	1219	1220	1221	734
Average B-factors				
Macromolecule	15.2	13.4	16.9	15.9
Solvent	23	21.9	25.2	22.5
RMSD bond lengths (Å)	0.015	0.017	0.015	0.015
RMSD angles (°)	1.72	1.81	1.70	1.73

Ramachandran plot (%) favoured / outliers	96.1/ 2	96.0/ 2	96.1/ 2	96.2/2
Clashscore	1.26	1.22	1.25	0.32

Values in parentheses refer to data in the highest resolution shell. See section 2.8 for more information about R_{merge} , R_{meas} , R_{pim} , R_{work} , R_{free} , and $CC_{1/2}$.

Table B.3. X-ray data collection and refinement statistics for Hyd-2 R479K-OP after various treatments with different gasses. Dr. Steve Carr solved all structures.

Sample treatment	5RED	5COF
Data collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions (Å)	a=99.24, b=99.97, c=168.7	a=99.56, b=100.02, c=168.77
Resolution (Å)	99.3 – 1.5 (1.53-1.5)	168.8- 1.6 (1.63-1.6)
Total reflections	1,331,490 (66,284)	2,704,947 (128,090)
Unique reflections	266,431 (13,126)	221,610 (10,889)
Completeness (%)	99.8 (99.9)	100 (100)
Multiplicity	5.0 (5.0)	12.2 (11.8)
$\langle I/\sigma \rangle$	9.1 (2.0)	6.0 (1.9)
R_{merge} (%)	9.1 (61)	25.3 (114)
R_{pim} (%)	4.9 (33.9)	7.8 (36.4)
$CC_{1/2}$	0.992 (0.442)	0.989 (0.500)
Refinement		
$R_{\text{work}}/R_{\text{free}}$ (%)	15.8/17.9	17.2/20.1
No. of atoms	13,710	13,631
Macromolecule	12,831	12,831
Solvent	879	800
Average B-factors		

Macromolecule	16.3	17.0
Solvent	20.7	20.5
RMSD bond lengths (Å)	0.02	0.019
RMSD angles (°)	1.99	1.87
Ramachandran plot (%) favoured / outliers	96.0 / 2	96.0/2
Clashscore	0.59	0.83

Values in parentheses refer to data in the highest resolution shell. See section 2.8 for more information about R_{merge} , R_{meas} , R_{pim} , R_{work} , R_{free} , and $CC_{1/2}$.

Table B.4. X-ray data collection and refinement statistics for Hyd-2-NOP after various treatments with different gasses. Dr. Steve Carr solved all structures.

Sample treatment	NAIR	NCOF	NREDO	NOX18
Data collection				
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions (Å)	a=99.64, b=100.50, c=168.92	a=99.30 b=100.35, c=168.32	a=99.34 b=100.48, c=168.40	a=98.74, b=99.92, c=167.82
Resolution (Å)	168.7 – 1.4 (1.42-1.4)	89.2- 1.4 (1.42-1.40)	168.4-1.5 (1.53-1.50)	167.8-1.6 (1.63-1.6)
Total reflections	1,713,316 (84,137)	2,084,784 (101,667)	1,364,619 (66,312)	1,531,964 (71,804)
Unique reflections	330,287 (16,22)	328,801 (16,136)	264,596 (12,905)	219,456 (10,712)
Completeness (%)	99.9 (99.9)	100 (99.9)	98.7 (97.8)	100 (100)
Multiplicity	5.2 (5.2)	6.3 (6.3)	5.2 (5.1)	7.0 (6.7)
$\langle I/\sigma \rangle$	8.8 (1.3)	9.1 (1.4)	5.8 (1.6)	6.1 (1.4)
R_{merge} (%)	7.5 (89)	9.7 (81.3)	13.6 (68.7)	15.2 (68)
R_{pim} (%)	4.0 (47.1)	4.6 (38.6)	7.4 (37.8)	6.5 (31.4)

CC _{1/2}	0.998 (0.696)	0.997 (0.522)	0.988 (0.51)	0.992 (0.703)
Refinement				
R _{work} /R _{free} (%)	15.7/17.7	15.4/17.7	16.5/18.9	17.5/20.4
No. of atoms	13,897	13,806	13,741	13,589
Macromolecule	12,753	12,816	12,835	12,835
Solvent	1144	990	906	754
Average B-factors				
Macromolecule	17.6	16.3	17.0	18.6
Solvent	24.1	18.4	20.9	21.0
RMSD bond lengths (Å)	0.015	0.016	0.019	0.017
RMSD angles (°)	1.71	1.75	1.86	1.72
Ramachandran plot (%) favoured / outliers	96.4/2	96.3/ 1	96.3/ 2	96.2/ 2
Clashscore	1.23	1.0	1.06	1.46

Values in parentheses refer to data in the highest resolution shell. See section 2.8 for more information about R_{merge}, R_{meas}, R_{pim}, R_{work}, R_{free}, and CC_{1/2}.