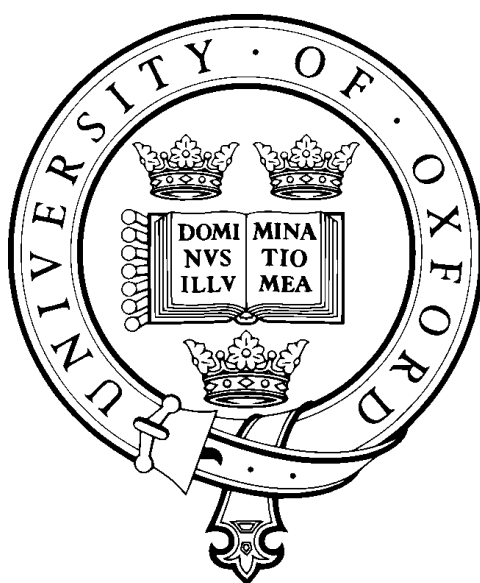

THE IMPORTANCE OF ELECTRON
TRANSFER IN DETERMINING
PROPERTIES OF [NiFe]-HYDROGENASES



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LINACRE COLLEGE

Trinity Term 2013

A thesis submitted to the Board of the Faculty of Physical
Sciences, for the degree of Doctor of Philosophy, University of
Oxford

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[NiFe] hydrogenases are microbial metalloenzymes that catalyse the reversible interconversion between molecular hydrogen and protons with high selectivity and efficiency. The catalytic properties of different [NiFe] hydrogenases vary according to the physiological roles they each play, yet all seem to be based upon an almost identical catalytic site architecture. Through efforts to understand the structural and mechanistic basis for the differing properties of [NiFe] hydrogenases, it has become increasingly evident that electron transfer to and from the active site, mediated by a set of Iron-Sulphur clusters, influences to a significant extent the observed catalytic properties of different hydrogenases. Here we present a comprehensive study of *E. coli* Hyd-1, an O₂-tolerant hydrogenase, by PFE with a focus on the properties that are characteristic of O₂-tolerant enzymes: overpotential requirement, lack of H₂ production, low $K_M^{H_2}$, and high E_{switch} . We show that Hyd-1 catalysis can be made reversible by increasing the equilibrium potential for the reaction through changes in substrate concentration, and that electron transfer into and out of the enzyme molecule, rather than active site properties, is responsible for the characteristics of overpotential and bias in Hyd-1. We present a set of experiments with Hyd-2 from *E. coli* in which surface-exposed cysteine residues are specifically introduced near the distal and medial Iron-Sulphur clusters to act as points of attachment for photosensitizer molecules, and a study of the kinetics of electron injection from photoexcited molecules to the enzyme and subsequent absorbance changes attributed to transient redox changes at the active site. We are able to show light-dependent H₂ production from a Hyd-2 + photosensitizer system. Finally, we present the first purification of the formate-hydrogen lyase (FHL) complex from *E. coli*, the complex responsible for H₂-production by this organism during fermentation, and we provide a characterisation of the complex by EPR and PFE. The properties of Hyd-3, the hydrogenase subunit of the FHL, seem to differ from those observed previously for other [NiFe] hydrogenases.

Collaborations

Several collaborators have contributed to the success of this work. Mass spectrometry analysis of mutated and labelled Hyd-2 variants in Sections 4.2.6 and 4.2.8 was carried out using a system and protocol established and managed by Dr. L. Gong, Central Mass Spectrometry Facility, Chemistry, University of Oxford. I prepared the samples and analysed the data for this MS work. Ruthenium polypyridyl photosensitizers were synthesized by D. Streich. Some of the labelling experiments and all of the flash photolysis and absorption spectroscopy experiments, the light-driven H₂ experiments, as well as the data analysis for these experiments was carried out by D. Streich, Uppsala, Sweden. The strain MG059e1, carrying an engineered HycE subunit bearing an internal affinity tag for purification of the FHL complex, was constructed by Professor Frank Sargent, University of Dundee. Metal ion analysis of the isolated FHL complex was carried out by Professor M. Haumann, Freie Universität Berlin. Dr. M. Rößler, University of Oxford, provided valuable advice and instruction for the characterisation of the FHL complex by EPR spectroscopy.

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List of Abbreviations

$[\text{Ru}(\text{dm-bpy})_2(\text{MI-phen})]^{2+}$ $[\text{Ru}(4,4'\text{-dimethyl-bipyridine})_2(5\text{-maleimido-phenanthroline})]^{2+}$

B-PER Bacterial Protein Extraction Reagent

bpy 2,2'-bipyridine

CHES 2-[N-cyclohexylamino]ethane-sulfonic acid

CV Cyclic Voltammogram

CW Continuous-wave

DDM β -dodecyl maltoside

DMK demethylmenaquinone

DTBM dithio-bis-maleimidoethane

Ech Energy-converting hydrogenase

EPR Electron Paramagnetic Resonance

FHL Formate Hydrogen Lyase

FT-IR Fourier transform Infrared

H₄MPT tetrahydromethanopterin

HEPES N-[2-hydroxyethyl]piperazine-N-[2-ethane-sulfonic acid]

LB	Luria-Bertani
LC-ESI-MS	Liquid chromatography-Electrospray Ionisation-mass spectrometry
MA	Modulation Amplitude
MES	2-[N-morpholino]ethane-sulfonic acid
MGD	Molybdopterin guanine dinucleotide
MK	menaquinone
MK	menaquinone
MLCT	Metal-to-ligand charge transfer
MO	Molecular orbital
MP	Microwave power
MWCNT	Multi-walled carbon nanotubes
OD	Optical Density
OH-NQ	4-OH-1,4-naphthoquinone
PCR	Polymerase Chain Reaction
PDH	Pyruvate Dehydrogenase
PFE	Protein Film Electrochemistry
PFL	Pyruvate:formate lyase
phen	1,10-phenanthroline
PMF	Proton-motive force
ROS	Reactive oxygen species

TAPS	N-tris[hydroxymethyl]methyl-3-amino-propane-sulfonic acid
TSB	Transformation and Storage Buffer
TXRF	Total Reflection X-ray fluorescence
UQ	ubiquinone
XAS	X-ray absorption spectroscopy

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Chapter 1

Introduction

1.1 Metabolism

1.1.1 Living electronics: Powering a cell

When, in 1828, Friedrich Wöhler synthesised urea [1], he reported to his mentor Jöns Berzelius

‘I cannot, so to say, hold my chemical water and must tell you that I can make urea without thereby needing to have kidneys’.

Wöhler’s discovery, the first synthesis of an organic compound, changed the way we think about life by challenging the widely-held belief that the fundamental distinction between living and non-living was a ‘vital force’ present in organic, but not inorganic matter. This discovery blurred the line between biology and chemistry. Since then, chemists have worked tirelessly to understand the way in which chemical transformations give rise to vitality.

An updated understanding of the organising principles of life, as I remember them from high school cell biology, are that: 1) Cells isolate themselves from the environment using a membrane; 2) Inside that membrane, *chemistry* happens, allowing energy to flow through the system from one form to another, and creating order in the process 3) At the centre of these energy flows lies a ‘common currency’ for energy, ATP (Adenosine Triphosphate).

What I didn’t appreciate at the time was the extent to which this mysterious *chemistry* resembled the batteries and fuel cells dominating parallel lessons in high-school chemistry lectures. Nor did I understand that the membrane served not only as separation from the external world, but indeed as a grid, electrically connecting the various ‘anodes’ and ‘cathodes’; that the energy contained in the apple I ate and the air I breathed could only be captured by connecting these through a complicated cellular circuit. Or that even these machinations would depend on an electrochemical gradient, a second ‘common currency’ for cellular energy, dammed by a membrane and exploited

with the use of a protein-based turbine. Finally, I did not understand that, viewed through the lens of these cellular electronics, my own ‘apple and air’ style of metabolism would seem a folksy and primitive mix, compared with the sophisticated machinery of the Gram-negative bacterium *Escherichia coli*.

The ‘anodes’ and ‘cathodes’ of the cell, the oxidase and reductase enzymes, are responsible for much of metabolism, from photosynthesis, through lithotrophy and other anaerobic respiration, to the aerobic respiration that couples the sugar in my apple to the oxygen in the air I breathe. The reaction of fuels with oxidants via well-controlled membrane-bound electron transfer chains leads to energy conversion by contributing to a transmembrane electrochemical gradient, often referred to as the proton-motive force [2]. This gradient stores energy and serves as a means of interconverting between different forms of energy: it can be increased by light-driven proton translocation, by mechanical proton pumping, and by scalar proton pumping (otherwise known as ‘redox loops’, in which a reaction consumes protons on one side of the membrane and releases them on the other side); this gradient can be used to drive ATP formation as well as the transport of molecules up a concentration gradient. Substrate-level processes can contribute to this gradient by driving production of ATP; a reversible membrane-bound turbine, the F_0F_1 -ATPase, can function bidirectionally, interconverting between energy stored as ATP and as electrochemical gradient. Through these connections, all metabolic processes are tied into the living circuitry of the cell.

E. coli is a widely-distributed bacterium, ubiquitous in the gastrointestinal tract of mammals, birds and reptiles as well as being widely distributed in the external environment. A rapidly changing environment means that *E. coli* must be flexible in its metabolism, to thrive under a number of different conditions; the diverse respiratory processes that mediate *E. coli*’s metabolic flexibility are dependent upon a variety of oxidases and reductases (Figure 1.1). Many different substrates can be used, and all can be connected through a common pool of membrane-bound quinones [2, 3], the compo-

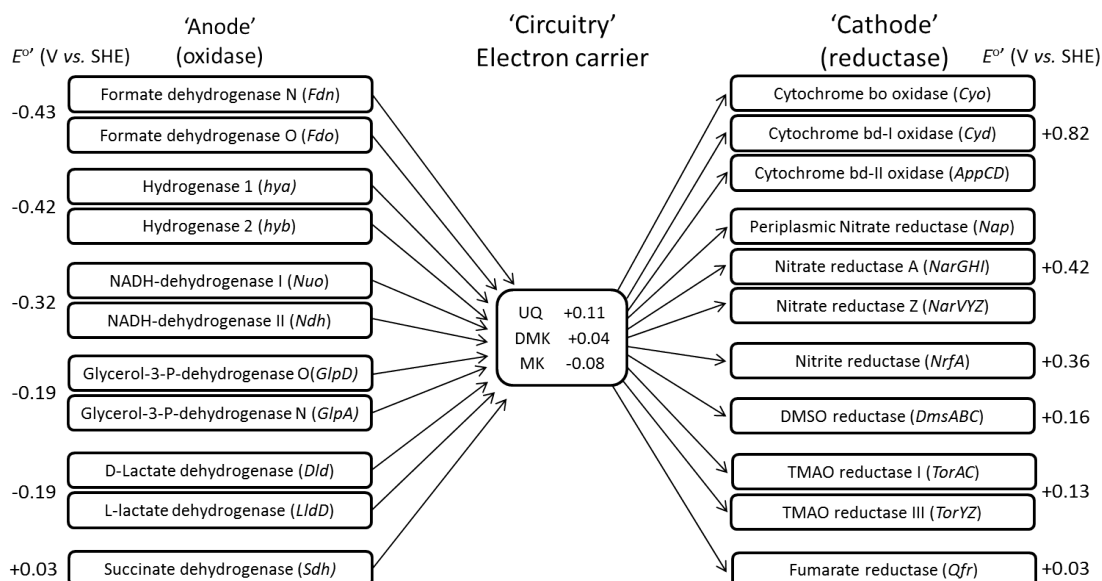


Figure 1.1: The respiratory pathways of *E. coli*. The standard biological reduction potential for the redox couple catalysed by each oxidase or reductase is shown.

sition of which can be adjusted to better match the potential of a given redox couple [4].

Perhaps more impressive still is the way in which isoenzymes, whose expression is tuned by environmental conditions, allow the same substrate to be used under different conditions, balancing the need for a high growth rate with the need for efficiency of energy extraction. In our analogy with an electrical circuit, this is akin to tuning the load on the circuit to suit the available voltage. For example, the NADH:Quinone couple, when catalysed by NADH dehydrogenase I, drives H^+ pumping across the membrane, but the same reaction when catalysed by NADH hydrogenase II does not pump H^+ [5, 6, 7]; downregulation of the NADH dehydrogenase II as cells enter late stationary phase allows for more energy to be extracted as the NADH/NAD⁺ ratio increases [8]. Similarly, on the side of the reductase enzymes, energy extraction can be optimised by differential expression. Cytochrome *bo* oxidase is expressed under high concentrations of O_2 , and is able to pump protons directly, as well as functioning as a scalar proton pump [9]; conversely, the cytochrome *bd-I*, which is expressed under O_2 -limited conditions, contributes to the electrochemical gradient only through scalar proton pumping, and

thus contributes to the movement of only one proton per electron [10], while the *bd-II* enzyme does not contribute to a PMF [11].

What is perhaps most notable is that the standard potential for all couples available for respiratory energy generation in *E. coli* lie within a range of only 1.25V; this range is narrowed to only 0.85V under anaerobic conditions. For any work to be done with such a small potential difference, the redox catalysts mediating these processes must be superbly efficient. Through billions of years evolution, organometallic-like complexes have been fine-tuned by their peptide scaffold for superb efficiency, providing some of the only examples of truly reversible catalysis in non-noble metals [12]. We can treat these components like we would any other electronic component - by ‘wiring’ them into a circuit, using the technique of Protein Film Electrochemistry (PFE), it is possible to study the electrochemical properties of these catalysts, with the hope that the principles underlying efficient and specific catalysis can be applied to biomimetic catalyst design. In some cases, these enzymes are not seen to be reversible; there are cases in which the evolutionary process seems to have sacrificed reversibility for other desirable properties, something which is also interesting to understand more fundamentally. This will be discussed in chapter 3 of this thesis in the context of Hydrogenase-1 from *E. coli*.

The many links with non-respiratory processes are too numerous to show in the above figure; nonetheless a few key points are worth mentioning. The production of formate from pyruvate, catalysed under anaerobic and microaerobic conditions by the pyruvate:formate lyase (PFL), serves as an alternative to the aerobic pathway via pyruvate dehydrogenase (PDH). The formate is then preferentially used for aerobic- or nitrate respiration as pictured above [13], but in the absence of these electron acceptors, formate is processed by a third formate dehydrogenase enzyme, the Fdh-H (*fdh* operon) that is directly coupled with a hydrogenase, Hyd-3, together making up the formate hydrogen lyase (FHL) complex [14, 15]. The disproportionation of formate to H_2 and CO_2 by Hyd-3 is responsible under most conditions for all H_2 production observable in

E. coli during fermentation [16], and some of the H_2 produced by FHL is reoxidised by the respiratory H_2 -oxidisers, Hyd-1 and Hyd-2 [13]. It is notable that the disproportionation of formate to H_2 and CO_2 is unfavourable under standard biological conditions, but conditions in the mammalian gut shift the equilibria sufficiently that a small driving force exists *in vivo*. This enzyme, which is the subject of Chapter 5 of this thesis, is a fantastic example of both the flexibility of the metabolic pathways of *E. coli* and of the tremendous efficiency of the catalysts involved.

1.1.2 Hydrogen metabolism

The utility of the oxidoreductases of Figure 1.1 is limited by the availability of their substrates. However, there exists one reductase enzyme whose substrate is never absent: Hydrogenase. H_2 can be produced by H^+ reduction and is often used as a means of venting excess reducing equivalents. Under anaerobic conditions, fermentation of various carbon sources to produce H_2 is seen across a large diversity of organisms. Fermentation of glucose via various organic acids to $6CO_2$ and $12H_2$ produces at most 4 moles ATP per mole glucose [17]. Some organisms, including *Carboxydotherrmus hydrogenoformans*, release H_2 as a product of CO respiration [18, 19, 20]. In photosynthetic organisms, both prokaryotic and eukaryotic, hydrogen production can be used during high-light conditions to prevent the photosystems from becoming over-reduced and thus sustaining damage [21, 22]. H_2 is also known to be produced as a necessary byproduct of N_2 reduction by nitrogenase [23], and more recent studies have revealed that low rates of H_2 production are seen for various other metalloenzymes whose mechanisms include a metal-bound hydride, including formate dehydrogenase [24], carbon monoxide dehydrogenase [25, 26] and Pyruvate:Ferredoxin oxidoreductase [26].

In almost every environment where H_2 is produced, there is an organism nearby able to use the H_2 as reductant. Most nitrogen-fixing bacteria are able to reoxidise the H_2 produced as a by-product of N_2 fixation, recapturing the reducing equivalents

it holds [27, 28]. H_2 and CO_2 produced as a result of fermentation can be used for methanogenesis; the energy derived from this reaction varies from 1.5 to as little as 0.5 mole ATP per mole CH_4 depending upon the organism, allowing the reaction to be favourable even under very low $\rho(\text{H}_2)$, down to approximately 10Pa [17]. Coupling H_2 oxidation with sulphate reduction is more exergonic than CH_4 production, thus sulphate reducers outcompete methanogens and the $\rho(\text{H}_2)$ required to sustain this metabolism in sulphate-rich environments can be as low as 1Pa [29]. H_2 respiration can also be coupled to nitrate and Fe^{3+} reduction [30]. Some organisms are able to grow in microaerobic environments where H_2 produced by fermentation or anaerobic respiration mixes with O_2 , as in the upper layers of lake or marsh sediment, and some parts of the digestive tract [31]; some of these organisms can grow on H_2 as sole electron source, using aerobic H_2 respiration to drive CO_2 fixation [32, 33, 34]. Abiotically-produced H_2 emerging at hydrothermal vents is oxidised and respiration with various compounds, including O_2 , is possible [35, 36, 37]. The atmospheric concentration of H_2 is roughly 0.5ppm; nonetheless, aerobic soils show H_2 uptake activity under atmospheric conditions [38]. Recent work has shown this activity to be due to *Streptomyces* species that express [NiFe] hydrogenases with extremely high H_2 affinities, exhibiting $K_M^{\text{H}_2}$ values in the range of tens of nM [39].

With its roles in anaerobic metabolism, it is no surprise that H_2 plays a role in the ecophysiology of gastrointestinal flora, both beneficial and pathogenic; the *extent* to which hydrogen-cycling plays a specific and crucial role in pathogenicity is, however, surprising. H_2 is essential to virulence in *Salmonella typhimurium* [31]. Mutations abolishing the H_2 -uptake activity of *Helicobacter hepaticus* abolish pathogenic activity in mice [40]; the same mutations in *H. pylori*, a causative agent of gastric ulcers and cancers, reduce colonization of mouse stomach by this pathogen [41]. H_2 produced during anaerobic metabolism in the gut enters the bloodstream and serves as an energy source that pathogens, but not human cells, can use in aerobic environments. Elevated H_2 in

the breath has been shown to be an effective test for lactose intolerance [42]. Studies in mice have shown that, with intestinal concentrations of H_2 in the hundreds of μM , concentrations in the liver and spleen are roughly $50\mu M$ [31, 43], levels well below the enzyme-specific and cellular affinities for H_2 in the enterobacteria [44, 31].

1.1.3 Evolution of H_2 metabolism

Significant evidence [45, 46] exists to suggest that the first living organisms evolved at hydrothermal vents, or ‘white smokers’, where H_2 -rich, basic waters from the vent met with CO_2 -rich, and thus acidic, ocean water. Under these conditions, reduction of CO_2 by H_2 is exergonic, providing a driving force for the accumulation of organic matter [47], something like a formate-hydrogen lyase complex operating in reverse; this early metabolic pathway is proposed to underlie the Wood-Ljungdahl pathway in which CO_2 reduction by H_2 yields methane or acetate, a pathway which seems to be conserved across the Bacteria and Archaea, suggesting that its precursor was present in the last universal common ancestor of all life forms [48]. If this hypothesis proves true, hydrogen-splitting is at the very core of the development of life on Earth. Hydrothermal vents also contain very high levels of Fe, S and Ni clustered in forms such as greigite [48], the elements proposed to have supported the buildup up prebiotic organic matter acting as catalysts [49]. As if in support of this theory, *Methanopyrus kandleri*, one of the most ancient organisms known, grows on H_2 and CO_2 , exploiting three distinct Ni-Fe-S based proteins for H_2 activation [50].

Much later, after the first primitive organisms were able to generate their own proton-motive force and move away from hydrothermal vents, hydrogen metabolism is believed once again to have played a critical role in the diversification of life. The ‘hydrogen hypothesis’ proposed by Martin and Müller in 1998 suggests a very special role for H_2 -cycling in the evolution of eukaryotic life: the endosymbiotic event believed to have eventually resulted in the development of mitochondria may have originally involved the

association of an anaerobic hydrogenotrophic archaeobacterium with a eubacterium that generated molecular hydrogen as a waste product of anaerobic respiration; thus H_2 was the currency of the exchange between symbiont and host [51, 52, 53]. This symbiosis is thought to have given rise to an early form that is ancestral to both mitochondriate and amitochondriate eukaryotes, in which the symbiont developed either into the mitochondrion or the hydrogenosome, or was lost entirely, depending upon selective pressure [54, 55].

A strong sequence homology exists between the energy-converting (Group IV) hydrogenases and Complex I (NADH:quinone oxidoreductase), suggesting that Complex I evolved from a hydrogenase complex - yet another crucial link between H_2 metabolism and the development of complex life forms. This homology will be discussed in Section 5.1.5.

1.2 Hydrogenase enzymes

1.2.1 Hydrogenases exploit Ni and Fe-based cofactors

The enzymes responsible for catalysing heterolytic H_2 cleavage, $H_2 \rightleftharpoons H^+ + H^- \rightleftharpoons 2H^+ + 2e^-$, are known as hydrogenases. Three separate classes of hydrogenase are known, distinguished by the metal content of their active sites: the [NiFe], [FeFe], and mono[Fe] enzymes [56]. The three classes seem to have evolved separately, with no apparent homology between the hydrogenase peptides or the pathways that allow their expression and maturation [50]; nonetheless, a remarkable structural consensus seems to have been reached through convergent evolution. In all three classes, a minimum structure of an Fe ion ligated by CO ligands is seen, and in the case of the [NiFe] and [FeFe] enzymes, CN is also present as a ligand (Fig. 1.2). Both CO and CN are incredibly rare in biology, due to their high toxicity and rapid diffusion rates, as well as a lack of a pre-existing biosynthetic pathway. We may therefore speculate that the π -acceptor nature of these

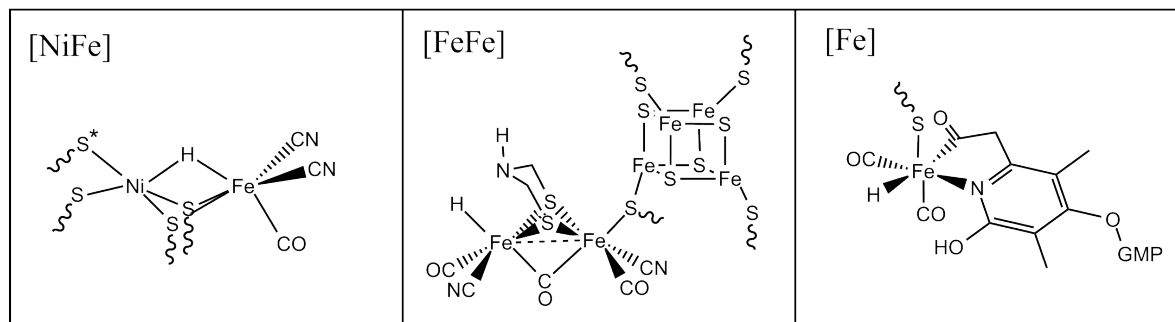


Figure 1.2: The active site structures of the three classes of hydrogenase. In all cases, the structure is presented with a metal-bound hydride at the site of binding. Wavy lines indicate points of attachment to the peptide backbone via cysteine residues; in the case of [NiFeSe] hydrogenases, one cysteine, marked with an asterisk (*), is replaced by a selenocysteine. GMP = guanylyl monophosphate.

ligands is essential to allow the first-row transition elements to adopt the low oxidation states seen in the catalytic cycle, allowing these readily available elements to mimic reactivity more commonly associated with Pt-type metals [57].

Not surprisingly, the three classes of hydrogenase are found to exhibit distinct catalytic properties. The [FeFe] hydrogenases are found in Bacteria and Eukarya [30] and are predominantly H_2 -producing enzymes in their physiological context. The [NiFe]-enzymes, the subject of this thesis, may play both H_2 -producing and H_2 -oxidising roles *in vivo* but generally show lower rates as well as more significant product inhibition for H_2 production relative to [FeFe] hydrogenases [58, 59]. These [NiFe] enzymes are more tolerant to inhibition by O_2 , forming reversibly-inactivated states upon reaction with O_2 [60]. Some [NiFe] hydrogenases are capable of sustained H_2 oxidation in the presence of high levels of O_2 ; the special properties of this sub-class of enzymes are discussed further in Chapter 3. In some organisms, the [NiFe] hydrogenase contains a selenocysteine residue in place of one of the cysteine residues; this is marked with an asterisk(*) in Figure 1.2. The [FeFe] enzymes tend to give higher turnover rates for H_2 production than for H_2 oxidation (a so-called ‘catalytic bias’ to H_2 production) but show the highest turnover frequencies of the three classes of enzyme in both directions [58] as well as the lowest activation barrier [59]. The Fe-Fe cluster is thought to cycle

through Fe(I)Fe(I), Fe(I)Fe(II) and Fe(II)Fe(II) states [61, 62], and a dithiomethylamine ligand is well-positioned to abstract a proton from the H₂-binding site, facilitated catalysis [63, 64]. The [Fe]-only hydrogenases are expressed by methanogenic archaea [56] and depend for activity upon the organic cofactor methenyltetrahydromethanopterin (methenyl-H₄MPT), whose carbocationic character makes it an excellent hydride acceptor [65] - this enzyme catalyses hydrogen splitting, but not the full oxidation to protons and electrons catalysed by [NiFe] and [FeFe] enzymes [30].

1.2.2 Classes of [NiFe] hydrogenase

The [NiFe] enzymes are subdivided into four classes. Class I, the membrane-bound H₂-uptake enzymes, include Hyd-1 and Hyd-2 from *E. coli* discussed in this thesis and facilitate respiration of H₂. Class II enzymes are found in the cytoplasm and include the cyanobacterial uptake hydrogenases as well as the H₂-sensing hydrogenases, those whose role is not to catalyse H₂ cycling but to regulate gene expression in response to H₂ levels. Class III enzymes often catalyse reversible H₂ cycling *in vivo*, mediating exchange of reducing equivalents between H₂ and a soluble redox cofactor such as NAD or the methanogenic electron carrier known as factor 420. Class IV enzymes are also known as the membrane-associated H₂-evolving hydrogenases or energy-converting hydrogenases, a group which includes Hyd-3 of *E. coli*, the subject of Chapter 5 of this thesis. These enzymes are found in direct complex with other redox proteins including formate dehydrogenase, carbon monoxide dehydrogenase and acetate dehydrogenase [66].

1.2.3 Catalytic cycle of [NiFe] hydrogenases

No consensus has been reached regarding the unstable catalytic cycle intermediates in H₂ cycling in [NiFe] hydrogenases; a cycle proposed by Siegbahn and Hall [67] is presented here (Figure 1.3).

Electron Paramagnetic Resonance (EPR) , Mössbauer, Fourier Transform Infra-red

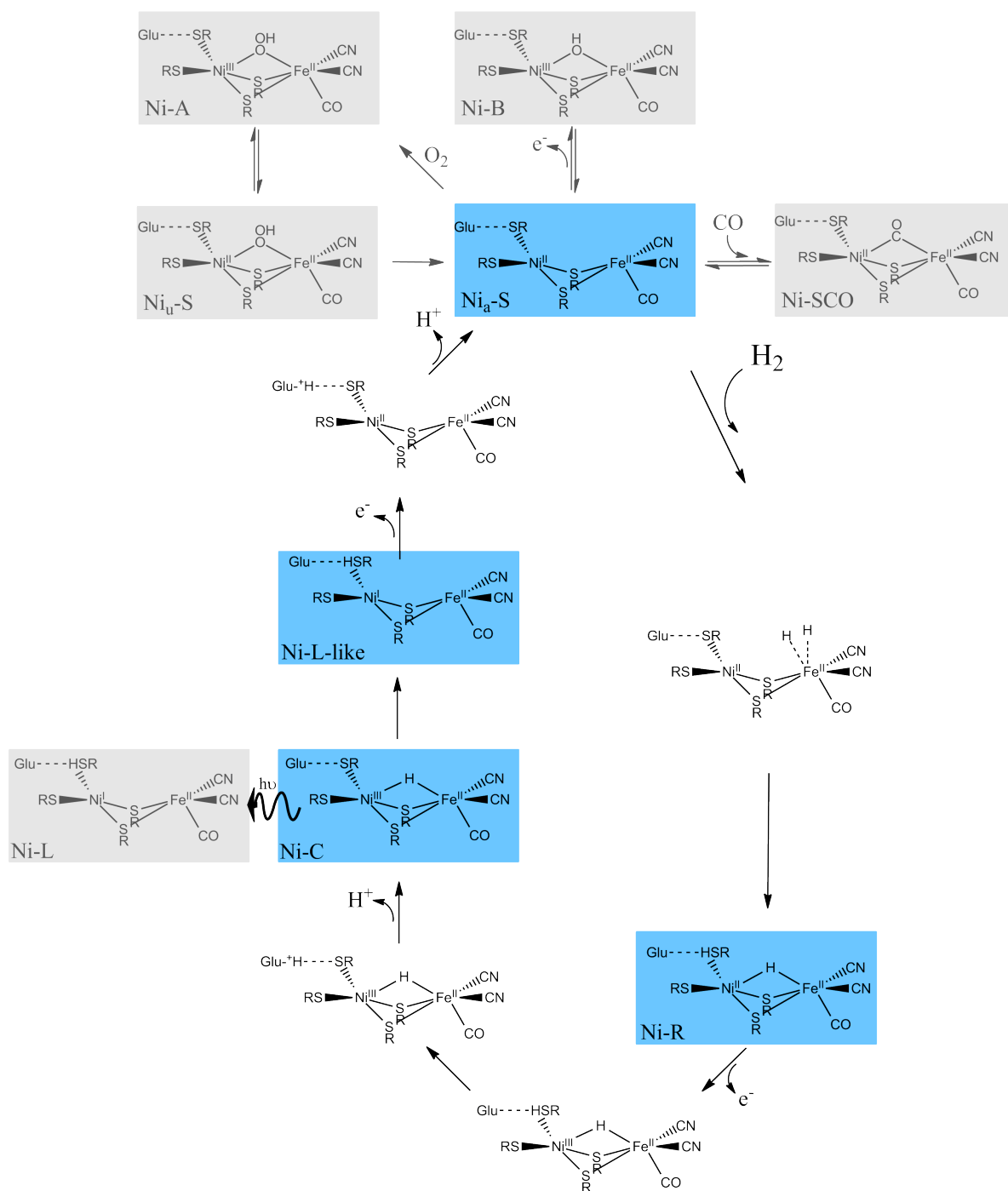


Figure 1.3: The catalytic mechanism of H_2 oxidation by [NiFe] hydrogenases, as proposed by Siegbahn et al. [67], with known inactive states shown. The spectroscopically-characterised states are indicated by a shaded rectangle, and states not directly involved in the catalytic cycle are shown in grey. The Ni-L state, formed by photolysis at low temperature, is displayed separately from the ‘Ni-L-like’ state observed for *E. coli* Hyd-1 due to a lack of consensus, for the time being, regarding the role played by Ni-L in the catalytic cycle.

(FT-IR) and X-ray absorption spectroscopies have all contributed to a characterisation of the observable states of the [NiFe] hydrogenase active site, identified by shaded rectangles in Figure 1.3. Observable states which are not believed to be involved in the catalytic cycle are shown in grey. Characterised states are generally organised, top-to-bottom, from most oxidised to most reduced; in other words, in the course of a potentiometric titration, Ni-S is generated upon reduction of Ni-A and Ni-B, etc.

For all observed states, the Fe ion has been shown to remain in the low-spin Fe(II) state by Mössbauer [68], EPR [69] and ENDOR [70] spectroscopy. The Ni is EPR-visible as an $S = 1/2$ species in the most oxidised states, Ni-A and Ni-B, as well as in the Ni-C state, which is two electrons more reduced than Ni-B. The Ni_a-S state, one electron more reduced than Ni-B, and the Ni-R state, three electrons more reduced than Ni-B, contain EPR-silent Ni(II) [71]. The stretch frequencies of the CN and CO ligands at the Fe offer an excellent handle to study the active site states that are invisible to EPR and to better understand changes at the Fe site. Crystal structures have also played a role in characterising the various enzyme states [69, 72, 73].

Recent studies of the O₂-tolerant hydrogenases from *E. coli* and *A. aeolicus* have brought into question the nature of the Ni-L state and its relationship to the catalytic mechanism of [NiFe] hydrogenases. Earlier studies identified Ni-L as a non-catalytic state, generated reversibly from the Ni-C state by illumination at low temperatures [74], and believed to be formed by photodissociation of the bridging hydride as H⁺, which would be expected to leave a formally Ni(I) species, although the formal oxidation state of the Ni has alternatively been proposed as Ni(III) [75]. Recent studies of the O₂-tolerant MBHs from *E. coli* and *A. aeolicus* show that, for these enzymes, Ni-L is observed even in the absence of low-temperature photolysis and suggest that the Ni-L state may have physiological relevance [76]. In the literature, this state is referred to as ‘Ni-L*’; here the state will be referred to as ‘Ni-L-like’ in order to avoid the resemblance to notation for an excited state. Chemical intuition tells us that a Ni-L type state would

be the intermediate generated by deprotonation of the Ni site during H₂ oxidation.

It is worth mentioning that, although specific bases are identified for proton abstraction, FT-IR and EPR studies show that different variants of the Ni-L [77, 78, 79, 80] and Ni-R [81, 82, 80] states, whose relative populations depend upon pH, suggest that several bases near the active site may play a role in accepting and donating H⁺.

The oxidised inactive states, Ni-A and Ni-B, contain bridging ligands at the dimetallic core, and are inactive. Ni-B, which is generated by both aerobic and anaerobic oxidation, contains a bridging hydroxide ligand [72]; this state is rapidly reactivated to re-enter the catalytic cycle when the potential is lowered. Conversely the Ni-A state, which contains a bridging peroxide [68, 73] and is believed to be generated by reaction of O₂ at the active site [74], is very slow to reactivate [83, 60]. This difference is important to O₂-tolerance, discussed further in chapter 3. Reduction of the Ni-A state rapidly gives rise to an EPR-silent Ni(II) state, termed Ni_u-S ('u' for 'Unready') that is inactive and can be reoxidised to the Ni-A state; reactivation of Ni_u-S to the active Ni_a-S is a slow, irreversible process and involves removal of the bridging peroxide ligand [84].

Carbon monoxide is a classical inhibitor of hydrogenases and a high tolerance to inhibition by CO has become a defining characteristic of O₂-tolerant [NiFe] hydrogenases. CO binds reversibly to the Ni_a-S state [85, 86].

1.2.4 A protein scaffold for Hydrogenases

A protein structure, often made up of a large and a small subunit [66], provides a scaffold for the cofactors mediating electron transfer and catalysis, holding these cofactors in place by ligation with cysteine, selenocysteine, histidine and glutamate residues. In addition to ligating the redox cofactors, this peptide-based frame provides well-placed acidic and basic groups to facilitate catalysis. Figure 1.4A shows the acidic and basic groups of *E. coli* Hyd-1 believed to be important for hydrogenase catalysis, including Glu-28, the analogue of which was shown to be essential to H⁺ transport in the [NiFe]

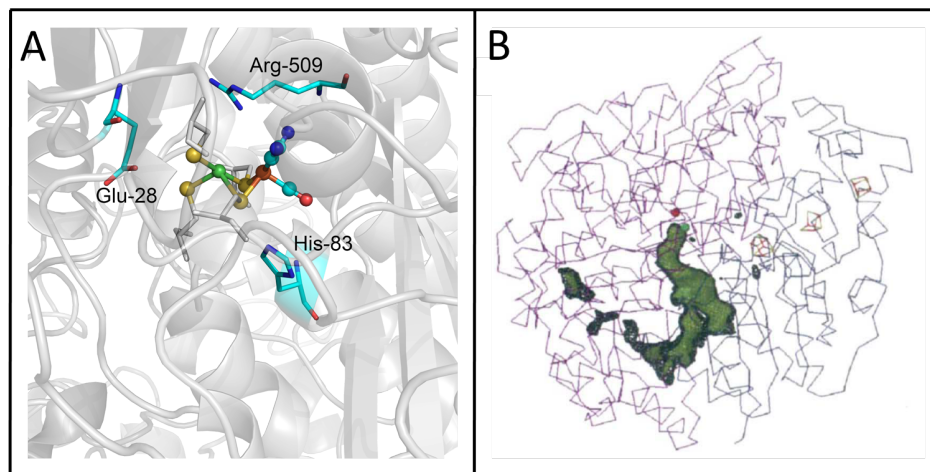


Figure 1.4: The protein scaffold provides structure, protection and proton transport. A) The active site of *E. coli* Hyd-1 with acidic and basic groups Glutamate-28, Histidine-83 and Arginine-509 thought to contribute to catalytic efficiency B) An H₂ probability map for the [NiFe] hydrogenase from *D. gigas*, based on X-ray crystallography of Xe-saturated enzyme molecules and molecular dynamics simulations. The large and small subunits are shown in magenta and blue, respectively, and the active site Fe and Ni are shown as red and green spheres respectively. FeS clusters are displayed in stick form. Figure reproduced from Montet *et al.* [88]

hydrogenase from *D. fructosovorans* [87].

The bulky protein scaffold also protects the enzyme cofactors from exposure to water and O₂; organometallic model complexes of hydrogenases often show sensitivity to both molecules [89], and recent work has shown that embedding organometallic complexes within 3D conductive structures can mimic this protective effect to some extent [90]. Molecular dynamics simulations have shown that small molecules such as H₂ readily permeate the protein structure of hydrogenases [91, 92]. X-ray crystallography studies using high pressures of Xe gas have shown that hydrogenases contain a hydrophobic ‘gas channel’ leading from the exterior of the protein to the Ni site [88], shown as an H₂ probability map in Figure 1.4B for the [NiFe] hydrogenase from *D. gigas*.

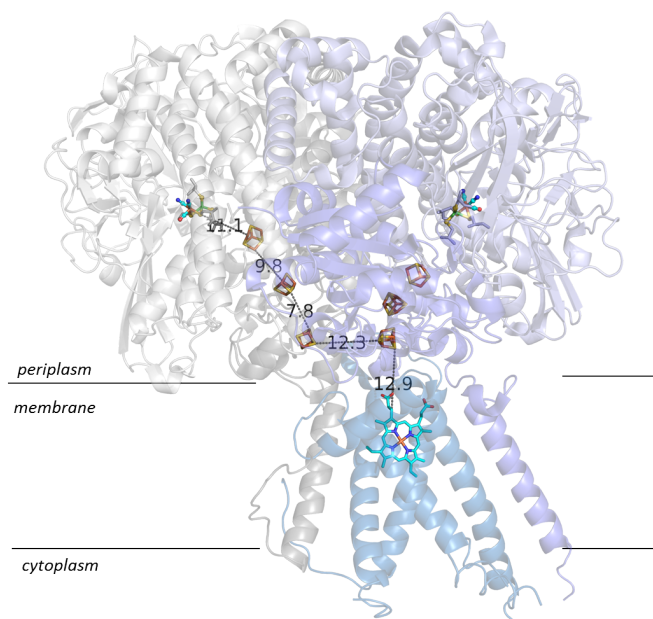


Figure 1.5: Electron transfer distances to and from the active site of *E. coli* Hyd-1, as revealed in a crystal structure of the P242C medial cluster mutant. Corner-to-corner distances between cofactors are displayed in Å with broken lines. PDB ID 4GD3 [93].

1.2.5 Iron-sulphur clusters transport electrons to and from the active site

Because the active site is buried deep within the protein scaffold, electron transfer to and from an external redox partner such as a membrane-bound cytochrome is impossible without intermediate redox cofactors; in the case of hydrogenases, Fe-S clusters play this role. Type-I hydrogenases, such as Hyd-1 and Hyd-2 from *E. coli*, normally have three FeS clusters, contained in the small subunit, that link the active site to the protein surface; these are named according to their relative position with respect to the active site as the proximal, medial, and distal clusters. Recent publication of the crystal structure of dimeric Hyd-1 in complex with a cytochrome subunit sheds light on the pathway of electron transfer for this enzyme in the cell (see Figure 1.5) [93]. A single cytochrome subunit accepts electrons from the distal clusters of both heterodimeric subunits, and is itself in turn oxidised by the membrane-bound quinone pool, although it is suggested that one cytochrome per hydrogenase is likely to be present *in vivo*. The relatively short distance between adjacent distal clusters suggests that electron transfer between these

clusters is likely to occur.

A series of FeS clusters within electron transfer distance has often been analogised as a ‘molecular wire’ connecting the surface to the active site. Recent work on hydrogenases [94, 95, 96, 59] and other proteins [97] suggests that, far more than being a simple conductive filament, these chains in fact contribute to the properties of the catalysts that they ‘wire’ into the cellular grid. The roles played by FeS clusters in determining the properties of the hydrogenases of *E. coli* is a major theme explored in this thesis.

1.3 Hydrogen metabolism in *Escherichia coli*

1.3.1 The hydrogenases of *E. coli*

The conversion of formate to H_2 by *E. coli* was first demonstrated over 100 years ago [98]; H_2 oxidation activity was demonstrated in the bacterium a few years later [99]. Some fifty years passed before three hydrogenase enzymes, immunologically distinct [100] and expressed under different conditions [101] were discovered and named, these are shown in Figure 1.6. Hyd-1 and Hyd-2 are Class I enzymes, membrane-bound H_2 -uptake respiratory hydrogenases that couple H_2 oxidation with quinone reduction [102]. The large subunits, HyaB and HybC, harbour [NiFe] cofactors while the small subunits, HyaA and HybO, each contain three FeS clusters. Each small subunit carries a C-terminal transmembrane α -helix that serves to anchor the protein to the membrane [103]. Quinone reduction occurs at a *b*-type cytochrome with four membrane-spanning domains [104, 93], termed HyaC, in Hyd-1. In Hyd-2, a 16Fe ferredoxin subunit, HybA, is thought to pass electrons to the HybB subunit, proposed by some to be a *b*-type cytochrome [105, 106] though that has been challenged on the basis of sequence homology [103] and is yet to be structurally resolved. Although the primary role of Hyd-2 seems to be H_2 oxidation, studies have shown that Hyd-2 also produces H_2 at low levels under certain conditions [107, 108]. Hyd-1 has the rather unique property of being O_2 -tolerant,

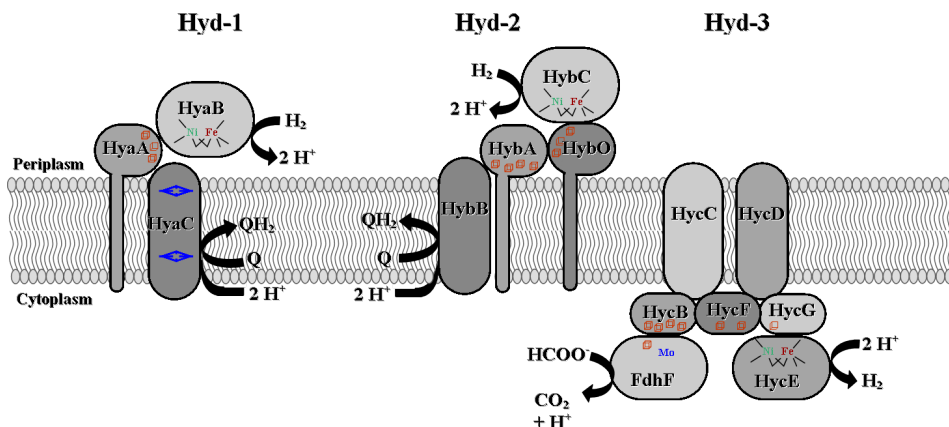


Figure 1.6: The hydrogenases of *E. coli*. In each case, the large subunit is indicated by the presence of a [NiFe] cofactor, and FeS clusters are represented as orange cubes. Hyd-1 interacts with the quinone pool via a *b*-type cytochrome, HyaC, believed to contain two haem groups, shown as blue diamonds. Hyd-2 contains a 16Fe ferredoxin, hybA, as well as a second membrane-bound subunit whose nature is debated, hybB. Orientation of the quinone-binding sites is not known for Hyd-1 or Hyd-2, but is discussed below. The FHL complex directly couples formate oxidation with H^+ reduction; the formate dehydrogenase enzyme FdhH contains a molybdenum-containing cofactor.

a property which will be discussed in Chapter 3.

Hydrogenase-3 of *E. coli* is part of the formate-hydrogen lyase (FHL) complex, and is a member of the Class IV hydrogenases, known as energy-converting hydrogenases (Ech), made up of complexes containing five to six subunits and bearing sequence homology to Complex I [109]. The complex is responsible for nearly all H_2 production in *E. coli* under a wide variety of conditions tested [16]. The formate dehydrogenase subunit, FdhF contains a molybdopterin guanine dinucleotide (MGD) cofactor and catalyses the oxidation of formate to CO_2 , H^+ and electrons. The electrons are passed via the FeS clusters of the many subunits of the complex to reach the hydrogenase large subunit, HycE, where they are used to reduce H^+ to H_2 . The five subunit functional complex is cytoplasmically-oriented and is membrane-associated through its interaction with the membrane-integral subunits HycC and HycD [110].

A fourth hydrogenase, encoded by the *hyf* operon, has been discovered in the *E. coli* genome. This operon seems to encode a second FHL complex (FHL-2), bearing close sequence similarity to the Hyd-3 FHL complex, with an additional three membrane-

bound components homologous to Complex I subunits [111]. Expression of the *hyf* operon seems to be at a very low level [112, 113], although more recent reports have suggested that the enzyme does play an important role under certain conditions with *hyf*-dependent H_2 production observed mostly at alkaline pH [114, 108]. The subunit configuration and the physiological role of the enzyme are not well understood, and this complex has therefore been left out of Figure 1.6. For the purpose of this thesis, references to the FHL complex, unless otherwise stated, refer to the FhlF-HycBCDEFG complex, elsewhere referred to as FHL-1.

1.3.2 Expression of hydrogenases in *E. coli*

Generally speaking, Hyd-1 and Hyd-2 are H_2 -oxidisers, while Hyd-3 and Hyd-4 are H_2 -producers. Unlike many organisms possessing O_2 -tolerant enzymes [115, 116], the expression of all *E. coli* hydrogenases is inhibited by O_2 [117, 118]. The transcriptional regulator *fnr* plays a key role in inducing hydrogenase expression under anaerobic conditions [101], and other studies have also implicated the ArcA and AppY systems in anaerobic induction or derepression [119, 120, 117]. When *E. coli* is grown anaerobically on non-fermentable carbon sources, exponential phase growth is accompanied by expression of Hyd-2 [100, 101, 117], and deletion of the *hyb* operon eliminates the ability of *E. coli* to grow on H_2 fumarate medium [105], confirming that *hyd-2* plays a role in respiration. The expression of Hyd-2 is repressed by nitrate via the NarX/L and NarQ/P systems [117]; nonetheless, H_2 -dependent nitrate respiration has been observed in *E. coli* [121], a process which can be mediated by either Hyd-1 or Hyd-2, but which only supports growth when Hyd-2 is the oxidising enzyme [122]. Hyd-2 expression seems also to be catabolite repressed [117].

In the absence of O_2 for aerobic respiration, or of other electron acceptors (fumarate, NO_3^- , NO_2^- , sulfur oxides) for anaerobic respiration, *E. coli* must resort to mixed-acid fermentation, a scheme that produces at most 3 moles of ATP per mole of glucose

consumed [123]. During strictly defined fermentation, all ATP is derived from substrate-level phosphorylation, and a transmembrane H^+ gradient is created by ATP hydrolysis. However, even during mixed-acid fermentation, several membrane-bound oxidases and reductases are functional [124], including Hyd-1.

Under anaerobic and microaerobic conditions, the pyruvate dehydrogenase complex that acetylates coenzyme-A bound for the citric acid cycle with concomitant NAD^+ reduction is replaced by the pyruvate formate lyase complex. This complex acetylates coenzyme A without NAD^+ reduction to produce formate instead of CO_2 and H_2 . Formate is exported from the cell as a waste product and builds up, acidifying the media [125]. When the periplasmic pH is at or below pH 6.8, formate is reimported into the cell [15]. Formate oxidation is preferentially coupled with oxygen or nitrate respiration [126, 127] via alternative formate dehydrogenases, but in the absence of these electron acceptors, formate builds up to a level sufficient to stimulate production of the transcriptional activator FhlA, which enables transcription of the formate hydrogen lyase complex [128, 129]. Thus, it is not surprising that the expression of FHL is suppressed in the presence of nitrate and promoted at low pH [130]. The expression of Hyd-1 (but not of Hyd-2) is also stimulated at low pH [131, 124] and in the presence of formate [101, 131]. This connection suggests the possibility that Hyd-1 may in some cases serve to reoxidise the H_2 produced by the FHL.

Each of the hydrogenases of *E. coli* is transcribed in a σ -54 dependent manner [132].

1.3.3 Energetics of H_2 -cycling in *E. coli*

The high sequence homology between the two H_2 -oxidising hydrogenases, Hyd-1 and Hyd-2, and between the two H_2 -producing formate-hydrogen lyase complexes containing Hyd-3 and Hyd-4, raises the question of the energetic advantage afforded to the bacterium by differential expression of the isoenzymes under varying conditions. Although this can be a difficult question to address, mutational studies combined with

assessment of the isoenzymes' properties allows for some predictions to be made. A fundamental question for both Hyd-1 and Hyd-2 is the site of quinone binding, a factor that determines whether or not turnover contributes to the membrane electrochemical gradient. H₂-oxidation releases one proton per electron, while quinone reduction takes up one proton per electron, thus, if H₂ oxidation occurs at the periplasmic face and quinone reduction occurs at the cytoplasmic face, the reaction contributes one proton per electron to the H⁺ gradient without any direct pumping mechanism; this type of 'redox-loop' was first described for the formate hydrogenase-N in *E. coli* [133, 134]. If both the hydrogen oxidase and the quinone reductase reactions take place on the same side of the membrane, no energy is stored. Sequence analysis of the HyaC subunit, as well as more recent crystallographic analysis [93] strongly suggest that this is a di-haem cytochrome *b*, despite only one haem being present in the crystallised form; cytochrome *b* proteins are often implicated in redox loops [135]. Although original sequence analysis also predicted the hybB subunit to be a di-haem cytochrome *b* [105, 106], other studies [103] challenge that assertion, suggesting that the subunit is rather more similar to the cytochrome *c*₃ known to interact with hydrogenases from sulphate-reducing bacteria. *E. coli* is known to grow on H₂-fumarate media, an ability which is reliant on a hydrogenase-mediated redox loop, since the fumarate reductase of *E. coli* does not contribute to the electrochemical gradient [136, 135]. Mutants in which Hyd-2 activity is knocked out show little to no growth on H₂-fumarate media [103].

The expression patterns of Hyd-1 and Hyd-2 differ sufficiently that one would suggest they should play specialised roles in the physiology of the cell. To summarise, Hyd-2 is expressed maximally during exponential phase and at high pH, in a manner independent of formate, while Hyd-1 is expressed during stationary phase and at low pH, and is upregulated by formate. In terms of physiological properties, Hyd-1 is unique from Hyd-2 in some interconnected ways [44]: Hyd-1 is capable of sustained catalysis under O₂, while Hyd-2 is O₂-sensitive. Hyd-1 is seen to require an overpotential for H₂-oxidation,

and undergoes oxidative inactivation at higher potentials than does Hyd-2 (discussed in-depth in Chapter 3). These differences led Lukey *et al.* [44] to speculate that Hyd-1 is an enzyme optimised for activity under microaerobic conditions, in which the cell potential is increased [44]. This interpretation was called into question by Volbeda *et al.* [93] who rightly point out that Hyd-1 expression is strongly inhibited in the presence of O₂. Thus, although it may be possible that Hyd-1 mediates H₂-dependent aerobic respiration, the expression profile does not suggest this to be its main function [118].

The localisation of the FHL at the membrane has led some [137] to suggest that the disproportionation of formate to H₂ and CO₂ may serve to drive proton translocation. Others suggest that the additional subunits of the *hyf* complex, which are absent in FHL-1 and are homologous to subunits of Complex I, may be responsible for H⁺ pumping [111]. H₂ production in the cytoplasm, followed by periplasmic H₂ oxidation and cytoplasmic quinone reduction *would* serve to contribute to the PMF, provided an electron acceptor were available to reoxidise the quinone pool; this could explain the concomitant expression of FHL and Hyd-1. FHL has been shown to be important to the acid tolerance of *E. coli* under anaerobic conditions [138], suggesting that one of the functions of this enzyme may in fact be the detoxification of formate.

1.4 Electron transfer in proteins

The challenge of carrying electrons to and from the active site of a redox protein can be accomplished by a variety of cofactors, most commonly haem groups or FeS clusters. Embedding these cofactors in a peptide medium, sheltered from H₂O, substantially lowers the reorganisation energy, λ upon reduction or oxidation [139]. Calculations and measured rates of electron transfer suggest that, for closely spaced redox cofactors (<14 Å edge-to-edge distance) in a protein environment, rapid electron tunneling occurs between cofactors at rates faster than active site turnover, irrespective of the particular

values of ΔG and λ [140]. This was taken to suggest that, for cofactors without a solvent shell, little to no selective pressure exists to tune the properties of redox cofactors or the protein environment that separates them to increase rates of electron transfer; simply placing cofactors sufficiently close to one another ensures robust electron transfer [140]. But this conclusion is seemingly at odds with the rather precise up-and-down potential steps seen for cofactor chains in many oxidoreductase enzymes (for examples see [140, 141]), a property which suggests a great deal of selective pressure. This pattern is still not understood, but it seems that the selective pressure to fine-tune properties of redox cofactors is not merely for the optimisation of electron transfer rates.

1.4.1 Iron-Sulphur Clusters

Electron transfer in hydrogenases relies upon redox cofactors made up of clusters of iron and sulphide. These complexes, which form spontaneously in reducing environments including at hydrothermal vents, are thought to be among the most ancient bioinorganic complexes [46, 142, 48]. Several basic forms of FeS cluster are known, shown in Figure 1.7. Ligation of the clusters, most often by cysteine or histidine residues, protects them from oxidation by O_2 and lowers solvent reorganisation energies for reduction or oxidation.

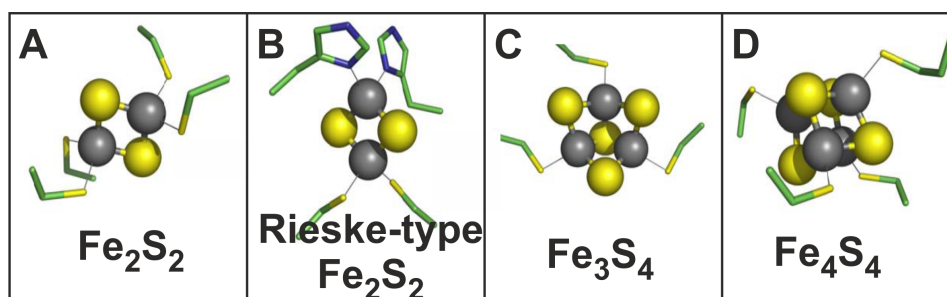


Figure 1.7: Four common types of Fe-S clusters found in proteins. Adapted from [143].

Evolution has coopted these structures to serve other functions: the redox-sensitivity of clusters may be used to coordinate redox-dependent changes in protein expression [144], or clusters may be used to sense intracellular Fe levels [145]. Modified FeS clus-

ters may also play a catalytic role, for example in CO oxidation in the Ni-containing carbon monoxide dehydrogenase [146] or for disulphide reduction [147]. Studies of the P-cluster of nitrogenase [148, 149] have revealed that this special cluster is engineered with conformational flexibility that allows ATP hydrolysis to contribute to the reducing power of an electron stored at the cluster, a feature that is necessary to drive the very difficult reduction of N_2 .

Recent work on hydrogenase enzymes [94, 95, 96] has suggested that the FeS clusters of these enzymes may play an important role in determining some of the properties of these enzymes, and this thesis aims to expand our understanding of the variations in catalytic properties across different [NiFe] hydrogenases, with a particular focus on the FeS clusters of the enzymes.

1.5 Topics addressed in this thesis

This thesis addresses the importance of electron transfer in determining the properties of hydrogenase enzymes from *E. coli*. Chapter 3 provides an in-depth characterisation of Hyd-1 from *E. coli*, with a special emphasis on the properties associated with O_2 -tolerance in membrane-bound hydrogenases. Detailed investigations of these properties suggest that differences in electron transfer between the H_2 -oxidising isoenzymes of *E. coli* underlie the significant catalytic differences observed for these two enzymes, and the strict correlation between these properties and O_2 -tolerance in MBHs from other organisms suggests that this difference in electron transfer properties contributes to the phenomenon of O_2 -tolerance. Chapter 4 presents experiments aimed at assessing rates of electron transfer by direct injection of photoexcited electrons. Using a method pioneered by Harry Gray [139], we have used click-chemistry to covalently attach a Ru-based photosensitizer to an inserted residue near the enzyme distal cluster. Chapter 5 details the purification of intact FHL complex from *E. coli*, over a hundred years

after the first observation of its activity. Characterisation of Hyd-3 from this complex reveals an enzyme with properties different from what has previously been seen for [NiFe] hydrogenases, and in some ways more similar to [FeFe] enzymes, an observation that is in line with the role of Hyd-3 as an H₂-producing enzyme but that raises the question of how a [NiFe] active site can be tuned to give properties more resembling those of an [FeFe] hydrogenase.

Chapter 2

Methods

2.1 Molecular Biology Methods

All hydrogenase proteins were expressed at wildtype levels from the native locus in order to avoid issues arising from the complicated regulation and maturation of these enzymes. Addition of a polyhistidine tag to the expressed sequence of *E. coli* Hyd-1, Hyd-2 and Hyd-3, yielding strains FTH004, FTH0013, and MG059e1, respectively, was carried out by Frank Sargent and Tracy Palmer (University of Dundee). These polyhistidine tags allowed for rapid purification of the enzymes by adsorption to a Ni-affinity column. In the case of Hyd-1 and Hyd-2, the hexa-histidine tag has been inserted at the extreme C-terminus of the hydrogenase small subunit, *hyaA* and *hybO*, respectively; this represents a standard approach to affinity tagging. A unique approach was required for affinity-tagging Hyd-3, due to the termini of both of the subunits playing important roles either structurally or for the maturation of the FHL complex. Thus, the hydrogenase large subunit, *hycE*, was instead modified by the addition of a decahistidine tag immediately after glycine-83. All proteins studied in this thesis were harvested from FTH004, FTH0013, MG059e1, or derivatives thereof (see table 2.1). Note that all amino acid numbering in this thesis refers to post-translationally-modified peptides (pre-modified numbering in parentheses where relevant).

2.1.1 Cell strains, plasmids, and oligonucleotides used in this thesis

Plasmids used in this thesis are detailed in Table 2.2.

Oligonucleotide primers used for PCR in this thesis are presented in Table 2.3. All primers were ordered from Life Technologies (Invitrogen).

Strain Name	Description	Source
<i>E. coli</i> DH5 α	<i>endA1 supE44 thi-1 hsdR17 recA1 gyrA96 relA1 δ lacU169 φ80 lacZδ M15</i>	[150]
<i>E. coli</i> XL-2 Blue	<i>endA1 supE44 thi-1 hsdR17 recA1 gyrA96 relA1 lac F proAB lacI^q ZΔM15 Tn10 (Tet^r)Amy Cm^r</i>	Stratagene
<i>E. coli</i> MC4100	F ⁻ [araD139] _{B/r} Δ (argF-lac)169 *lambda ⁻ e14 ⁻ flhD5301 Δ (fruK-yeiR)725 (fruA25) $\ddagger$ relA1 rpsL150(strR) rbsR22 Δ (fimB-fimE)632(::IS1) deoC1	[151]
FTH004	As MC4100, <i>hyaA</i> 6 $\times$ His	[103]
FTH013	As MC4100, <i>hybO</i> 6 $\times$ His	[44]
MG059e1	As MC4100, <i>hycE</i> 10 $\times$ His, internal	This thesis
BJM01	As FTH013, <i>hybO</i> D239C	This thesis
BJM02	As FTH013, <i>hybO</i> E297C	This thesis
BJM03	As FTH013, <i>hybO</i> 310C	This thesis
BJM04	As FTH013, <i>hybO</i> V312C	This thesis
BJM06	As FTH013, <i>hybO</i> S333C	This thesis
BJM08	As FTH013, <i>hybO</i> R373C S374C	This thesis
BJM09	As FTH013, <i>hybO</i> V312C S319C	This thesis
MLJL1	As FTH004, <i>hyaA</i> C20S	This thesis
MLJL2	As FTH004, <i>hyaA</i> C115S	This thesis

Table 2.1: Bacterial Strains used in this thesis.

Plasmid Name	Description	Source
pBluescript	Amp ^R	Stratagene, La Jolla, USA
pMAK705	Cam ^R	[152]
pML12	pBluescript carrying <i>EcoRI-EcoRI hybO</i> +500 bp flanks from <i>E. coli</i> FTH013	[44]
pBJM01	As pML12, <i>hybO</i> D239C	This thesis
pBJM02	As pML12, <i>hybO</i> E297C	This thesis
pBJM03	As pML12, <i>hybO</i> 310C	This thesis
pPW01	As pMAK-705, carrying the <i>XbaI/KpnI</i> 2.1kbp fragment from pML12	This thesis
pMAK_BJM01	As pPW01, <i>hybO</i> D239C	This thesis
pMAK_BJM02	As pPW01, <i>hybO</i> E297C	This thesis
pMAK_BJM03	As pPW01, <i>hybO</i> 310C	This thesis
pMAK_BJM04	As pPW01, <i>hybO</i> V312C	This thesis
pMAK_BJM06	As pPW01, <i>hybO</i> S333C	This thesis
pMAK_BJM08	As pPW01, <i>hybO</i> R373C S374C	This thesis
pMAK_BJM09	As pPW01, <i>hybO</i> V312C S319C	This thesis
pMLJL1	As FTH004, <i>hyaA</i> C20S	This thesis
pMLJL2	As FTH004, <i>hyaA</i> C115S	This thesis

Table 2.2: Plasmids used in this thesis.

Primer Name	Sequence	Function
hybO_F	5'-TATTGAATTCCGCTATATATGA CAATATATAGCGAATGAGG-3'	Sequencing and Colony PCR
hybO_R	5'-TATTGAATTTCGCGCACCAAACGG GTTGTTGTAGTCGTACTTCG-3'	Sequencing and Colony PCR
hybOseq hybOexR	5'-GCCGCTGCAGAGATGGCCGAATCGG-3' 5'-ATAATCTAGATTAGTGATGGTGA TGGTGATGAGATCTTTC-3'	Sequencing Sequencing
hybO_E297C_F	5'-CCCTTGCTATGGCTGTAAC TGCG AAGGTATCGGCTTCC-3'	Quick-change PCR
hybO_E297C_R	5'-GGAAGCCGATACCTTCGCAGTT ACAGCCATAGCAAGGG-3'	Quick-change PCR
hybO_310C_F	5'-GGCATCCATCAGCTTTGCGCC AACGTCGAAAATCAAAC TCC-3'	Quick-change PCR
hybO_310C_R	5'-GGAGTTTGATTTTCGACGTTGG CGCAAAGCTGATGGATGCC-3'	Quick-change PCR
hybO_V312C_F	5'-GCTTGCCAAC TGCGAAAA TCAAAC TCCGCG-3'	Quick-change PCR
hybO_V312C_R	5'-CGCGGAGTTTGATTTTC GCAGTTGGCAAGC-3'	Quick-change PCR
hybO_S319C_F	5'-CAAAC TCCGCGTTGTCAGAAACCGG-3'	Quick-change PCR
hybO_S319C_R	5'-CCGGTTTCTGACAACGCGGAGTTTG-3'	Quick-change PCR
hybO_S333C_F	5'-GGCGGCAACGTCTGTG CAGGCGCTATTGG-3'	Quick-change PCR
hybO_S333C_R	5'-CCAATAGCGCCTGCACA GACGTTGCCGCC-3'	Quick-change PCR
hybO_R373C_S374C_F	5'-CTCACGGGGAGAATGTT GTCATCACCATCACC-3'	Quick-change PCR
hybO_R373C_S374C_R	5'-GGTGATGGTGATGACAAC ATTCTCCCCGTGAG-3'	Quick-change PCR
hybC_F	5'-TATTGAATTCCTCGAT TGTCATCTTTGAAG-3'	Sequencing and Colony PCR
hybC_R	5'-TATTGAATTCCTTGCT ATCTCTTCAGTCAT-3'	Sequencing and Colony PCR
hybC_C21V_F	5'-GGGGCATTTACGCATCGATG TCGAAATCGAAAATGGCGTCG-3'	Quick-change PCR
hybC_C21V_R	5'-CGACGCCATTTTCGATTTTCG ACATCGATGCGTAAATGCCCC-3'	Quick-change PCR
hybCseqBM1 hybCseqBM2	5'-GCTGAAAGGCGTTTCGACCTGGC-3' 5'-GTCTGACGACGGGAA ATATTCCTGGG-3'	Sequencing Sequencing

Table 2.3: Oligonucleotide primers used in this thesis.

2.1.2 Routine methods in cell growth and culture

Bacterial cells were grown in Luria-Bertani broth, consisting of 10g/L peptone, 5g/L yeast extract, and 5g/L NaCl, prepared in deionised water and autoclaved. For solid media, 1.5% (w/v) agar (Melford) was added to the media prior to autoclaving. Antibiotics were made up in 1000* concentrated stocks, filter-sterilised, and stored at -20 °C. Where relevant, Chloramphenicol (Melford) was used at a final concentration of 25 $\mu\text{g}/\text{mL}$, denoted here as 25Cm, while Carbenicillin was used at a final concentration of 100 $\mu\text{g}/\text{mL}$, denoted here as 100Cb.

Preserving cell strains as glycerol stocks

Glycerol stocks of cell strains were prepared according to the following protocol and stored at -80 °C.

- A 5mL LB broth culture, containing antibiotic where appropriate, was inoculated from a single colony and grown overnight at 37 °C with shaking.
- 1 mL of this growth was used to inoculate 5mL fresh liquid LB, with antibiotic where appropriate, and the culture was grown at 37 °C with shaking to an $\text{OD}_{600\text{nm}}$ of 0.6 cm^{-1} .
- This culture was placed on ice for 15 minutes.
- Cells from 1mL of this culture were precipitated by centrifugation at $3000\times g$ for 5 minutes, and supernatant was poured off.
- The cell pellet was resuspended in 1mL pre-chilled 50% glycerol (Fisher Scientific) solution and left on ice for 10 minutes.
- The cell suspension was flash frozen in liquid N_2 and stored at -80 °C.

2.1.3 Transformation of cells with plasmid DNA

Two methods were used for the preparation of competent *E. coli* cells: the RbCl method, and the Transformation and Storage Buffer (TSB) method. In general, the TSB method produces higher numbers of transformants; however, for some transformations the RbCl method produced transformants while the TSB method did not.

RbCl method [150, 153]

- A 5mL liquid LB culture was inoculated from a single colony and grown overnight at 37 °C with shaking.
- 1mL of this culture was used to inoculate a 50mL LB culture, which was grown at 37 °C with shaking to an OD_{600nm} of 0.4 cm⁻¹.
- The culture was cooled on ice for 30 minutes.
- Cells were pelleted by centrifugation at 3000×*g* for 5 minutes at 4 °C, and the supernatant was poured off.
- The cell pellet was resuspended in 30mL TFB I (Table 2.4), and incubated on ice for 15 minutes.
- Cells were pelleted by centrifugation at 3000×*g* for 5 minutes at 4 °C, and the supernatant was poured off.
- The cell pellet was resuspended in 2mL TFB II (Table 2.4), and incubated on ice for 15 minutes.
- Cell suspensions were divided into 50μL aliquots and flash frozen in liquid N₂, and stored at -80 °C.

Transformation and Storage Buffer (TSB) method [154]

- A 5mL liquid LB culture was inoculated from a single colony and grown overnight at 37 °C with shaking.
- 250μL of this culture was used to inoculate a 25mL LB culture, which was grown at 37 °C with shaking to an OD_{600nm} of 0.4 cm⁻¹.
- The culture was cooled on ice for 20 minutes.
- Cells were pelleted by centrifugation at 3000×*g* for 5 minutes at 4 °C, and the supernatant was poured off.
- The cell pellet was resuspended in 1mL pre-chilled TSB (table 2.4), and incubated on ice 10 minutes.
- Cell suspensions were divided into 50μL aliquots and flash frozen in liquid N₂, and stored at -80 °C.

Buffer name	Composition
TFBI	30mM KOAc, 100mM RbCl, 10mM CaCl ₂ , 50mM MnCl ₂ , 3mM [Co(NH ₃) ₆]Cl ₃ , 15% glycerol, titrated to pH 5.8 with acetic acid
TFBII	10mM MOPS, 10mM RbCl, 75mM CaCl ₂ , 15% glycerol, titrated to pH 6.5 with KOH
TSB	50mM MgSO ₄ , 5% (v/v) DMSO , 10% (w/v) PEG-6000 in LB

Table 2.4: Buffers used for inducing competence in *E. coli*.

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

Frozen competent cells were transformed by the following protocol:

- Frozen cells were thawed for 5 minutes on ice.
- Plasmid DNA was added to the cells (0.5 μ L in the case of mini-prepped plasmid, 2-5 μ L in the case of transformation with the products of a PCR or ligation), and cells were mixed by gently flicking the eppendorf tube in which they were held; the cell-plasmid mix was incubated on ice for 30 minutes.
- The cells were heat-shocked in a water bath at 37 °C for 5 minutes in the case of transformations with pMAK705, or at 42 °C for 30 seconds, in the case of transformations with other plasmids.
- Cells were returned to ice for 2 minutes, after which time 700 μ L of sterile antibiotic-free LB media was added.
- Cell cultures were grown at 30 °C for 1.5 hours, in the case of transformations with pMAK705, or at 37 °C for one hour, in the case of transformations with other plasmids.
- Cells were gently pelleted by centrifugation at 3000 $\times g$ for 5 minutes, and the supernatant was poured off.
- Pellets were resuspended in 50 μ L LB and plated on LB agar plates containing the appropriate antibiotic. Plates were incubated overnight at 30 °C in the case of transformation with pMAK705 and 37 °C in the case of transformation with other plasmids.

2.1.4 Routine methods in molecular biology

Plasmid DNA was purified from 5mL liquid cultures using QIAprep® Spin Miniprep kit or EconoSpin Prepacked Spin Columns, according to the manufacturer's instructions. All restriction enzymes were purchased from New England Biolabs and used according to the manufacturers' instructions. Ligations were carried out using T4 DNA Ligase (New

England Biolabs) according to the manufacturer's instructions. For ligation reactions, restriction enzyme-digested plasmids were 5'-dephosphorylated with New England Biolabs Antarctic Phosphatase prior to ligations, to reduce the incidence of self-ligation; the Antarctic Phosphatase was heat-inactivated according to the manufacturer's instructions prior to mixing with insert DNA for ligation.

Agarose gel electrophoresis of DNA

Agarose gels (1%) were used to separate DNA fragments by size. Gels were cast by melting a solution of agarose in TAE buffer (40mM Tris, 40mM glacial acetic acid, 1mM EDTA, pH 8.3) and adding 0.005% (v/v) SYBR Safe® DNA Gel Stain (Invitrogen) to allow visualisation of DNA under UV light. Samples were prepared for loading by addition of 6* DNA loading dye (New England Biolabs), and a 1kb DNA ladder (New England Biolabs) was run as a standard. Gels were submerged in TAE buffer and run at 100V for 60-80 minutes. Gel slices were extracted using a scalpel and DNA was recovered using the QIAquick Gel Extraction Kit (QIAGEN), or EconoSpin Prepacked Spin Columns, according to the manufacturers' instructions.

DNA Sequencing

Sequencing of plasmid-based mutations was carried out by the Source BioScience sequencing service on purified plasmid using primers M13R and M13F, or gene-specific primers within the gene, to confirm insertion of the mutations and to affirm that the rest of the gene sequence was uncompromised. To confirm correct insertion of the mutation onto the chromosome, colony PCR was carried out (section 2.1.4) and the PCR product was run on an agarose gel and purified (section 2.1.4) before sequencing with gene-specific primers.

Polymerase Chain Reaction

Polymerase Chain Reaction allows for rapid and accurate replication of fragments of DNA, as well as the insertion of mutations by the use of engineered primer sequences. PCR was used in this thesis for the amplification and mutation of hydrogenase gene sequences in two plasmids, pBluescript and pMAK705. PCR in pBluescript is very standard and reliable, however in many cases cloning of mutated sequences from pBluescript into pMAK705 for chromosomal integration proved extremely problematic. Therefore, in some cases, PCR was carried out directly on pMAK705 derivative plasmids; this reaction required significantly altered PCR conditions to account for the length of pMAK705 and for the seemingly low binding affinity of the primer to the target sequence.

Typical conditions for each of these types of reaction follow. Successful amplification of target DNA frequently requires modification of the concentration of many components of the reactions; therefore, the reaction mixtures are often annotated with a range of the concentrations of component used.

PCR of pBluescript derivative using KOD Hot Start DNA Polymerase

Component	Volume	Final Concentration
10* Manufacturer-supplied KOD Buffer I	5 μ L	
MgCl ₂ , 25mM	0.25 μ L	0.125 μ M
DMSO	0-5 μ L	0-10%
dNTPs, 2mM each	0.5 μ L	200 μ M each
Forward primer, 100 μ M	0.25-0.75 μ L	0.5-1.5
Reverse primer, 100 μ M	0.25-0.75 μ L	0.5-1.5 μ M
DNA template, mini-prepped	0.25 μ L	
KOD Hot Start Polymerase	0.4 μ L	
Molecular Biology Grade H ₂ O	To 50 μ L	

Table 2.5: Components in a 50 μ L KOD-based quick-change PCR

The temperature was cycled as follows:

94 °C	5 minutes	
94 °C	30 seconds	
60 °C	30 seconds	x35
72 °C	30 seconds/kbp	
72 °C	10 minutes	

Direct mutagenic PCR of pMAK705 derivatives using Herculanase DNA Polymerase

Carrying out mutagenic PCR directly on pMAK705 derivatives bypasses the need to ligate mutated gene products into empty pMAK705 for chromosomal mutagenesis, and also cuts out one sequencing phase from the protocol. As the ligation step into pMAK705 is extremely unreliable for unknown reasons, this is a very favourable prospect. However, direct PCR of pMAK705 is complicated by its relatively large size (5.5kb without insert), and success with this reaction is only achieved with very long, low-temperature extension phases. Herculanase II *Pfu* DNA polymerase (Agilent) seems to work more reliably for this reaction than does KOD Hot Start polymerase.

Component	Volume	Final Concentration
5* Herculanase buffer	10 μ L	2mM Mg ²⁺ , other components not specified
dNTPs, 5mM each	1 μ L	100 μ M each
Forward primer, 100 μ M	1 μ L	2 μ M
Reverse primer, 100 μ M	1 μ L	2 μ M
DNA template, mini-prepped	0.5 μ L	
Herculanase II Polymerase	1 μ L	
Molecular Biology Grade H ₂ O	To 50 μ L	

Table 2.6: Components in a 50 μ L Herculanase II-based quick-change PCR

The temperature was cycled as follows:

95 °C	2 minutes	
95 °C	30 seconds	
46 °C	1 minute	x16
68 °C	15 minutes	

Colony PCR using *Taq* polymerase

This technique is useful for one-step amplification of a region of chromosomal DNA for sequencing. In this technique, a single colony is picked from an agar plate and is resuspended in 100 μ L of molecular-grade H₂O with vortexing. This mixture serves as the source of template DNA for the reaction. W1 detergent, as well as the heat of the PCR cycle, ensure that cells are lysed and DNA released for the reaction.

Component	Volume	Final Concentration
10* <i>Taq</i> buffer	5 μ L	20mM Tris, 50mM KCl, pH 8.4
W1 detergent	2.5 μ L	0.05% polyoxyethylene ether
50mM MgCl ₂	1-2 μ L	1-2mM MgCl ₂
dNTPs, 2.5mM each	0.5-1 μ L	25-50 μ M each
Forward primer, 100 μ M	0.5-1 μ L	1-2 μ M
Reverse primer, 100 μ M	0.5-1 μ L	1-2 μ M
<i>Taq</i> polymerase	0.5 μ L	
Resuspended colony mixture	1 μ L	
Molecular Biology Grade H ₂ O	To 50 μ L	

Table 2.7: Components in a 50 μ L *Taq*-based colony PCR

The temperature was cycled as follows:

94 °C	5 minutes	
94 °C	30 seconds	
50 °C	30 seconds	x35
72 °C	30 seconds / 500 bp	
72 °C	7 minutes	

2.1.5 The pMAK cycle: homologous recombination for native expression of recombinant proteins

Chromosomal mutations were introduced by homologous recombination with a version of the gene shuttled by the pMAK705 plasmid, whose pSC101 origin of replication allows for selection of chromosomal integration [152] by modulating temperature. The procedure for generating chromosomal mutations is summarised in Figure 2.1.

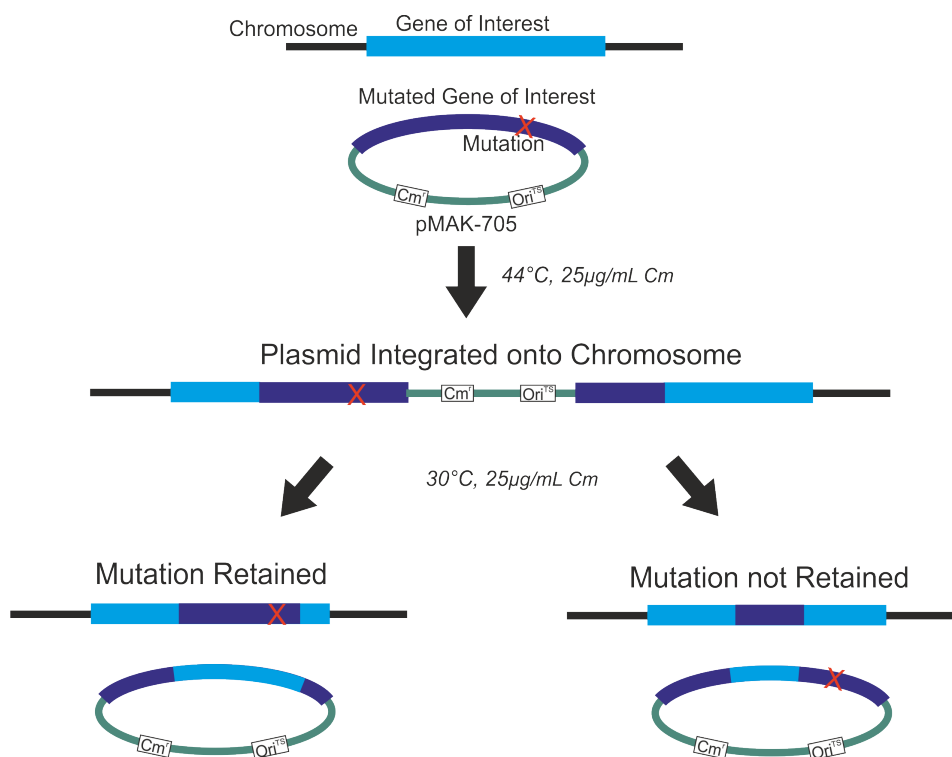


Figure 2.1: Homologous recombination in the pMAK cycle

First, a version of the gene was cloned into a vector by amplification with the polymerase chain reaction (PCR), digestion with restriction enzymes, and ligation into similarly-prepared sites in the vector. This step was carried out by Dr. Michael Lukey for both the *hybC* and *hybO* genes. Once this construct had been generated, specific site-directed mutagenesis was carried out by quick-change PCR of this plasmid, using complementary forward and reverse primers bearing the mutation. For some mutants, this mutagenesis was carried out in a pBluescript derivative (section 2.1.4), and the resulting gene was then excised using restriction enzymes and ligated into pMAK705.

In later experiments, the mutagenic quick-change PCR step was carried out directly on pMAK705 constructs carrying *hybO* and *hyaA* genes plus 500bp flanking regions (section 2.1.4). These unmutated pMAK705-based plasmids, known as pPW01 and pPW02, respectively, were constructed by P. Wulff.

Following successful construction of a pMAK705 derivative carrying the mutated gene of interest, the following protocol was followed to select for the two recombination events required to introduce the mutation onto the chromosomal copy of the gene:

- The pMAK derivative carrying the mutated version of the gene was transformed into the corresponding His-tagged strain (FTH004 or FTH013) by the 37/30 protocol described in section 2.1.3, plated onto LB+25Cm (25 μ g/mL chloramphenicol), and incubated at 30°C overnight.
- A single colony was used to inoculate a 5mL LB+25Cm liquid culture, which was grown overnight with shaking at 30°C.
- The overnight culture was diluted by a factor of 10^4 , plated onto LB+25Cm, and incubated at 44°C overnight to select for cells in which the plasmid was integrated into the chromosome. A control plate was made from a 10^7 factor dilution, plated onto LB+25Cm, and incubated at 30°C.
- Colonies from the 44 °C plates were picked into 5mL liquid LB+25Cm tubes; three tubes were grown, and each tube was inoculated with four different colonies. The cultures were grown overnight at 30°C with shaking.
- 10 μ L of each growth was subcultured into 10mL LB+25Cm and again grown overnight at 30°C.
- The next day, the flasks were again subcultured as above.
- The liquid cultures were streaked for single colonies on LB+25Cm plates and grown overnight at 30°C.
- Single colonies were inoculated into 10mL LB without antibiotics and grown overnight at 44°C with shaking; this was carried out in duplicate for each plate.
- The cultures were streaked for single colonies on LB without antibiotics and grown overnight at 44°C.
- Single colonies were replica-patched onto LB only and LB+25Cm plates and grown overnight at 30 °C.
- Colonies showing sensitivity to chloramphenicol were chosen for colony PCR of the gene of interest, carried out according to the protocol described in section 2.1.4. The resulting PCR product was purified by agarose electrophoresis and extraction with a QIAquick column (section 2.1.4). These gene fragments were then sent for sequencing.

2.2 Purification of Hydrogenase Enzymes

2.2.1 Aerobic Isolation of hydrogenases, Hyd-1, Hyd-2, and intact FHL from *E. coli*

His-tagged enzyme was purified by single-step affinity chromatography on a 5mL HisTrap column (GE Healthcare). This column is sepharose-based, and contains nitrilo-triacetic acid groups that provide partial coordination of Ni^{2+} ions. The purification of Hyd-1 and Hyd-2 from *E. coli* was carried according to the following protocol [44]:

- A glycerol stock of the relevant strain expressing a His-tagged hydrogenase was used to streak an LB agar (Melford) plate for single colonies.
- Single colonies were used to inoculate 5mL starter cultures of LB (Melford) broth.
- Each 5mL starter culture was used to inoculate 6L of growth medium. For Hyd-1 and Hyd-2 preparations, cells were grown in LB broth supplemented with 0.5% glycerol (Fisher Chemical) as carbon source and 0.4% w/v sodium fumarate (Alfa Aesar) as electron acceptor, filter-sterilised with a Steritop filter (Millipore). For Hyd-3 preparations, cells were grown in LB broth supplemented with 0.5% glucose (Duchefa Biochemie) as a carbon source.
- Cells were incubated at 37 °C without shaking. For Hyd-1 or Hyd-3, cells were grown to late stationary phase (12 hours after inoculation), while for Hyd-2 purification, cells were grown to late exponential phase (9 hours after inoculation).
- Cells were harvested by centrifugation at $3000\times g$ for 12 minutes and resuspended in Buffer 1 (see table 2.9), approximately 3.5mL for each litre of growth media used.
- Cells were lysed by three passages through a Constant Cell Disruption System (Constant Systems Limited).
- Unlysed cells were removed by centrifugation at $3000\times g$ for 12 minutes.
- Membranes were pelleted by centrifugation at $150,000\times g$ for 60 minutes and resuspended in Buffer 2 (table 2.9), approximately 6mL for each litre of growth media used.
- Triton X-100 detergent was added (Fisher Scientific) to a final concentration of 3% to solubilise membrane-bound components, and the mixture was stirred at 4 °C for one hour.
- Insoluble components were removed by centrifugation at $150,000\times g$ for 60 minutes.

- The supernatant was supplemented to 1mM DTT and 40mM imidazole and loaded onto a 5mL HisTrap HP column (GE Healthcare), pre-equilibrated with Buffer A (table 2.9) at a flow rate of 1.5mL/minute
- The column was washed with 100mL (20 column volumes) of Buffer A and eluted with a linear gradient from 100% Buffer A to 100% Buffer B (table 2.9) over 20mL.
- In the case of enzyme preparations for electrochemistry, the buffer was exchanged into Buffer C (table 2.9) by subsequent dilution and reconcentration using Vivaspin concentrator columns (Sartorius Stedim Biotech.) and centrifugation at $3000\times g$.
- In the case of preparations of Hyd-2 or mutants thereof for labelling with maleimido-compounds, the enzyme was exchanged into sulphido-free buffer (table 2.9) using a PD-10 size exclusion column (GE Healthcare) in an anaerobic glovebox ($[O_2]<2\text{ppm}$).
- In the case of preparations of Hyd-3, the enzyme was exchanged into EPR buffer (table 2.9) using a PD-10 size exclusion column (GE Healthcare) in an anaerobic glovebox ($[O_2]<2\text{ppm}$).
- Enzyme samples were flash-frozen and stored in liquid N_2 .

2.2.2 Anaerobic Isolation of Hyd-3 from *E. coli*

Unfortunately, the purification protocol used by Professor Sargent (University of Dundee) was not feasible on the scale of several mg of enzyme, which was needed for spectroscopic studies; therefore, a new protocol was optimised. This protocol was then modified for use in an anaerobic glovebox (Glovebox Technologies and M. Braun), as both the hydrogenase and the formate-dehydrogenase of the enzyme complex are thought to be O_2 -sensitive. Although every effort was made to minimise contact with O_2 , the centrifugation steps were carried out in sealed plastic bottles; thus, we must assume that the harvesting stage is carried out under microaerobic conditions ($[O_2]<50\text{ppm}$), while the remainder of the preparation was carried out under anaerobic conditions ($[O_2]<10\text{ppm}$).

The protocol is detailed below.

- A glycerol stock of MG059e1, expressing His-tagged FHL, was used to streak an LB agar (Melford) plate for single colonies.
- Single colonies were used to inoculate 5mL starter cultures of LB (Melford) broth.
- Each 5mL starter culture was used to inoculate 6L of growth medium. Cells were grown in LB broth supplemented with 0.5% glucose (Duchefa Biochemie) as

carbon source, filter-sterilised with a Steritop filter(Millipore). Cells were grown at 37 °C, without shaking, to late stationary phase (12 hours after inoculation).

- The growths were transferred to an anaerobic glovebox, and all further steps were carried out under anaerobic conditions, except during centrifugation, when sealed centrifuge bottles were used to prevent contact with air.
- Cells were harvested by centrifugation at $3000\times g$ for 12 minutes, brought back into the glovebox, and resuspended in Buffer 1 (see table 2.9), approximately 3.5mL for each litre of growth media used.
- Cells were lysed by the addition of Lysozyme enzyme from egg white (Sigma-Aldrich) and Triton X-100 (0.3 g TX-100 per L cell growth), supplemented with 0.5mM DTT, and left stirring 90 minutes.
- Insoluble components were removed by centrifugation at $200,000\times g$ for 60 minutes.
- The supernatant was supplemented to 40mM imidazole and loaded onto a 5mL HisTrap HP or FF crude column (GE Healthcare), pre-equilibrated with Buffer A (table 2.9) at a flow rate of 1.5mL/minute.
- The column was washed with 20mL (4 column volumes) of Buffer A and eluted in a batch elution with Buffer B (Table 2.9).
- The enzyme was exchanged into EPR buffer (table 2.9) using a PD-10 size exclusion column (GE Healthcare) in an anaerobic glovebox ($[O_2] < 10\text{ppm}$) (Glovebox Technologies or M. Braun).
- Enzyme samples were flash-frozen and stored in liquid N_2 .

2.2.3 Variations of Isolation Methods for purification of Hyd-3 from *E. coli*

A number of methods were designed for the purification of FHL complex from *E. coli*. These are listed as variations of the two methods described above. The method used by Sargent and coworkers, our collaborators for this project, is also described, and is titled 'Aerobic BPER method'. This method was not used extensively by us due to the high cost of the lysis solution required. All protocols are numbered for easy referencing with the results section.

No.	Protocol name	Description of Protocol
1	Aerobic (French press lysed)	According to Section 2.2.1
2	Anaerobic, TX-lysed	According to Section 2.2.2
3	Aerobic, BPER (Method performed in Dundee)	Cells were grown as described in Section 2.2.2, harvested by centrifugation at $3000 \times g$ for 30 minutes and resuspended in BPER solution (Pierce) containing 50 mM imidazole, lysozyme, and DNase. The solution was stirred 30 minutes at room temperature and centrifuged at $17\,000 \times g$ for 12 minutes to remove cell debris. The supernatant was loaded directly onto a HisTrap Ni-NTA column (GE Healthcare) pre-equilibrated with Buffer D1. The column was washed with 10 column volumes Buffer D1 and eluted in a linear gradient to 100% Buffer D2.
4	Aerobic harvest, Anaerobic prep, BPER	Cells were harvested aerobically according to section 2.2.1 and subsequent steps were carried out in an anaerobic atmosphere, $\rho(\text{O}_2) < 10$ ppm. Cell lysis and membrane solubilisation were carried out in a single step using B-PER, with addition of DTT and imidazole to match concentrations detailed in Section 2.2.1. Buffers contained 0.03% DDM in place of TX-100.
5	Anaerobic, BPER	All steps were carried out under an N_2 atmosphere, $\rho(\text{O}_2) < 10$ ppm. Centrifuge bottles and tubes were filled, balanced and sealed under an N_2 atmosphere, but after sealing these were taken out of the glovebox and centrifugation steps took place in a lab atmosphere. Cells were lysed with BPER and buffers contained DDM as above.
6	Ferricyanide harvest, BPER	Prior to pelleting cells by centrifugation, $\text{K}_3\text{Fe}(\text{CN})_6$ was added to bring the potential of the solution from an original value of approximately -220 mV <i>vs.</i> SHE to stabilise at approximately 260 mV <i>vs.</i> SHE. Cells were then harvested aerobically, and subsequent steps were carried out in an anaerobic atmosphere, $\rho(\text{O}_2) < 10$ ppm. Cells were lysed with BPER and buffers contained DDM as above.

Table 2.8: Modifications of the purification protocol attempted to give better yields of FHL complex

Buffer name	Components
Buffer 1	100mM Tris pH 7.5, 350mM NaCl, 1mM EDTA, DNase, Lysozyme
Buffer 2	100mM Tris pH 7.5, 350mM NaCl
Buffer A	20mM Tris pH 7.2, 350mM NaCl, 60mM imidazole, 1mM DTT*, 0.02% Triton X-100
Buffer B	20mM Tris pH 7.2, 350mM NaCl, 750mM imidazole, 1mM DTT*, 0.02% Triton X-100
Buffer C	20mM Tris pH 7.2, 350mM NaCl, 1mM DTT*, 10% glycerol, 0.02% Triton X-100
Sulphido-free	20mM Tris, pH 7.2, 350mM NaCl, 5mM Na-ascorbate*, 10% glycerol, 0.02% Triton X-100.
Buffer D1	20 mM Tris pH 7.5, 150 mM NaCl, 0.02% DDM, 50 mM imidazole
Buffer D2	20 mM Tris pH 7.5, 150 mM NaCl, 0.02% DDM, 1 M imidazole
EPR Buffer	100mM MES, pH 6.0, 150mM NaCl, 10% glycerol, 0.02% DDM
Tris, NaCl, DTT and DDM were purchased from Melford. Imidazole was purchased from Alfa-Aesar.	
*In all cases, DTT and ascorbate were added immediately prior to use. For anaerobically-prepared enzyme, [DTT] was decreased to 50 μ M to prevent reduction of the Ni ²⁺ of the HisTrap column.	

Table 2.9: Buffers used in hydrogenase purifications

2.2.4 The Bradford Assay: Determination of Protein Concentration by a Colorimetric Method

The concentration of protein samples was carried out using Bradford's method [155], in which the binding of Coomassie Brilliant Blue G-250 to protein amino groups leads to an increase in the dye's absorbance at 595 nm. Bradford Assay Reagent (Sigma) was used according to the manufacturer's instructions, and a calibration curve was constructed with samples of bovine serum albumin of a known concentration (Sigma).

2.2.5 Mediator-based assays of H₂ production and oxidation

The H₂ oxidation and production activity of hydrogenase enzymes was assessed using a viologen-based solution assay. Both assays were carried out in 50mM Tris buffer, pH 6.0, 100mM NaCl. For H₂ oxidation, a solution of 1mM benzyl viologen in this buffer was saturated with 100% H₂ and a small amount of enzyme was added. For H₂ production, a solution of 600 μ M methyl viologen, reduced by addition of equimolar dithionite, was saturated with 100% Ar and a small amount of enzyme was added. In both cases, absorption at 604nm was monitored to determine the rate of catalysis, and molar absorptivities of 7.4 mM⁻¹cm⁻¹ and 13.7 mM⁻¹cm⁻¹ were used for benzyl and methyl viologen, respectively. For assays of Hyd-3 activity in Chapter 5, lower pH values were desired and assays were therefore conducted in 100mM MES buffer, in place of Tris buffer.

2.2.6 Poly-Acrylamide Gel Electrophoresis

Isolated protein was analysed for identity and purity by SDS-PAGE. Prior to loading, sample buffer was added to samples and they were heated to 70 °C for 10 minutes. Pre-cast Novex NuPAGE 10% Bis-Tris gels (Life technologies) were run according to the manufacturer's instructions using MOPS running buffer, or in the case of small proteins (<10 kDa), using MES running buffer (Table 2.10). Gels were run at 200V for 45 minutes. After electrophoresis, gels were stained using Coomassie Blue staining solution and destained using destaining buffer (Table 2.10).

Buffer Name	Composition
MES running	50 mM MES, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.3
MOPS running	50 mM MOPS, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.7
Coomassie stain	0.25% Coomassie Brilliant Blue, 50% Ethanol, 10% glacial acetic acid (Fisher Scientific)
Destain	20% Ethanol (Sigma-Aldrich), 10% glacial acetic acid
NativePAGE running	50 mM Bis-Tris, 50 mM Tricine, pH 6.8
NativePAGE sample	50 mM Bis-Tris, 50 mM NaCl, 10% w/v Glycerol, 0.001% Ponceau S, pH 7.2
NativePAGE Cathode additive	0.4% Coomassie G-250

Table 2.10: Buffers for Poly-Acrylamide Gel Electrophoresis

2.3 Protein Film Electrochemistry

Protein Film Electrochemistry began with early experiments showing that non-covalent adsorption of proteins to carbon electrodes allowed for direct electron transfer between the electrode and redox-active cofactors of the protein. This provided an excellent technique for studying redox transitions of the cofactors of metalloproteins [156] in the form of non-turnover signals. More importantly, for redox catalysts, this technique allowed fast and potential-controlled assessment of the rate of enzymatic turnover [157, 158].

2.3.1 Electrochemical cell setup

Figure 2.2 provides a schematic of the standard three-electrode setup used for Protein Film Electrochemistry. A potential is applied to the working electrode, measured with respect to the reference electrode. At a third electrode, termed the counter or auxiliary electrode, a current flows which is equal in magnitude, and opposite in direction, to the flow of current at the working electrode; the current flow between working and counter electrode is recorded as the output of the experiment. Several different materials may be used for the working electrode, including graphite, graphene, carbon nanotubes, gold, and semiconductors including TiO_2 and CdS . The counter electrode normally consists of a platinum wire, and the reference is often a calomel or Ag/AgCl electrode.

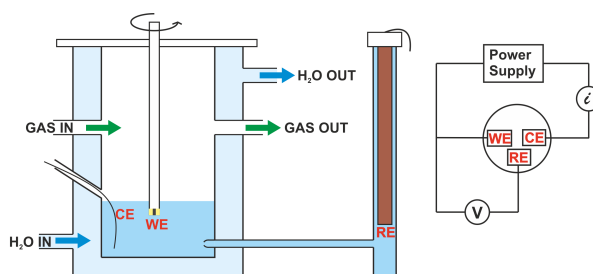


Figure 2.2: A standard three-electrode setup for Protein Film Electrochemistry Experiments. WE = working electrode, CE = counter electrode, RE = reference electrode.

Much of the mechanistic information available from PFE experiments comes from the ability to very precisely control temperature, pH, and substrate and inhibitor concentrations. The electrochemical cell is water jacketed, so that a thermostatted water circulator may be attached. The cell buffer is under a continuous flow of gases, whose composition can be controlled by mass flow controllers. Rotation of the electrode prevents the depletion of substrate or the buildup of product at the electrode. Unless otherwise stated, all PFE experiments herein are carried out in mixed hydrogenase buffer (0.1M NaCl, 15mM each sodium acetate, MES, HEPES, TAPS, and CHES, all purchased from Sigma, titrated to the desired pH at the temperature of the experiment with HCl and NaOH) [83].

2.3.2 Non-Faradaic processes at the electrode

The currents observed during a PFE experiment are due to a combination of non-Faradaic and Faradaic processes. Non-Faradaic processes are those in which no charge transfer occurs at the electrode-solution interface, while Faradaic processes involve a transfer of charge between the working electrode and the solution.

Experiments have shown that the working electrode-solution interface behaves as a capacitor, with the formation of an electrical double layer near the electrode: when the electrode is positively charged, an inner ‘*Helmholtz*’ layer of specifically adsorbed anions will form, surrounded by an outer layer of solvated cations. The capacitance thus created at the working electrode-solution interface will lead to the recording of current with changes in potential, even when no current flows from electrode to solution. Figures 2.3 and 2.4 illustrate this capacitance effect through simulated potential sweep and potential step procedures applied to a simple RC circuit, that is, a circuit in which a resistor of resistance R_s and a capacitor of capacitance C_d are connected in series with a current source.

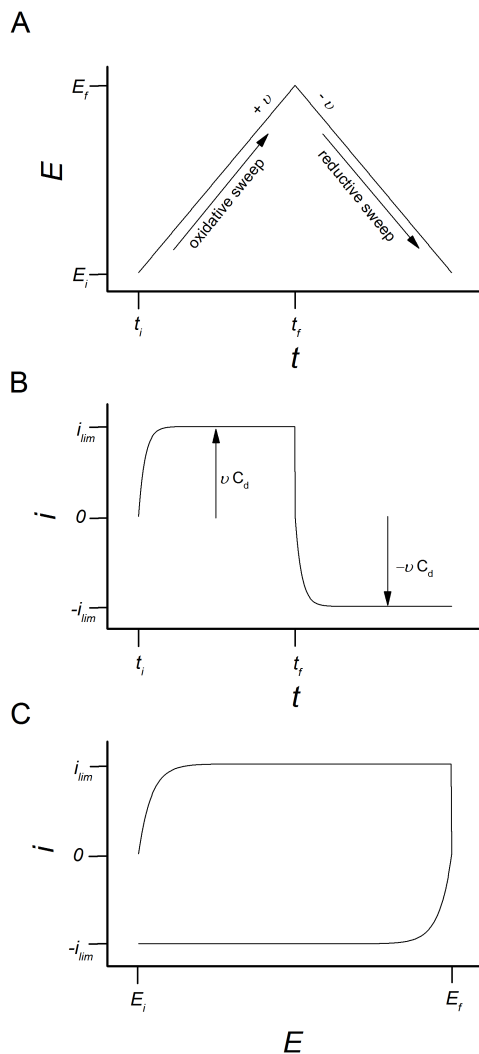


Figure 2.3: A cyclic potential sweep applied to an RC circuit (A) Potential-time trace (B) Current-time trace, wherein the limiting current is equal to the scan rate, v times the capacitance, C_d (c) Current-potential trace

In a cyclic voltammetry experiment, the potential is swept from a potential E_i to a potential E_f at a consistent scan rate of v , and then back to E_i at a scan rate of $-v$ (Fig. 2.3A). The initial acceleration of the scan rate from zero to v results in a growing current derived from capacitance, with a limiting value of vC_d ; when the direction of the scan is reversed, the current becomes negative (Fig. 2.3B). Plotting the current vs. potential trace (Fig. 2.3C) approximates the shape of a ‘blank’ cyclic voltammogram (CV), recorded at a clean PGE electrode to which no enzyme is adsorbed.

In a chronoamperometry experiment, the electrode potential is poised at a constant potential, and may be changed in discrete steps (Fig. 2.4A). The capacitance effect due to this change will be a spike in current of maximum magnitude $\Delta E/R_s$, and with a decay time constant equal to the product of the resistance and the capacitance, $\tau = R_s \times C_d$ (Fig. 2.4B).

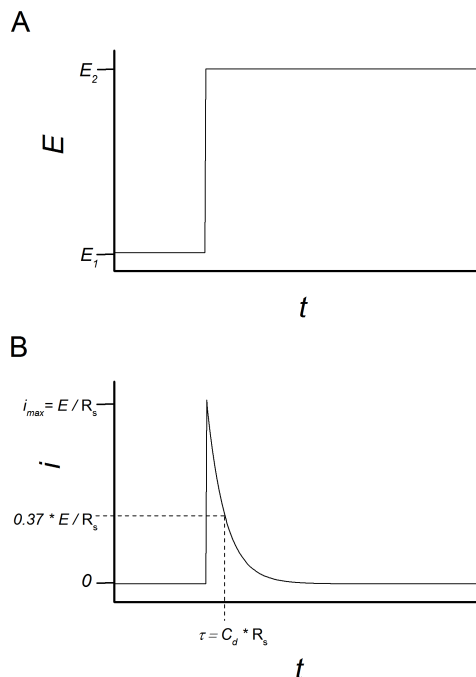


Figure 2.4: A potential step applied to an RC circuit (A) Potential-time trace (B) Current-time trace, wherein the maximum current is equal to the potential step divided by the resistance, and the time constant for decay is equal to the resistance times the capacitance.

2.3.3 Faradaic processes at the electrode

Faradaic processes involve charge transferred from the working electrode to the solution and/or adsorbate at the electrode. The meaningful signals studied by PFE fall into two categories: ‘non-turnover’ signals caused by an oxidation or reduction step at a protein cofactor, and ‘turnover’ signals due a steady flow of electrons to or from a cofactor that is catalysing substrate turnover. ‘Non-turnover’ voltammetry requires the use of high scan rates to generate a good signal-to-noise ratio, since the total number of oxidations or reductions is limited to the number of cofactors adsorbed within electron transfer

distance. By contrast, the ‘turnover’ voltammetry used to study catalytic processes gives the highest signal-to-noise ratio at lower scan rates and serves as a much more detailed version of an enzyme assay, allowing the exploration of both thermodynamics and kinetics of various processes including turnover and inhibition.

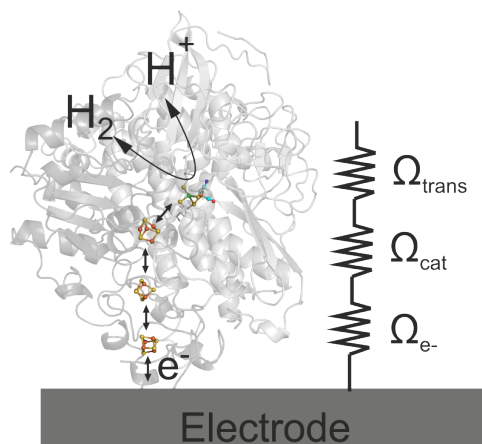


Figure 2.5: A schematic of a PFE experiment, in which the electron transfer, catalytic turnover and substrate and product diffusion are theorised as resistors [159]

When enzyme is adsorbed at the electrode, electrons pass between the electrode and a surface-exposed redox cofactor (interfacial electron transfer), and then between internal redox cofactors (intramolecular electron transfer), providing driving force for catalysis at the active site, which is also dependent upon access of the substrate to, and removal of product from, the active site. The experiment was conceptualised as the application of a potential across a set of resistors (Figure 2.5) [159], herein we will attempt to expand this analogy to better reflect the electronic roles played by FeS clusters, especially in Hyd-1 from *E. coli*.

2.3.4 Enzyme stabilisation

Traditional PFE experiments rely on non-specific protein adsorption to a sanded pyrolytic graphite edge (PGE) electrode, graphite whose graphene layers are oriented perpendicular to the surface of the electrode. Sanding the electrode breaks the graphene

sheets, exposing valence unsaturated carbons that rapidly react with solvent water, forming various oxidised species. The mixture of hydrophilic oxygen groups combined with hydrophobic graphene planes presumably forms a useful surface for the non-specific adsorption of many protein molecules. The normal method of ‘growing a film’ is to sand or polish with an alumina powder slurry, followed with a rinse in distilled water and wiping with cotton wool, followed by pipetting 2-5 μL of enzyme onto the electrode surface, pipetting up and down around 30 times and then rinsing [160]. This method is useful for most proteins tested to date. Zwitterionic coadsorbate such as the antibiotic polymyxin have been used [161] and may often allow larger currents to be registered or increase the stability of enzyme adsorption at the electrode [162]. This system has the advantage of being particularly simple and quick, but since adsorption is non-specific, this leads to a heterogeneity in the interfacial electron transfer distance, and for some proteins, this method does not yield sufficient current density or stability to be useful.

Alternate electrode materials open up new possibilities. Multi-walled carbon nanotubes can be covalently attached or non-covalently adsorbed to a range of electrode materials to increase surface area and thus current response from adsorbed enzyme [163, 164, 165, 166]. In this work, MWCNTs were prepared in the following way: 30 μL of a 10mg/mL slurry of MWCNTs (outer diameter 10-15 nm, inner diameter 2-6 nm, length 0.1-10 μm obtained from Sigma) in DMF, sonicated for 15 minutes, were deposited onto a sanded, sonicated, rinsed and dried PGE electrode and the DMF was allowed to dry for 2 to 12 hours. After rinsing, a ‘film’ was grown as above.

This approach may be combined with covalent enzyme attachment by the addition of 1-pyrenebutyric acid, 10mM in DMF, applied to the MWCNTs and presenting a carboxylic acid group. Modification of this carboxylic acid using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxy sulfosuccinimide (NHSS), 0.4 M and 0.1 M, respectively, in H_2O , activates the carboxylic acid group of the pyrene butyric acid to allow for peptide bond formation between this carboxylic acid and exposed

amine groups of the protein [167].

2.3.5 Electrochemistry on Gold Electrodes

The use of Au electrodes, commonly in conjunction with thiol-based self-assembled monolayers (SAMs), allows for a wide range of chemical groups to be used for covalent attachment or non-covalent interaction with protein molecules. The procedure for electrochemistry on Au electrodes used in this thesis is as follows: Au-coated silica wafers were prepared by Dr. R. Jacobs (Surface Analysis Facility, University of Oxford). The gold surface was cleaned immediately prior to SAM formation by rinsing with ethanol, then water, and immersing in Piranha solution (3:1 18 M H_2SO_4 :30% H_2O_2) for fifteen minutes, and finally a second rinse with ethanol and then distilled H_2O . A SAM was grown by soaking the cleaned electrode in a 1mg/mL solution of dithio-bis-maleimidoethane (DTBM) in DMSO for 1 hour. The electrode was then rinsed thoroughly with distilled H_2O and 20 μL of enzyme solution (1-4 mg/mL) was deposited onto the surface, which was stored under a beaker to prevent drying out. After 30 minutes, the electrode was immersed in solution using a Crocodile clip for electrical connection to the cell. The electrode was not rotated.

2.4 Potentiometric titration of enzyme samples

All samples for analysis by EPR spectroscopy were prepared in EPR buffer (Table 2.9). Where enzyme was not sufficiently concentrated, it was anaerobically concentrated using 500 μL Amicon spin concentrators with a molecular weight cutoff of 50 kDa (Millipore). Enzyme samples were titrated in a specially constructed glass cell allowing a two-electrode setup to measure the solution potential in a minimal volume. A stationary Pt electrode (Mettler Toledo, InLab Redox Micro) served as a working electrode while a micro Ag/AgCl electrode (WPI, DRIFEF-2) served as both a reference and a

counter electrode. A small stirrer bar ensured homogeneity through the solution and a temperature-controlled water jacketed cell kept the enzyme at 20 °C throughout the titration. Enzyme was added to the cell and, except in the case of ‘as-isolated’ samples, the enzyme was exposed to a stream of H₂ for three to four hours to activate inactive states, until a stable low potential was reached. The cell was then flushed continuously with Ar to remove all H₂. 40 μM each of the redox mediators methyl viologen, benzyl viologen, phenazinemethosulfate, indigotetrasulfonate, 1,2-naphthoquinone, and 2-hydroxyl-1,4-naphthoquinone were added, and the potential was adjusted by small additions of 400 mM sodium dithionite or K₃Fe(CN)₆ as reductant or oxidant, respectively. When a stable potential was obtained, enzyme was injected rapidly into the EPR tube using the pressure of the gas flow above the solution, the ‘shooting’ method described elsewhere [168], and were frozen rapidly in an isopropanol slush or acetone and dry ice bath.

2.5 Electron Paramagnetic Resonance Spectroscopy

EPR spectra were collected using a Bruker EMX spectrometer (Bruker BioSpin GmbH) with an X-band super-high-sensitivity probehead (Bruker) cooled with a low-temperature helium flow cryostat (Oxford Instruments CF935). For all spectra, background scans were recorded under identical conditions and subtracted from the experimental scan. The magnetic field was calibrated at room temperature by Dr. M. Rößler using 2,2-diphenyl-1-picrylhydrazyl ($g = 2.0036$). Spin quantification was carried out using a calibration line obtained by Dr. M. Rößler using copper perchlorate samples measured under nonsaturating conditions.

2.6 Mass spectrometry

Intact labelled and unlabelled protein was analysed by inline LC-ESI-MS (Liquid chromatography-ElectroSpray Ionisation-Mass Spectrometry). Protein samples were exchanged into pH 7.4 50mM ammonium acetate buffer by repeated dilution and concentration using an Amicon Ultra centrifugal filter unit (Millipore, 3kDa molecular weight cutoff) and 5 μ L were injected onto a nanoAcquity C4 reversed phase column, (Waters) and eluted with a linear gradient from 5% to 80% acetonitrile in water, with 0.1% formic acid in both the acetonitrile and water at a flow rate of 0.3mL/min over 7 minutes. The chromatography was run using an Acquity UPLC system (Waters). The eluate from the column was run directly into a Micromass LCT Premier ESI-TOF mass spectrometer (Waters Inc.) for analysis. The settings used are summarised in Table 2.11. The LC-MS systems were controlled by Micromass MassLynx software version 4.1 (Waters Inc.).

Parameter	Value
Ionisation Mode	Positive
Capillary Voltage	3000V
Sample Cone Voltage	100V
Tube Lens	186V
Desolvation Temperature	250°C
Source Temperature	120°C
Cone Gas Flow	30 L/hour
Desolvation Gas Flow	550 L/hour
Mass Range	510 - 3000 m/z
Data mode	Continuum

Table 2.11: Mass spectrometry settings used for LC-ESI-MS of intact labelled and unlabelled peptides.

Spectra were summed over individual elution peaks, and were then background-subtracted and analysed using the MaxEntI algorithm [169, 170, 171] provided with MassLynx in order to determine the uncharged isotopic average mass of the eluted species. An example of this process is illustrated in Figure 4.10. The predicted average and monoisotopic masses of labelled and unlabelled peptides were calculated using the BioLynx add-in to the MassLynx package.

2.7 Covalent attachment of Ru-polypyridyl complex

$[\text{Ru}(4,4\text{-dimethyl-bipyridine})_2(5\text{-maleimido-phenanthroline})]^{2+}$ was synthesized and purified by Daniel Streich, Uppsala University, Sweden. The relative purity of the compound was assessed to be greater than 60% on the basis of ESI-MS results. All steps in the labelling of enzyme with $[\text{Ru}(4,4\text{-dimethyl-bipyridine})_2(5\text{-maleimido-phenanthroline})]^{2+}$, as well as light-driven H_2 production and transient absorption spectroscopy of these samples, were carried out under an anaerobic atmosphere. Purified enzyme samples (typically 50 μM) were thawed and a 20-50 fold molar excess of ruthenium polypyridyl complex was added. After 2-3 hours dark incubation the reaction mixture was purified on a Superdex PD-10 gravity flow column (Amersham Biosciences) equilibrated with 0.1 M sodium acetate buffer, pH 6, 150 mM NaCl, 1 mM DTT, 0.02% DDM. Alternatively, reaction mixtures were purified on a Superdex 200 HR 10/30 column (GE Healthcare) equilibrated with 0.1 M sodium acetate buffer, pH 6, 350 mM NaCl, 1 mM DTT, 0.02% DDM. Fractions containing enzyme were pooled, flash-frozen in liquid nitrogen and stored at -80°C .

For control experiments in which β -maleoyl alanine was used to assess cysteine labelling, a three to five-fold excess of β -maleoyl alanine was added to the enzyme, which was incubated for one hour at room temperature.

2.8 Light-driven H_2 production

Labelled enzyme, or enzyme in solution with free photosensitizer, was irradiated with a halogen lamp (Schott KL2500 LCD, with Osram Xenophot HLX, 24V, 250W), filtered with BG19 and GG455 filters and a 1cm path-length water-filled quartz cuvette to produce light of 455-850nm. Throughout light-driven H_2 production experiments, the solution was stirred with a magnetic stir bar. The cuvette was gas-tight and was directly connected to a CP4900 Micro-GC (Varian) gas chromatography unit equipped

with a MolSieve 5 PLOT column and a thermal conductivity detector for *in situ* H₂ measurements.

Quantum yield calculations were carried out as follows: absorbed photons were calculated based upon the absorbance of the excited state at 460nm immediately following a laser flash, with $\epsilon = 14000 \text{ M}^{-1} \text{ cm}^{-1}$; the quantum yield was calculated as the number of H₂ molecules produced per two absorbed photons.

2.9 Transient absorption spectroscopy

Labelled enzyme, or enzyme in solution with free photosensitizer, was thawed and transferred to a gas-tight cuvette (10x2 mm). A frequency tripled Nd-YAG laser (Spectra-Physics) tuned to 470nm by an optical parametric oscillator (OPO) was used to excite the sample along the short pathlength, while spectroscopic measurements were carried out using a Xe lamp (Xe900, Edinburgh Instruments) and a CCD camera (iStar, Andor) along the long pathlength. Scattering was minimised using a 2mm cardboard mask attached to the cuvette on the detector side of the sample. Kinetic traces were acquired using a photomultiplier tube (LP920-K, Edinburgh Systems) connected to a 500 MHz oscilloscope (TDS 3052B, Tektronix). The laser and detection system were controlled by L900 software (Edinburgh Instruments).

Calculations were carried out as follows: charge recombination of $[\text{Ru}(\text{bpy})_3]^{1+}$ with ascorbyl radical was estimated from the initial slope of a curve recorded at 510nm (reduced state), with $\epsilon = 6700 \text{ M}^{-1} \text{ cm}^{-1}$. Because the concentrations of ascorbyl radical and reduced state can be assumed to be the same, the rate constant for recombination is calculated as the linear fit of $1/[\text{Ru}(\text{bpy})_3]^{1+}$ vs. t . An upper limit for the rate of electron injection from $[\text{Ru}(\text{bpy})_3]^{1+}$ to Hyd-2 is calculated assuming that all depletion of the reduced state is due to electron injection: a linear fit to $\ln[\text{Ru}(\text{bpy})_3]^{1+}$ vs. t in the presence of Hyd-2 gives a pseudo first-order rate constant of 1680 s^{-1} . A lower limit

is calculated by assuming an equal contribution of recombination in the conditions with and without Hyd-2: a linear fit to $\ln[[\text{Ru}(\text{bpy})_3]^{1+}]_{\text{Hyd-2}}/\ln[[\text{Ru}(\text{bpy})_3]^{1+}]_{\text{noHyd-2}}$ vs. t gives a pseudo first-order rate constant of 530s^{-1} . Assuming that the concentration of oxidised Hyd-2 is consistent throughout the experiment (because the total Hyd-2 concentration is $13\mu\text{M}$, and the concentration of $[\text{Ru}(\text{bpy})_3]^{1+}$ is approximately $1\mu\text{M}$, as estimated from ΔA , when the rate of electron injection begins to compete with the rate of recombination), we can calculate limits on the rate constants for injection of an electron from $[\text{Ru}(\text{bpy})_3]^{1+}$ to Hyd-2, $4.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} < k_{ei} < 1.3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. The efficiency of electron injection was defined as the rate of electron injection divided by the sum of the rate of electron injection and the rate of recombination, summed over the time period $t = 0\text{-}3\text{ms}$. Again assuming an unvarying concentration of oxidised Hyd-2 and assuming that the concentration of ascorbyl radical is equal to the concentration of $[\text{Ru}(\text{bpy})_3]^{1+}$ at any given time, the injection efficiency is calculated to be approximately 3%. Alternatively, the efficiency of electron injection can be determined as the quantum efficiency for H_2 production, determined as above, divided by the quench efficiency, which gives a result of $7 \pm 2\%$.

2.10 UV-Vis absorption spectroscopy

For routine activity and protein concentration assays, UV-Vis spectroscopy was carried out using a Cary UV-Vis spectrophotometer. For UV-Vis measurements of enzyme in light-driven H_2 experiments, a diode array UV-Vis spectrophotometer (Agilent) in an anaerobic glovebox was used.

2.11 Detection of H₂ from Formate by Gas Chromatography

The production of H₂ was measured using a 7890A gas chromatography system (Agilent Technologies) equipped with a ShinCarbon ST micropacked column (Restek Corporation) and a thermal conductivity detector. N₂ was used as a carrier gas, and N₂ containing 2% CH₄ provided an internal standard. The column was held at 34 °C throughout the run and was heated to 200 °C between each sample to purge the column. Baselines were fitted by eye and peak areas were determined by analysis using ImageJ 1.45s software (Wayne Rasband, NIH, USA). H₂:CH₄ peak area ratios were converted to absolute H₂ concentrations by the construction of a calibration curve of samples from 0.3 to 2% H₂ mixed using mass flow controllers (Sierra Instruments).

Chapter 3

E. coli Hyd-1 : Characteristics of
O₂-tolerant enzymes and the
relationship with electron transfer

3.1 Introduction

3.1.1 Properties of O₂-tolerant enzymes

The ability to continuously catalyse H₂ oxidation in the presence of high levels of O₂ is an uncommon property among hydrogenases, and has mostly been identified and characterised over the last 10 years in a group of membrane-bound Group I hydrogenases that couple H₂ oxidation with quinone reduction. In the characterisation of these enzymes (most prominent of which are the *R. eutropha* and *R. metallidurans* MBHs, the *A. aeolicus* HaseI and Hyd-1 from *E. coli*), a set of common properties has emerged that seems to be characteristic, distinguishing the O₂-tolerant enzymes from their O₂-sensitive Group I counterparts.

In order to better characterise the unique features of O₂-tolerant MBHs, let us compare the O₂-tolerant Hyd-1 with the O₂-sensitive Hyd-2 from *E. coli*. A simple comparison of cyclic voltammograms recorded at pH 7, 37 °C and 10% H₂ reveals striking differences in the catalytic properties of the two enzymes. Hyd-1, the O₂-tolerant enzyme, exhibits little to no H₂-production capability (the so-called ‘catalytic bias’); indeed, no O₂-tolerant MBH has ever been reported to catalyse H₂ production at a considerable rate. A second characteristic feature is the overpotential requirement - under these conditions, H₂-oxidation activity does not begin until nearly 0.1 V above the Nernstian equilibrium potential, (marked with a |) for Hyd-1, while Hyd-2 is seen to cut the zero-current axis cleanly, exhibiting minimal overpotential requirement for catalysis in either direction [44]. This chapter explores in detail the overpotential requirement and catalytic bias of Hyd-1 and the relationship between the two.

A third important feature is the potential at which reversible oxidative inactivation is seen. All [NiFe] hydrogenases exhibit a characteristic drop in activity at oxidising potentials, resulting in a very distinctive shape for hydrogenase CVs. The characteristic decrease in activity seen at high potential is widely held to reflect the conversion of active

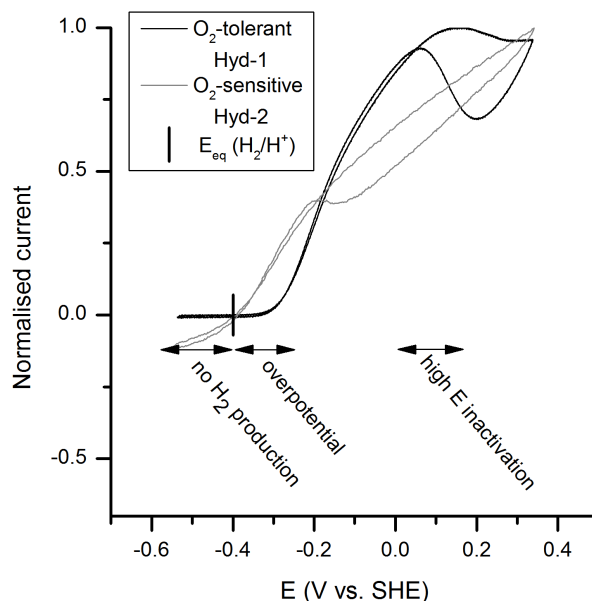


Figure 3.1: The properties of an O_2 -tolerant hydrogenase, Hyd-1, as revealed through comparison with a standard hydrogenase, Hyd-2. Both scans recorded at pH 7, 37°C , 10% H_2 , scan rate 5mVs^{-1} , $\omega = 2000\text{rpm}$.

enzyme to the inactive hydroxo-bridged $\text{Ni}^{\text{III}}\text{-B}$ (see section 1.2.3). Under identical conditions, the potentials at which this inactivation and subsequent reactivation occur are much higher for O_2 -tolerant hydrogenases than for their O_2 -sensitive, ‘standard’ counterparts. The significance of the potential, E_{switch} , measured for this reactivation will be discussed.

3.1.2 A definition for O_2 -tolerance in [NiFe] hydrogenases

The ability of [NiFe] hydrogenases to recover activity after inhibition by O_2 distinguishes them from the [FeFe] hydrogenases [172, 173] and makes the [NiFe] enzymes likely candidates for many biotechnological applications. Upon exposure to O_2 , [NiFe] hydrogenases are known to produce several states including the spectroscopically-defined Ni-A and Ni-B, which are broadly accepted to correspond to the kinetically-defined ‘Unready’ and ‘Ready’ states due to their different rates of reactivation (see section 1.2.3). Both of these states can be reductively reactivated by removal of O_2 and exposure to reductants such as H_2 or dithiothreitol [174], but the rate of reactivation is very slow for ‘Unready’

states. Most [NiFe]-hydrogenases are therefore purified aerobically [175, 176, 177] and subsequently reductively activated.

Recovery from inhibition, however, is distinct from what is termed ‘O₂-tolerance’ within the [NiFe] hydrogenases. This property, much more unique among these enzymes, is defined as the ability to sustain H₂ oxidation activity in the continued presence of O₂ [178]. Over the past ten years, several O₂-tolerant hydrogenases have been identified and characterised, including the membrane-bound hydrogenases from *R. eutropha* [179], *R. metallidurans* [180], *E. coli* [44] and *A. aeolicus* [164]. In many cases, the ability of these enzymes to maintain H₂-oxidation activity under O₂ is essential to their physiological function, as is the case for *Ralstonia* species, in which H₂-oxidation is coupled to aerobic respiration [181].

3.1.3 A model for O₂-tolerance in membrane-bound [NiFe] hydrogenases

A model for O₂-tolerance [182], summarised in Figure 3.2, allows us to separate the reaction with oxygen into distinct phases and to analyse the characteristics that allow for continued activity in the presence of O₂. The oxidation of H₂, shown in the left cycle, is competitive with attack by O₂; the extent of inactivation due to O₂ is lessened in increased concentrations of H₂ [182], and O₂ and H₂ are believed to bind at the same site. O₂-tolerance is therefore facilitated by tight binding of H₂ at the active site, a property that is associated with a low Michaelis-Menten constant, $K_M^{H_2}$, which will be discussed further in section 3.2.4.

Upon reaction of O₂ at the active site, one of at least two distinct species may be produced; the proportion of different states formed plays a critical role in O₂-tolerance, as these states exhibit drastically different rates of reductive reactivation. These states are defined by their properties as observed in PFE experiments, and are known as ‘Ready’ and ‘Unready’ for states with fast and slow reactivation kinetics, respectively.

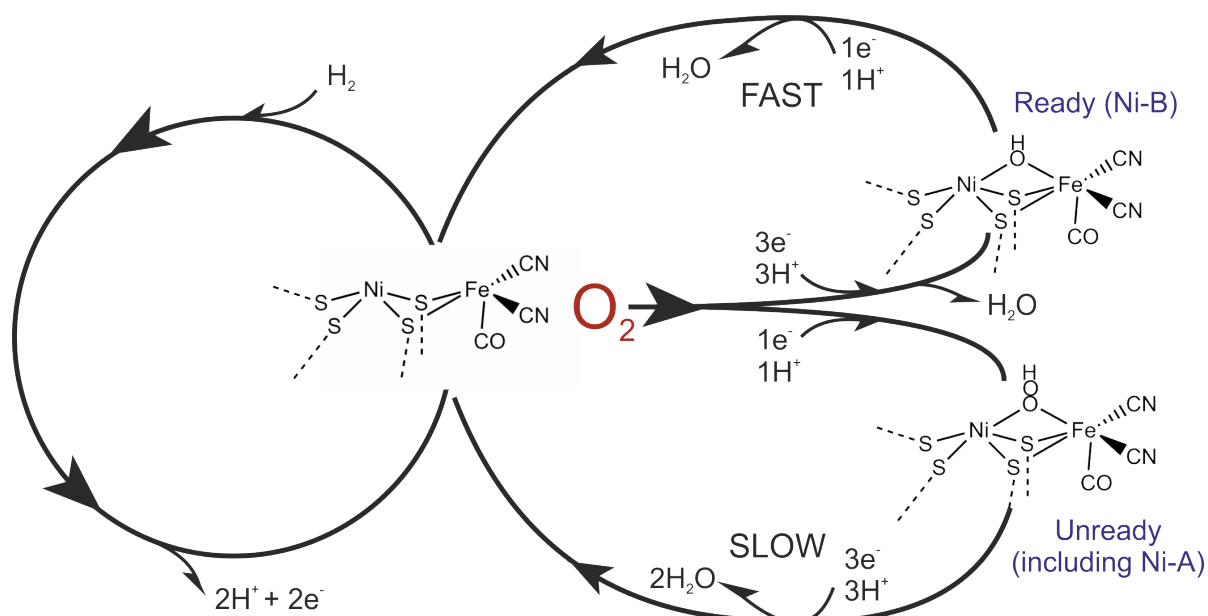


Figure 3.2: A model for the O₂-tolerance of [NiFe] MBHs, proposed by Cracknell et al. [182]. The left cycle represents catalytic oxidation of H₂, while the two cycles on the right are the reaction with O₂ to form Ready (top) and Unready (bottom) species, and the fast and slow recovery of these species, respectively. The rapid availability of electrons upon reaction of O₂ is critical in determining whether Ready or Unready states are produced. The ‘Ready’ state is widely believed to correspond to the spectroscopically-defined Ni-B state [182] while the ‘Unready’ state is thought to include the spectroscopically-defined Ni-A state but may also include other states with slow reactivation properties [96].

These states are believed to correspond to the spectroscopically-defined Ni-B and Ni-A states respectively [182], although ‘Unready’ states may include a number of slow-to-activate states, as the reactivation is too slow to accurately assess the rate order [96]. There is evidence that in some cases, partial reduction of O₂ leads to release of radical-oxygen species (ROS), causing damage at the active site including the oxidation of ligating thiolate groups [183]. Here the ‘Ready’ and ‘Unready’ labels will be used to avoid confounding the kinetic data from PFE experiments with spectroscopic data. The availability of electrons and protons for reduction of the attacking O₂ plays a pivotal role in the mechanism [182], since the production of the rapidly reactivated ‘Ready’ species requires the immediate supply of three electrons and three protons. Under electron-poor (more oxidising) conditions, the O₂ is partially reduced to ‘Unready’ states, shown here as the peroxo-bridged species Ni-A in the lower right cycle. The partially-reduced states are very slow to reactivate, therefore if they are produced at any appreciable rate they will build up, precluding continued catalysis in the presence of O₂. Under electron-rich (more reducing) conditions, O₂ reacting at the active site can be reduced by three electrons to a hydroxo-bridged ‘Ready’ state, the spectroscopically-defined Ni-B; the reactivation of this state is sufficiently fast that it may compete with the rate of reaction with O₂ even at high concentrations of O₂, allowing continuous turnover under aerobic conditions. In short, a critical feature of O₂-tolerant MBHs is that they are able to form almost exclusively ‘Ready’ states upon reaction with O₂, a feature which is at least in part due to an ability to supply three electrons to the active site rapidly upon reaction with O₂.

From the Ni-B stage, a further one-electron reduction is required to recover active enzyme, and the rate and potential of this reduction also play an important role in determining an enzyme’s ability to function in oxygen. For O₂-tolerance, this reactivation must occur in the presence of O₂, in other words, at relatively high potentials. Enzymes in which the Ni-B reduction reaction occurs only at very low potentials will not be able

to sustain oxidative catalysis even if all reactions with O_2 lead to the formation of the ‘Ready’ state.

3.1.4 A role for Fe-S clusters

In their role mediating electron transfer to and from the active site, Fe-S clusters are indispensable to the catalytic capability of [NiFe] hydrogenases. More detailed characterisation of these clusters, however, has shed light upon an important function for FeS clusters in mediating O_2 -tolerance, by ‘hosting’ electrons for rapid delivery back to the active site in the case of O_2 reaction there. The FeS clusters of Hyd-1 are demonstrated in Figure 3.3.

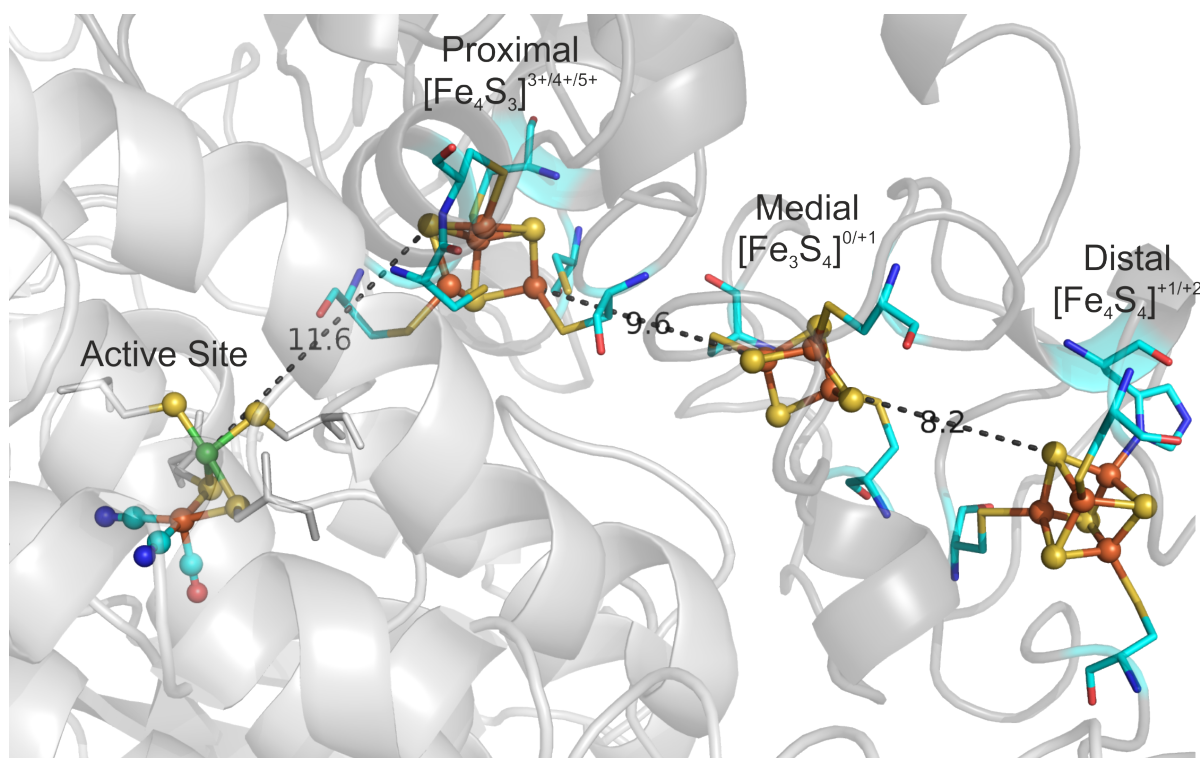


Figure 3.3: The Fe-S clusters and active site of *E. coli* Hyd-1, PDB ID 3UQY [184]. Corner-to-corner distances between cofactors are displayed in Å with broken lines.

The Fe-S clusters of Hyd-1 are similar in many ways to those of ‘standard’ membrane-bound [NiFe] hydrogenases: the small subunit ligates a string of three clusters, well within rapid electron transfer distance of one another [140]. The distal cluster is ligated by three cysteine thiolates and the imidazolium group of a histidine residue (His-187

in *E. coli* numbering) similar to ‘standard’ hydrogenases. By analogy with Fe_2S_2 clusters, which can either be all-Cys ligated (thioredoxin-type) or $2\times\text{His}$, $2\times\text{Cys}$ ligated (Rieske-type), we might expect the ligation by a histidine to cause an upshifting in the reduction potential of the cluster relative to all-Cys ligation [142] as well as introducing a pronounced pH-dependence of the reduction potential [185]. Recent investigations by Rößler *et al.* [76] reveal that this cluster is EPR-silent in both of its oxidation states; the +1 oxidation state, often visible for Fe_4S_4 clusters, is not seen in the wild-type Hyd-1 or in mutants designed to silence signals from the other clusters. This cluster is presumably invisible due to fast relaxation of an $S \succ 1/2$ ground state [76], a property shared by other Fe_4S_4 clusters ligated by three cysteines and a histidine or aspartate residue, including clusters found in nitrate reductase [186] and some ferredoxin proteins [187, 188]. The lack of EPR signals from this cluster makes it difficult to assess whether or not the cluster differs from analogous clusters in O_2 -sensitive hydrogenases.

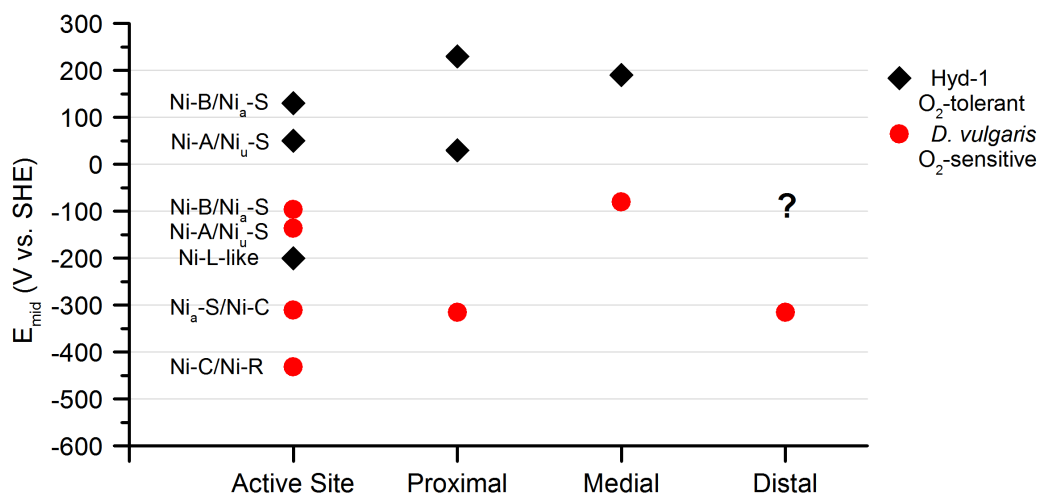


Figure 3.4: Mid-point potentials of the redox cofactors of Hyd-1 and the O_2 -sensitive [NiFe] hydrogenase from *Desulfovibrio vulgaris*. The potential for the Ni-L-like state is not a midpoint potential, but rather the potential of maximum intensity, as no mid-point potentials for the formation and disappearance of this state are available [189, 82, 76, 168].

The medial cluster is a Fe_3S_4 cluster in most MBHs characterised thus far with the

exception of the [NiFeSe] enzymes [190]. The reduction potential of a Fe_3S_4 cluster is normally several hundred millivolts higher than that of a Fe_4S_4 cluster [191], leading to an up-and-down energy profile for electron transfer via the three clusters of hydrogenases, seen in Figure 3.4. In the O_2 -tolerant enzymes, the potential of the medial Fe_3S_4 cluster is further upshifted by 100 mV or more relative to that of standard MBHS [184, 192, 193]. The importance of this high potential cluster in delivering electrons to neutralise attacking O_2 was shown by Evans *et al.* [96]. Indeed, the midpoint potential for all of the redox transitions measured to date seems to be upshifted in O_2 -tolerant hydrogenases, relative to their O_2 -sensitive counterparts. Figure 3.4 compares the available midpoint potential data for Hyd-1 with those for the standard hydrogenase from *D. vulgaris*. High-potential FeS clusters, because they are more likely to be reduced at any given time, are able to act as a good source of electrons to neutralise O_2 reacting at the active site; thus, the high potential clusters observed for O_2 -tolerant enzymes fit well with our model for O_2 -tolerance, in which the availability of electrons promotes the rapid reduction of O_2 leaving only ‘Ready’ inactive species bound at the active site.

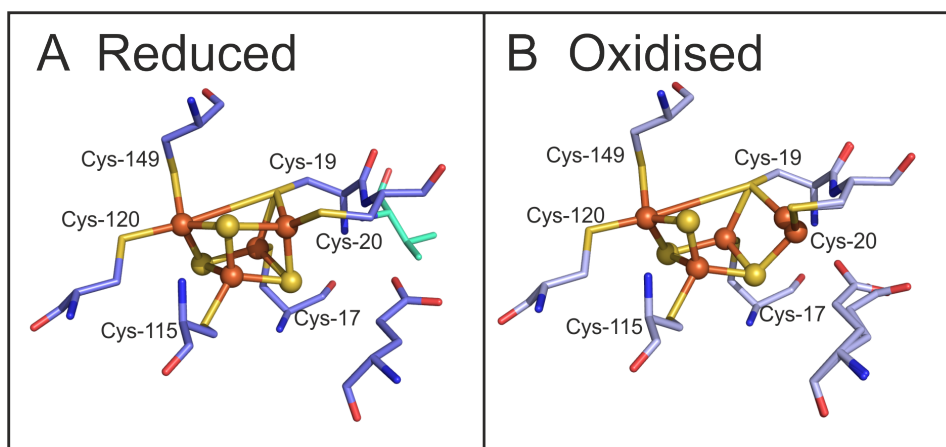


Figure 3.5: The proximal Fe_4S_3 cluster of Hyd-1, (A) H_2 -reduced, (B) oxidised with OH-NQ and $\text{K}_3\text{Fe}(\text{CN})_6$, PDB files 3UQY and 3USC [184]

The proximal cluster is the cluster which is the most obviously different between O_2 -tolerant and O_2 -sensitive MBHs. Indeed, this unique Fe_4S_3 cluster has only recently been discovered in these enzymes [94, 194] and is unknown in other proteins. Six cysteine

residues surround the cluster, one of which (Cys-19) replaces an inorganic S^{2-} at one corner of the cubic cluster. Two other cysteinyl residues, Cys-120 and Cys-149, together ligate a single Fe ion, while the remaining three cysteine residues (Cys-17, -20, and -115) each ligate an Fe ion as usual. In its most reduced form, the formal charge of the cluster is +3 (including the negatively charged thiolates this would be -3); this state can undergo a one-electron oxidation as a normal Fe_4S_4 cluster would, without significant rearrangement. The ‘intact’ cluster in either the +3 or +4 state is shown in Figure 3.5A. Under very oxidising conditions, such as when O_2 reacts at the active site, the cluster can undergo a second oxidation step, concomitant with a structural rearrangement (Figure 3.5B). In this ‘super-oxidised’ +5 state, one Fe ion, that which is ligated by Cys-20, moves out of the cluster and is ligated by the amide nitrogen of the adjacent residue and by the $O\epsilon$ of Glu76. Two conformations of this super-oxidised state are observed, in which the position of the Fe differs by $0.7\text{\AA}$. The cluster rearrangement, with ligation by the hard O and N ligands in place of S, allows the Fe to become oxidised to a +3 state, providing an additional electron to the active site for rapid reduction of bound oxygen species. This second oxidation is thought to be facilitated by deprotonation of the amide N of Cys-20, perhaps by Glu-76, which maintains local electroneutrality for this step and thus minimises reorganisation energy [184].

3.1.5 Overpotential requirement

For a redox process under a given set of conditions, the Nernst equation can be used to calculate the equilibrium potential, the potential at which the direction of net catalysis changes, as a function of the standard potential and the activities of the reactant and product species [195]. For the oxidation of molecular hydrogen, $H_2 \longleftrightarrow 2H^+ + 2e^-$ at 37°C and assuming activity coefficients of unity this equation simplifies to

$$E_{eq} = (pH * 61.5mV) - (\log[H_2] * 30.8mV) \quad (3.1)$$

For a reversible catalyst, when $E = E_{eq}$ the exchange current is high, with both oxidative and reductive catalysis proceeding at high but equal rates [195]. Shifting the electrode potential even marginally in either direction from E_{eq} results in a shift in the ratio of forward and reverse rates, and a steep increase in the recorded current. A voltammogram recorded for such a catalyst reveals a current trace that cuts cleanly through the zero current axis, as exemplified by Hyd-2 in Figure 3.1. In the case of irreversible systems however, the exchange current is low and a significant overpotential, $\eta = E - E_{eq}$, must be applied before any significant current is recorded. As will be seen later in sections 3.2.1 and 3.2.2, Hyd-1 is an example of the ‘irreversible’ case, exhibiting a significant overpotential requirement.

3.1.6 Understanding catalytic bias

The term ‘catalytic bias’ refers to an assessment of the relative rate of net turnover in one direction *vs.* the other with the application of a given overpotential η in either direction. Hyd-1 is said to be biased strongly toward H_2 -oxidation, because under most conditions, Hyd-1 oxidises H_2 at high rates but does not produce H_2 at measurable levels, even with the application of a large overpotential. The term has come into usage in the study of redox enzymes due to the observation that, under identical conditions, different enzyme variants exhibit different ratios of oxidative to reductive catalysis as observed by cyclic voltammetry. However, it is important not to misunderstand this term. By definition, a catalyst lowers the activation barrier for a reaction without shifting the position of equilibrium, K , which can be expressed as the ratio of the rates of forward and reverse catalysis, $k_{forward}/k_{back}$. The overall driving force for the reaction, expressed as the Gibbs free energy or overpotential ($\eta = E - E_{eq}$) determines the ratio of these rates (equation 3.2). Thus, under a given set of conditions, a catalyst can be more or less efficient (lowering the activation barrier to a greater or lesser extent) but this difference will affect both forward and reverse rates, with the ratio being consistent.

$$\frac{k_{forward}}{k_{back}} = \frac{-\Delta G}{RT} = \frac{nF\eta}{RT} \quad (3.2)$$

This understanding is seemingly at odds with the voltammetric profile of Hyd-1 (section 3.1.1), in which substantial H₂ oxidation but almost no H⁺ reduction is observed. This is explained by the fact that although the ratio of $k_{forward}/k_{back}$ is set by the driving force, the absolute value of these rates can, and often does, vary according to potential. In other words, a potential-dependent change in the lowest-energy pathway between reactants and products affects both the forward and reverse rates of reaction, leaving their ratio unchanged. Thus, the measured current, which is proportional to the difference between forward and reverse rates, $k_{forward}-k_{back}$, is often seen to change as a function of potential. In the case of Hyd-1 and other O₂-tolerant MBHs, a dramatic decrease in the absolute values of $k_{forward}$ and k_{back} is apparent below approximately -400mV vs. SHE; this decrease is observed as a lack of reductive activity at potentials below $E_{eq}^{H^+/H_2}$ and as an overpotential for H₂ oxidation at potentials above $E_{eq}^{H^+/H_2}$.

Several structural features may underlie a potential-dependent alteration in the absolute values of $k_{forward}$ and k_{back} . At the catalytic site itself, changes in the redox state of the active site metal ions will significantly alter energies of substrate binding and product release, altering $\Delta G^\ddagger$. This redox state change may or may not be accompanied by chemical changes, such as the binding of an intrinsic or extrinsic ligand to the active site. It is also possible for factors outside the active site to play a role in the potential dependence of catalytic effectiveness. For redox catalysts that depend upon discrete cofactors as mediators of electron transfer to and from the active site (*e.g.* Fe-S cluster relays in many redox enzymes), the potential of these cofactors will determine the range of potentials within which electron transfer will be favourable; outside these limits, electron transfer and thus turnover will be slow. The driving force provided to the active site will be mediated through discrete centres; thus, the electrochemical potential of

electrons reaching the active site may not be that which is set by the electrode, but may instead be lower. Understanding the structural basis for the overpotential requirement and catalytic bias that is common to all O₂-tolerant MBHs may allow a more complete understanding of the features that mediate this tolerance, and to assess whether it would be possible to design or discover an O₂-tolerant H₂-producing enzyme.

3.1.7 Differences between O₂-tolerant and -sensitive hydrogenases at the [NiFe] active site

X-ray diffraction studies of O₂-tolerant hydrogenases [196, 194, 184] reveal a [NiFe] core whose structure is virtually unchanged, relative to O₂-sensitive variants [197, 198, 183, 199]. Nonetheless, some lines of evidence point to subtle differences in properties at the active site that may contribute to O₂-tolerance. As discussed in section 3.1.3, the formation of ‘Unready’ states upon reaction with O₂ precludes O₂-tolerance. The best-characterised ‘Unready’ state, the peroxo-bridged Ni-A, is reported not to be formed in the O₂-tolerant enzymes from *R. eutropha* and *A. aeolicus* [180, 200]. While this is likely to be due, at least in part, to the supply of electrons to fully reduce reacting O₂, there may be other factors involved. Variants of *R. eutropha* MBH in which mutation of proximal cluster supernumerary cysteines leads to loss of O₂-tolerance *do* produce ‘Unready’ species as seen by PFE, but no Ni-A was visible using either FT-IR or EPR spectroscopy [94]. *E. coli* Hyd-1 does produce Ni-A in the presence of O₂, especially when the availability of electrons is low [44]. Thus it seems that the absence of the ‘Ni-A’ state may be correlated with O₂-tolerance, though it is not strictly necessary, nor is it sufficient, to guarantee O₂-tolerance.

EPR-investigations of *A. aeolicus* Hase I and *E. coli* Hyd-1 have revealed a second difference at the active site involving a species similar to the previously reported Ni-L. Studies in O₂-sensitive enzymes have shown that illumination at low temperature leads to conversion of the Ni-C state to a state named Ni-L, which is reconverted to Ni-C with

warming of the sample [78]. This conversion is believed to be due to the photolabilisation of the bridging hydride at the active site as H^+ [201]. For both *E. coli* Hyd-1 and *A. aeolicus* Hase I, however, substantial levels of a Ni-L-like species are seen even without illumination [76, 202]. Indeed, in the case of Hyd-1, no Ni-C is ever seen, while for HaseI, ENDOR studies of the Ni-C state have shown that the hydride is much more weakly bound to the Ni in this enzyme than in standard [NiFe] hydrogenases.

PFE studies with the inhibitor carbon monoxide also point to differences between the active sites of O_2 -tolerant and O_2 -sensitive enzymes. CO is a classical inhibitor of both [NiFe] and [FeFe] enzymes and has been used as a tool for assessing the rate of diffusion of an O_2 -sized molecule to the active site. PFE [85] and FT-IR [203, 86] studies suggest that CO binds to the Ni-SI state of hydrogenases. Whereas the sensitivity to CO is quite high for standard [NiFe] hydrogenases [85], the O_2 -tolerant MBHs from *R. eutropha*, *A. aeolicus*, and *E. coli* all show minimal inhibition by CO [86, 179, 44].

3.1.8 Aims of this chapter

The ability of a hydrogenase to operate under ambient concentrations of O_2 is truly a remarkable feat, and it should not be surprising that many individual structural or mechanistic features may be necessary to facilitate this ability. This chapter explores the features of *E. coli* Hyd-1 in detail, with a special focus on the properties common to all O_2 -tolerant enzymes characterised to date. Experiments herein carried out at very low pH show Hyd-1 acting as a reversible electrocatalyst, producing H_2 at relatively high rates; this has not before been seen for an O_2 -tolerant hydrogenase. This experiment serves as a lovely demonstration of the fact that catalytic bias is not a direction-dependent effect but is in fact due to a potential-dependent change in the effectiveness of a catalyst. Detailed experiments evaluating the onset potential under various conditions give insight into the links between onset potential and reversibility, and the structural features of Hyd-1 responsible for these properties.

Site-directed mutagenesis has proven an indispensable technique in understanding the role played by the proximal and medial clusters in O₂-tolerance of Hyd-1. Mutations at the proximal cluster certainly interfere with the ability of the hydrogenase to deal with O₂, however no mutation at the proximal or the medial cluster has ever resulted in alteration of the other properties normally associated with O₂-tolerance, including a high potential for reductive reactivation of Ni-B, overpotential requirement for H₂ oxidation, negligible H₂ production activity, low $K_M^{H_2}$ and minimal sensitivity to inhibition by CO. In addition to the important roles played by the high potential of the medial cluster and by the double redox transition of the proximal cluster, it thus seems very likely that other structural features help to mediate tolerance to O₂ in Hyd-1 and other O₂-tolerant MBHs, and that these structural features underlie the common properties of this group of hydrogenases mentioned above.

3.2 Results and Discussion

3.2.1 Hyd-1 oxidises H₂ in the presence of atmospheric levels of O₂

The ability of a [NiFe] hydrogenase to sustain catalytic turnover in the presence of O₂ is a rather unique property, as discussed in section 3.1.2. This ability has been demonstrated by simple O₂ injection experiments [44] in which an aliquot of oxygen-saturated buffer is injected into the cell during the forward sweep of a cyclic voltammetry experiment under constant flow of gas, so that the enzyme is exposed to a transient burst of O₂ at mid to high potential, and the O₂ is flushed from the cell by the time the scan direction is reversed.

This type of experiment has become the standard test for O₂-tolerance in hydrogenases. In this type of experiment, an O₂-sensitive hydrogenase will show an immediate

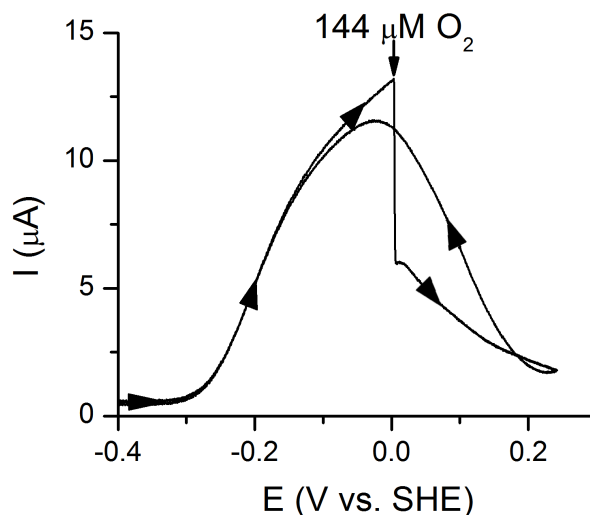


Figure 3.6: Hyd-1 O_2 -tolerance exhibited in the standard O_2 injection cyclic voltammetry experiment at pH 6, 30 °C, 100% H_2 , gas flow 1000 sccm, scan rate 1mVs^{-1} , $\omega = 3500$ rpm. Arrows indicate the direction of scan. At 0 V on the forward scan, O_2 -saturated buffer was injected to give an O_2 concentration of $144\text{ }\mu\text{M}$.

drop in H_2 -oxidation activity to zero, with recovery requiring full flushing of O_2 and the application of a relatively reducing potential [85, 44]. By contrast, an O_2 -tolerant enzyme will often exhibit only partial inactivation due to the burst of O_2 , followed by rapid reactivation as the O_2 is flushed from the cell, even at quite high potentials.

The sustained H_2 -oxidation activity upon injection, as well as the complete recovery of inactivated enzyme at high potential, are characteristic of oxygen tolerance. While this experiment gives a good first impression of the reaction with O_2 , it does not offer important information about the long-term stability under O_2 , nor does it allow us to deconvolute the effects of changing potential and changing O_2 concentration. As more advanced studies of O_2 -tolerance have evolved, more complete methods for testing O_2 -tolerance have been adopted. Figures 3.7 and 3.8 present more robust experiments designed to circumvent these shortcomings, allowing a better assessment of the potential-dependence of inhibition as well as the long-term effect of exposure to O_2 .

Figure 3.7 presents an alternative cyclic voltammetry experiment [204] in which a consistent concentration of O_2 allows us to measure the striking potential dependence of O_2 inhibition without a competing effect of changing O_2 concentration. Here, a scan is

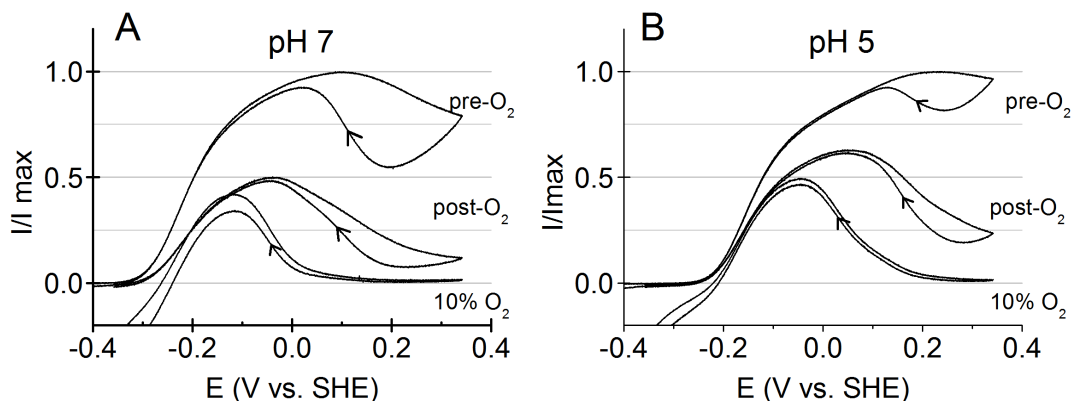


Figure 3.7: Cyclic voltammetry experiments demonstrating the ability of *E. coli* Hyd-1 to oxidise H_2 in the sustained presence of 10% O_2 . Experiments carried out at the stated pH and 10% H_2 , 25 °C, 1mVs^{-1} , gas flow rate = 1000 sccm, $\omega = 4500\text{rpm}$.

first recorded without O_2 , to record the initial level of activity. O_2 is then introduced into the gas mixture, while the H_2 level is kept constant. When the gas is fully equilibrated with the cell solution, a second ‘with O_2 ’ scan is recorded, giving a measure of the extent of inactivation across the full potential range. Finally, the O_2 is removed from the gas mixture and the cell buffer is again allowed time to equilibrate before a ‘post- O_2 ’ scan is recorded to quantify the extent of Unready species formed. We observe a striking dependence of inhibition on potential. Between -50 and -100mV, the scan conducted in the presence of 10% O_2 shows remarkable levels of activity - nearly 50% of the uninhibited enzyme in the pH 5 experiment, while at potentials above 200mV vs. SHE, the activity drops to zero. The consistent $[\text{O}_2]$ allows an uncomplicated assessment of the effect of potential. By comparing scans conducted at different pH values, we see that at lower pH, a lesser degree of inhibition is apparent for both the ‘with O_2 ’ and ‘post- O_2 ’ scans; in other words, the H_2 oxidation current observed during and after O_2 exposure as a proportion of the current recorded prior to O_2 exposure is greater for scans carried out at pH 5 than at pH 7. The observations from this simple experiment - that O_2 -tolerance is enhanced at low potential and low pH - provide sound support for the theory of O_2 -tolerance presented in section 3.1.3, in which the availability of protons and electrons play a role in determining whether ‘Ready’ or ‘Unready’ state is formed upon

reaction with O_2 . Upon removal of O_2 , only partial recovery of activity is observed in this experiment, in contrast with an O_2 -injection experiment, in which Hyd-1 shows full recovery of activity. This is likely to be due to the potentials at which the enzyme is exposed to O_2 ; exposure at high potentials is more likely to result in the formation of ‘Unready’ states.

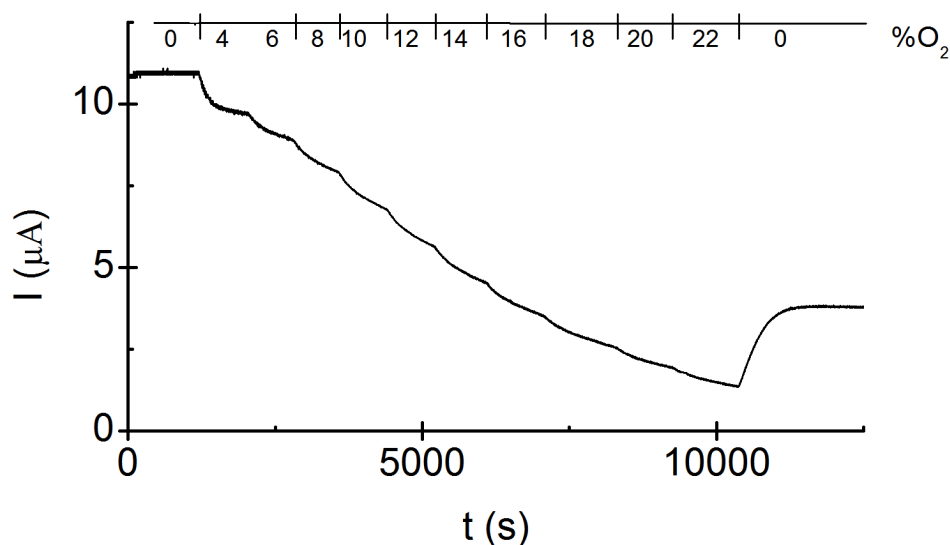


Figure 3.8: The response of Hyd-1 to increasing concentrations of O_2 at $-0.1V$, 10% H_2 , $37^\circ C$, pH 7, gas flow = 1000 sccm $\omega = 3500$ rpm. The prolonged exposure to O_2 allows detection of build-up of ‘Unready’ states over time.

It is also important to assess the response to prolonged exposure to O_2 as even slow accumulation of Unready states can prevent an enzyme from sustaining catalysis in O_2 . This can most easily be accomplished by chronoamperometry. An experiment examining the effect of increasing concentrations of O_2 is shown in Figure 3.8. Even under ambient O_2 and 10% H_2 , significant oxidation activity remains. Upon each subsequent addition of O_2 , a relatively steady current is observed, indicating a ‘steady-state’ level of activity in which the rate of inactivation due to reaction with O_2 matches the rate of reactivation. Exposure to very high concentrations of O_2 results in the appearance of a persistent slope in the extent of activity, indicative of the accumulation of inactive species; thus, at pH 7, Hyd-1 is not indefinitely stable at 22% O_2 , although it is at lower concentrations of O_2 (*e.g.* 4%). This is an important observation that can only be made with the use

of long-term experiments. Thus, the experiments shown in Figures 3.7 and 3.8 should be included as standard tests of O₂-tolerance, in addition to shorter-term experiments such as those based upon injection of O₂ with flushing.

Having established a more discriminating set of experiments to characterise O₂-tolerance in hydrogenases, it is now possible to move to an in-depth characterisation of the other properties associated with oxygen tolerance: an overpotential requirement for H₂ oxidation, a lack of H₂ production, a low $K_M^{H_2}$, a high tolerance to inhibition by CO, and a high E_{switch} .

3.2.2 An investigation of the overpotential requirement and catalytic bias of Hyd-1

[H⁺] but not [H₂] shifts the onset potential of Hyd-1 near neutral pH

Unlike Hyd-2 and other ‘standard’ O₂-sensitive hydrogenases, but similarly to all known O₂-tolerant MBHs, Hyd-1 does not catalyse H₂ oxidation near the Nernstian equilibrium potential near physiological pH [44]. Under 100% H₂ and at pH 7, an overpotential of roughly 0.1 V is required in order to drive any significant H₂ oxidation activity. The onset of activity does not shift with [H₂], but is instead fixed, resulting in *increasing* overpotential requirement as substrate concentration is increased (Fig. 3.9, panel A). The onset potential *is*, however, found to shift with pH (Fig 3.9, panel B), a surprising observation in light of its insensitivity to H₂.

As discussed in section 3.1.6, the limitation of catalytic turnover outside a given potential window can be due to redox-dependent changes in the active site cofactor, or a potential-dependent effect on the rates of electron transfer or substrate/product transport, factors due to centres outside the active site. In the interest of characterising the structural features responsible for O₂-tolerance, we would like to be able to identify the centre whose reduction potential sets a lower limit on the potential window for

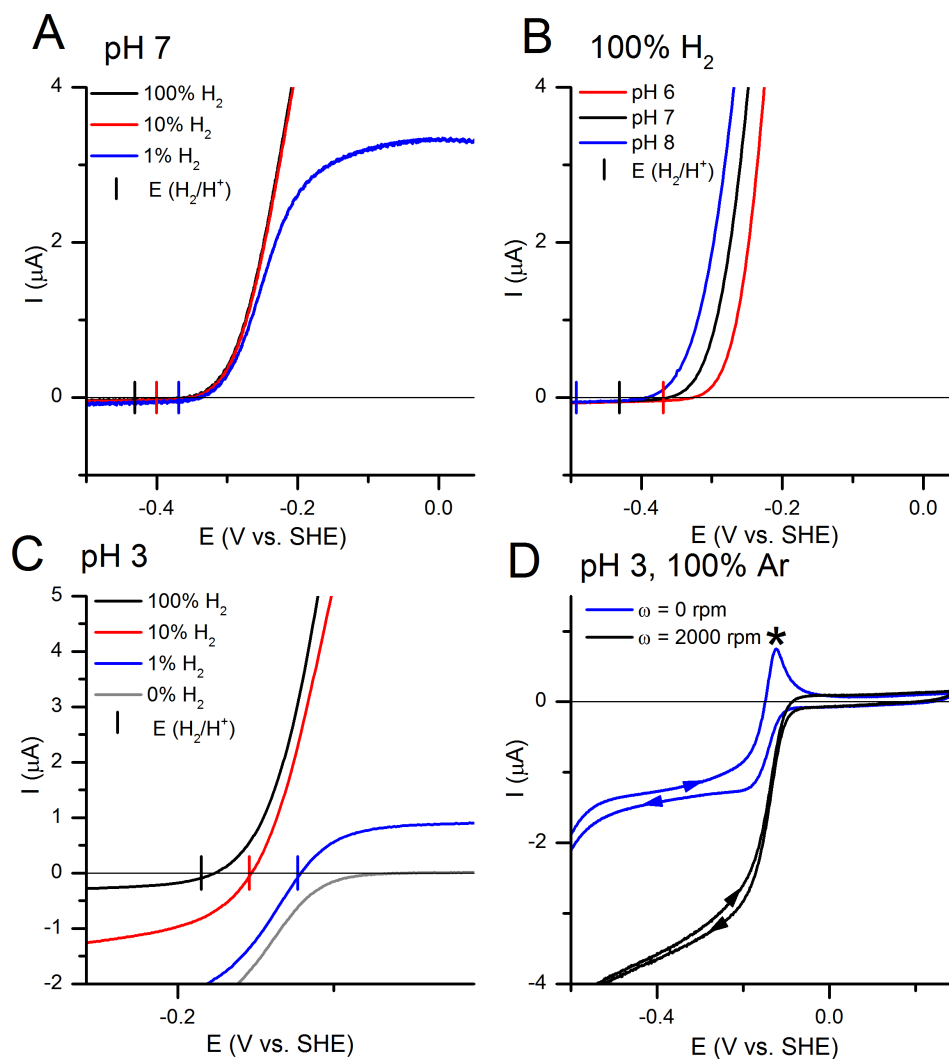


Figure 3.9: Irreversible (A, B) and Reversible (C, D) catalysis by *E. coli* Hyd-1. (A) At pH 7, changes in the $\rho(\text{H}_2)$ have no effect on onset potential. (B) Near neutral pH, changing the pH causes a 40 mV/unit shift in onset potential. (C) At very low pH, overpotential is eliminated and current due to H^+ reduction becomes significant. (D) A comparison of voltammograms recorded at rotating and stationary electrodes at pH 3 confirm that the reductive current is due to H_2 production. All scans recorded at 37 °C, 10 mVs^{-1} under a 1000 sccm flow of gas; $\omega = 2000$ rpm except for the stationary scan, panel D.

catalysis in O₂-tolerant MBHs. Taken together, the set of results presented in Figure 3.9 panels A and B indicate that the reduction potential of this centre is shifted by changes in the availability of H⁺ but not H₂. Because we know that the active site *must* favourably interact with both H₂ and H⁺, the reduction potential for transitions at the active site will be affected by changes in $\rho(\text{H}_2)$ and $\rho(\text{H}^+)$. Given that the onset potential is insensitive to $\rho(\text{H}_2)$, the limited activity at low potential cannot be due to redox transitions at the active site, as was suggested by Pandelia et al. [200] for the *A. aeolicus* enzyme. Rather, the site limiting catalysis at low potential must have a redox potential which is sensitive to protonation but which does not bind H₂.

It is possible that the lack of activity at low potential is related to the high potential of one or more FeS clusters, limiting the rate of electron transfer to or from the active site at low potential. A model for the voltammetric profiles of various [FeFe] and [NiFe] hydrogenases developed by Hexter et al. [59] suggests that the distal FeS cluster acts as an electron ‘control’ centre whose potential sets the relative bias toward oxidation or reduction for a particular enzyme. The fact that the onset potential is sensitive to $\rho(\text{H}^+)$ is consistent with a role for the distal cluster, as the proton-accepting nature of the histidine residue ligating the distal cluster should mean that the potential of this cluster changes with pH. This effect is well-documented for Rieske-type Fe₂S₂ clusters [185]. In the cell, electrons from Hyd-1 are passed to the membrane-bound quinone pool via a di-haem *b*-type cytochrome (Fig. 1.5) and recent crystallographic [93] and spectroscopic (P. Wulff, personal communication) studies have shown that this cytochrome often co-purifies with Hyd-1. Thus, it is also possible that the potential of the *b*-type haem serves as the point of entry and exit of electrons to and from the enzyme and limits reversible activity in Hyd-1. The pH-dependence of the cytochrome *b* redox potential is well-documented [205].

In light of our understanding that catalytic bias is a manifestation of potential-dependent change in catalytic effectiveness, we may speculate that the overpotential

requirement and the lack of H_2 production, or ‘catalytic bias’ toward H_2 -oxidation, are related properties, both being a demonstration of inefficient catalysis at low potentials. We have hypothesised that, if we could bring $E_{eq}^{H^+/H_2}$ to a potential above the lower limit on catalysis, we would eliminate the overpotential requirement and see, for the first time, efficient reversible catalysis performed by an O_2 -tolerant MBH. We can test this hypothesis by drastically increasing the concentration of H^+ in order to make $E_{eq}^{H^+/H_2}$ much more positive.

Experiments at very low pH reveal reversible, efficient electrocatalysis

Cyclic voltammograms recorded at very low pH (Fig. 3.9C) reveal a catalytic profile entirely unlike anything previously seen for O_2 -tolerant MBHs. Under all concentrations of H_2 , oxidative current rises sharply at potentials immediately above $E_{eq}^{H^+/H_2}$ for the given conditions - in other words, minimal overpotential requirement is observed. More dramatic still is the appearance of significant negative current, due to the reduction of H^+ to H_2 . Appreciable proton reduction activity has never before been observed for an O_2 -tolerant MBH. At low pH, Hyd-1 acts as a reversible catalyst.

Further confirmation that the reductive current observed at low pH is due to H_2 production can be obtained by comparing cyclic voltammograms recorded under stationary and rotating conditions (Fig. 3.9D). Without rotation of the electrode, movement of substrate and product toward and away from the electrode is slow, leading to a buildup of H_2 near the electrode during the reductive phase of the voltammogram. As the potential is scanned toward more positive potentials and crosses $E_{eq}^{H^+/H_2}$, we see a large positive peak, marked by an asterisk (*), due to reoxidation of accumulated H_2 . It is also notable that the magnitude of the reduction current is less in the stationary scan than in the rotating electrode scan, also due to accumulation of H_2 at the electrode leading to product inhibition of proton reduction. This simple experiment provides conclusive proof for the production of substantial levels of H_2 by Hyd-1.

Product inhibition remains substantial for Hyd-1 under these conditions. A chronoamperometry based assessment of the $K_I^{H_2}$, the inhibition constant measuring product inhibition, is shown in Figure 3.10. A calculation of the $K_I^{H_2}$ assuming saturation of the enzyme with substrate ($\rho(H^+) \gg K_M^{H^+}$) [85] gives a value of $125 \pm 4 \mu\text{M}$ at pH 3, 37 °C and -0.536V *vs.* SHE. For comparison, the $K_I^{H_2}$ of *E. coli* Hyd-2 is $210 \mu\text{M}$ at pH 6, 30 °C and -0.6V *vs.* SHE [44]. Thus, although substantial product inhibition is seen for Hyd-1, the value is comparable with that for a standard MBH, providing further demonstration that the observed bias of Hyd-1 is a potential-dependent property not likely to be related to properties of the active site itself. For this calculation and all subsequent equilibrium constants, error is stated as standard error, propagated disregarding covariance of the slope and intercept for the plot used [206].

This experiment also gives an excellent demonstration of the rather remarkable stability of a Hyd-1 film even at pH 3. The decrease in overall current over the course of the experiment is less than 2%, thus the data have not been corrected for film loss.

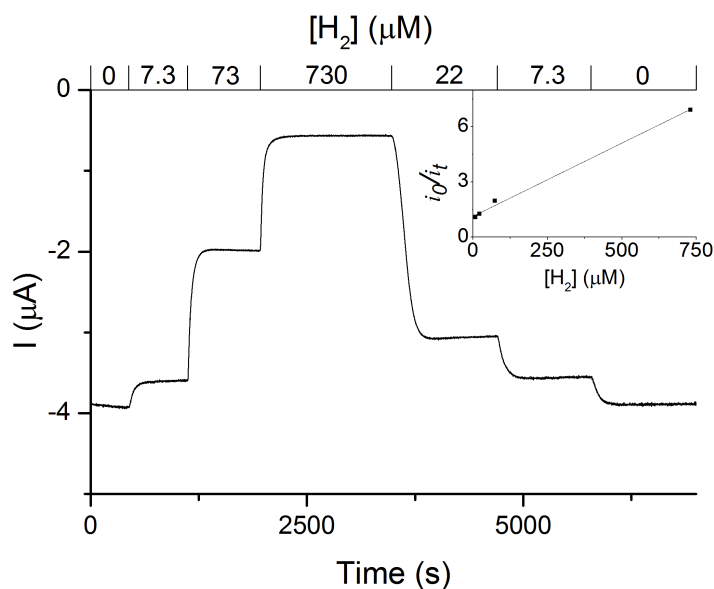


Figure 3.10: Product inhibition of hydrogen production catalysed by Hyd-1, measured by chronoamperometry. pH 3, 37 °C, -536 mV *vs.* SHE, gas flow 1000 sccm, $\omega = 5000\text{rpm}$.

Cyclic voltammograms carried out across a wide range of pH values are shown in Figure 3.11A. In order to more easily assess the overpotential for each scan, the same current traces are plotted as a function of the overpotential, calculated as the difference between the applied potential and the $E_{eq}^{H^+/H_2}$ for the conditions of the scan. In this panel, we can easily see that at and below pH 5, the onset of H_2 oxidation activity coincides with E_{eq} , and application of even a slight overpotential results in a rapid climb in the activity of the measured current. Above pH 5, the onset of oxidation is sluggish at small overpotential and requires application of increasing driving force as we move to higher pH. It is also important to note that scans exhibiting minimal overpotential requirement ($pH \leq 5$) show significant negative current due to H_2 -production activity, while those requiring an overpotential for oxidation activity ($pH > 5$) also show almost no reductive catalysis, confirming that overpotential requirement and catalytic bias are indeed manifestations of the same limitation on low-potential catalysis and attributable to the same structural feature.

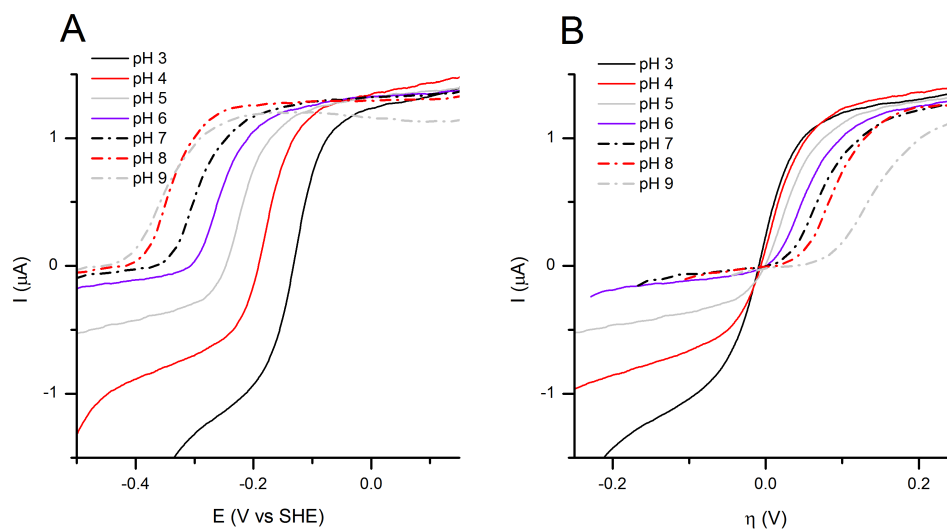


Figure 3.11: Voltammograms recorded at 1% H_2 across a wide range of pH values, at 37 °C, scan rate = 10 mVs^{-1} , ω = 2000rpm, gas flow rate = 1000 sccm. A) Scans are plotted conventionally, as a function of potential with respect to an external reference B) Scans are plotted as a function of overpotential, η , calculated by subtracting the equilibrium potential of the H^+/H_2 couple under the scan conditions from the applied potential.

The coincidence of these two features confirms that the appearance of H_2 production activity at low pH is not simply due to increased substrate concentration, but is rather related to a more fundamental change in reversibility. Raising $E_{eq}^{H^+/H_2}$ by drastically altering the pH allows Hyd-1 to catalyse efficiently and reversibly.

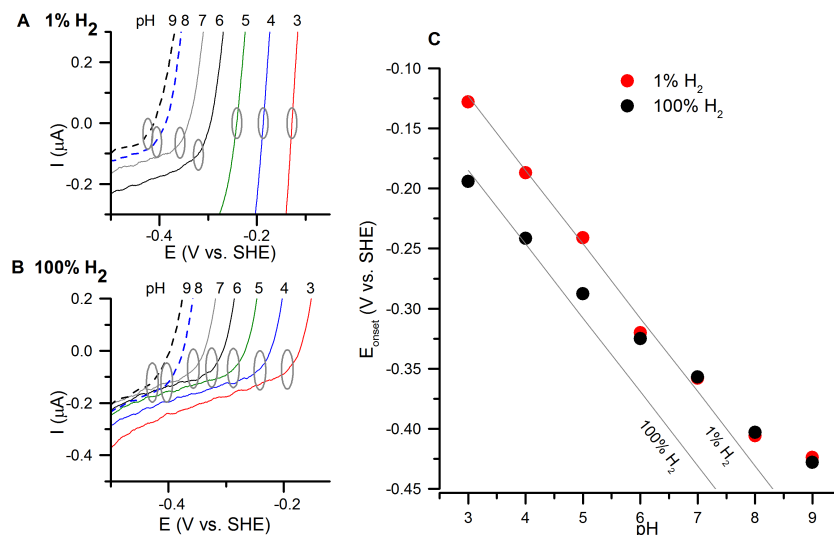


Figure 3.12: A summary of the onset potentials observed for Hyd-1 at varying pH and % H_2 . Scans were carried out at 37 °C under a 1000 sccm flow of gas, $\omega = 2000\text{rpm}$, scan rate = 10 mVs^{-1} . A) Scans under 1% H_2 . B) Scans under 100% H_2 . Forward and reverse scan currents were averaged, and the onset potential (marked with a grey oval) was assessed as follows: For scans in which significant reductive activity was visible, the zero-current intercept marks the onset of oxidation activity, and for scans in which little to no reductive activity is observed, the potential at which the slope of the CV deviates significantly from the slope of a blank scan marks the onset of oxidation activity. The error in this method is estimated as 0.01 V and the grey ovals are 0.02 V wide to indicate the assessed onset potential plus or minus estimated error. C) The onset potentials obtained from scans in A and B. The $E_{eq}^{H^+/H_2}$ for 1% and 100% H_2 are shown as diagonal grey lines.

A summary of the onset potential under a range of pH and % H_2 values is presented in Figure 3.12. For scans exhibiting reversible catalysis with significant reductive current, the onset potential is defined as the zero-current potential (ie. the potential at which the net direction of turnover changes from reduction to oxidation). However, for scans in which little to no reductive current is observed, the zero-current potential is highly sensitive to small effects from capacitance and current offset, and no longer represents the potential at which significant turnover rates are observed; for these scans, a different

method must be used to assess onset. We begin with the observation that, when little to no activity is observed, the CV shows a slope that is similar to the slope of a blank scan, and when activity begins in earnest, the slope of the experimental CV rises above the slope of a blank. A range is established for the slope of a blank scan as the mean plus or minus three standard deviation units, in this case, the slope of a blank scan lies in the region $2.1 \times 10^7 \pm 6.85 \times 10^7$. In both cases, the datum taken as onset potential is clearly marked on the scan with a grey oval whose width represents the estimated error range for the value. At and below pH 5, the onset potential is predicted by the Nernst equation (shown as grey diagonal lines). At and above pH 6, the data for scans at 1% and 100% H_2 converge, with the appearance of an overpotential requirement. Nonetheless, as observed in Figure 3.9B, the pH continues to affect the onset potential. The slope of a linear fit to all data for $pH \geq 6$ is 40 mV/pH unit.

For the purpose of comparison, similar experiments were conducted for Hyd-2, an O_2 -sensitive enzyme. A summary of the results is shown in Figure 3.13. Although Hyd-2 is normally characterised as a ‘reversible’ hydrogenase, here we see that alteration of the conditions to lower $E_{eq}^{H^+/H_2}$ causes this enzyme to act as an irreversible catalyst. Above pH 7, the onset potential begins to deviate from the Nernstian equilibrium potential; simultaneously, H_2 production activity is attenuated. The limiting potential, at which the onset deviates from the equilibrium potential, is roughly -420 mV *vs.* SHE, compared with a potential of -325 mV *vs.* SHE for Hyd-1. Here again, we are able to rule out the possibility that a lack of H^+ reduction activity is due simply to a lowering of substrate concentration as pH is increased, since this effect would not extend to the H_2 oxidation region in the form of an overpotential requirement. Thus, although Hyd-1 and Hyd-2, when compared at neutral pH, appear to have very different properties, here we see that both enzymes transition from irreversible to reversible catalysis as the pH is lowered; the principal difference is the value of $E_{eq}^{H^+/H_2}$ (and hence the pH) at which this transition occurs. The difference in observed properties is thus likely due to a difference, by as

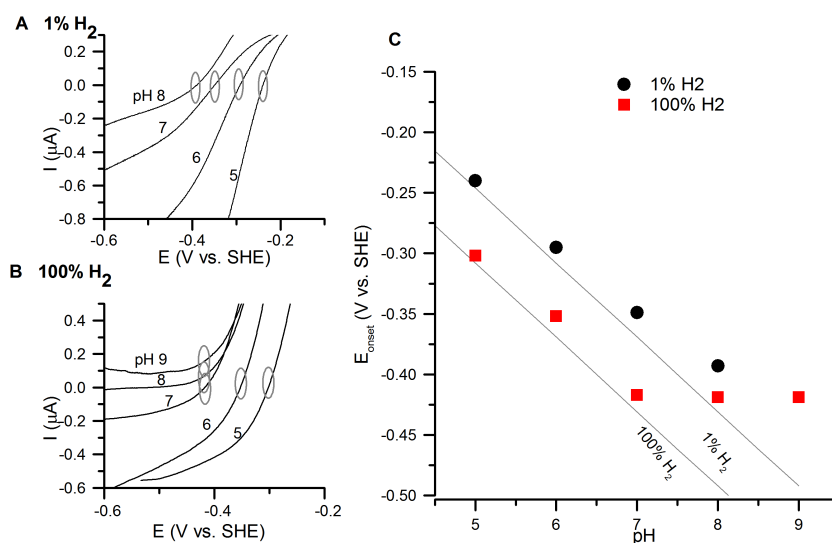


Figure 3.13: A summary of the onset potentials observed for Hyd-2 at varying pH and % H₂. Scans were carried out at 37 °C under a 1000 sccm flow of gas, $\omega = 2000$ rpm, scan rate = 10 mVs⁻¹. A) Scans under 1% H₂. B) Scans under 100% H₂. Forward and reverse scan currents were averaged, and the onset potential (marked with a grey oval) was assessed as follows: For scans in which significant reductive activity was visible, the zero-current intercept marks the onset of oxidation activity, and for scans in which little to no reductive activity is observed, the potential at which the slope of the CV deviates significantly from the slope of a blank scan marks the onset of oxidation activity. The error in this method is estimated as 0.01 V and the grey ovals are 0.02 V wide to indicate the assessed onset potential plus or minus estimated error. C) The onset potentials obtained from scans in A and B. The $E_{eq}^{H^+/H_2}$ for 1% and 100% H₂ are shown as diagonal grey lines.

little as 0.1 V, of the midpoint potential for the electrochemical control centre, thought to be the distal FeS cluster for these enzymes.

It is worth noting here that, once the $E_{eq}^{H^+/H_2}$ is made sufficiently positive to allow H_2 production, scanning to more negative potentials does not attenuate this activity (see for example Figure 3.11). This observation provides further unambiguous proof that the overpotential requirement is not due to redox transitions at the active site. If active site transitions were responsible for limiting activity at low potential, we would expect that, upon bringing $E_{eq}^{H^+/H_2}$ to a more positive potential, we would observe a window of reductive activity and a subsequent decrease in activity as the active site redox transition converted the enzyme to an inactive state. A rough simulation of this behaviour is shown in Figure 3.14. This demonstration was generated by applying a Nernstian one-electron reduction wave of active to inactive enzyme onto the CV recorded for WT Hyd-1 at pH 3, 1% H_2 . The midpoint potential used was -250mV, an estimate obtained by visual extrapolation of the irreversible onset potentials presented in Figure 3.12.

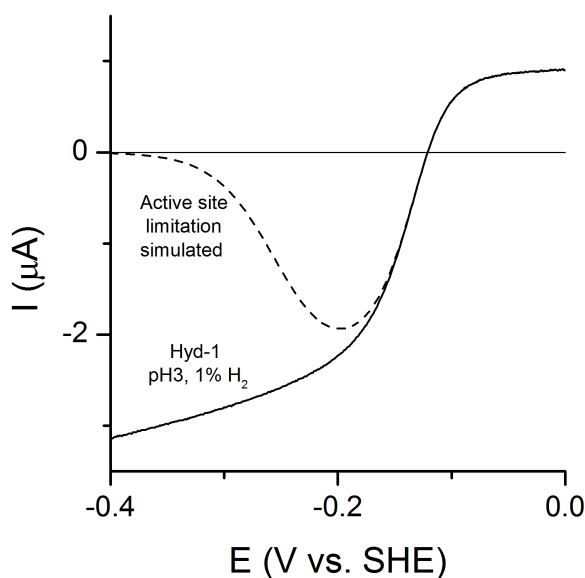


Figure 3.14: A rough simulation of the behaviour of Hyd-1, if activity at low potential were limited by an active site transition

A schematic is provided in Figure 3.15 to depict, qualitatively, the effect of increasing the $E_{eq}^{H^+/H_2}$ on the observed activity for Hyd-1, both for H_2 production (panel A) and

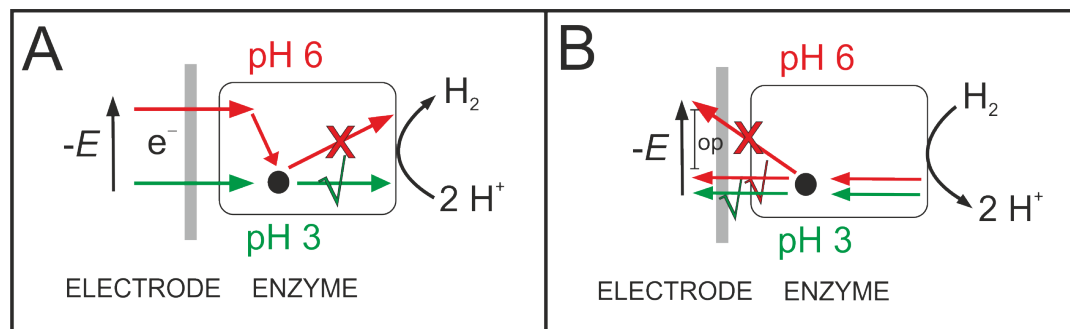


Figure 3.15: A schematic illustrating the effect of a high-potential electrochemical control centre upon catalytic activity, and the effect of changing pH (and thus $E_{eq}^{H^+/H_2}$) on observed catalysis. A) During H_2 production electrons must travel from the electrode to the active site while maintaining sufficient reducing power to drive H^+ reduction. B) During H_2 oxidation, electrons produced by catalytic turnover must be transferred from the enzyme active site to the electrode; ‘op’ signifies overpotential requirement. Figure courtesy of F. Armstrong.

H_2 oxidation (panel B). Note that, in this figure, the y-axis is $-E$, which is proportional to free energy, G . Thus, ‘downhill’ processes are spontaneous and ‘uphill’ processes are non-spontaneous. For H_2 production, electrons must travel from the electrode to the electrochemical control centre of the enzyme, while maintaining sufficient reducing power to drive H_2 reduction (ie. the electrons must be at a Fermi level below the equilibrium potential, $E_{eq}^{H^+/H_2}$). At pH 6, this is not the case, and H_2 production is therefore very slow, but by decreasing the pH to 3, thereby raising $E_{eq}^{H^+/H_2}$, the reducing power of an electron at the electrochemical control centre is sufficient to drive H^+ reduction. For H_2 oxidation activity (Figure 3.15B), electrons must travel from the enzyme to the electrode; in this case, interfacial electron transfer is very slow when the electrode potential is much lower than the potential of the electrochemical control centre. Near neutral pH, the effect of this limited electron transfer is seen above $E_{eq}^{H^+/H_2}$, leading to an overpotential requirement; at more acidic pH, the higher potential of $E_{eq}^{H^+/H_2}$ means that this limit is below the range in which H_2 oxidation is thermodynamically favoured, and thus minimal overpotential requirement is observed.

3.2.3 Assessing the reductive reactivation of Ni-B: The meaning of the switch potential, E_{switch}

The model of O₂-tolerance emphasises the importance of avoiding the formation of ‘Un-ready’ states upon reaction of O₂ at the active site (see Section 3.1.3); after this crucial step, however, there is still a one-electron reduction process, of Ni-B back to Ni-SI, that is necessary in order to resume activity. This reduction must be favourable at sufficiently high potential so that enzyme can reactivate in the continued presence of O₂, and must occur at a rate sufficient to compete with the rate of inactivation by O₂ [182, 96]. Indeed, site-directed mutagenesis studies with the O₂-sensitive hydrogenase from *D. fructosovorans* have shown that by increasing the rate of Ni-B reactivation, tolerance to O₂ is drastically increased [207]. As Ni-B is formed under both aerobic and anaerobic conditions, the formation and reactivation of Ni-B can be measured with standard CV experiments in the absence of O₂. The parameter E_{switch} serves as a measurement of the potential of reductive reactivation of the Ni-B species, as measured during a slow scan from high to low potential, following aerobic or anaerobic Ni-B formation. E_{switch} is defined as the minimum point in the first derivative of the voltammogram.

The interpretation of E_{switch} as a thermodynamic parameter has recently come under scrutiny [208], on the basis that the observed value is different from the mid-point potentials observed for the Ni-B to Ni-SI transitions using EPR and FT-IR-based potentiometric titrations, and due to the fact that the measured value for E_{switch} is dependent upon the scan rate and the extent of inactivation in the experiment. Fourmond et al. [208] therefore propose a strictly kinetic treatment of the inactivation and reactivation processes observable by PFE. As with the question of catalytic bias, it is useful to remember that, although the kinetics and thermodynamics of an observed process cannot be strictly correlated, a more in-depth treatment in which forward and reverse rates of a process are evaluated will give thermodynamic information.

In order to illustrate this, a slight modification of the E_{switch} experiment is demonstrated. Some of the difficulty in evaluating the reactivation process directly from the CV comes from the fact that the shape of the reactivation trace is a function of two separate effects. As discussed in section 2.3, the heterogeneity of enzyme orientation on the electrode means that as the overpotential increases, interfacial electron transfer becomes favourable for a larger number of enzyme molecules, giving rise to a persistent slope in the oxidative current, termed ‘dispersion’. This basic waveshape can be seen at high scan rates, where inactivation processes are too slow to be seen. The reactivation trace, at slow scan rate, is a superposition of the fraction of active enzyme, which increases as the scan sweeps to successively lower potentials, and the ‘dispersion’ slope due to heterogeneity of interfacial electron transfer distance. Figure 3.16 illustrates the deconvolution of these two factors.

Following anaerobic inactivation at high potential, a slow (0.1 mVs^{-1}) scan toward low potential traces the reactivation profile seen in black in Figure 3.16A. Immediately following this scan, a fast scan (200 mVs^{-1}) in which no inactivation is observed traces out the turnover as a function of potential, when all enzyme is active. This scan, shown with dashed lines, exhibits a very large capacitance current due to the fast scan rate used; this can be eliminated by taking the average of the forward and reverse scans, shown in blue. Traditional analysis involves taking the derivative of the slow scan, shown in panel B. The minimum of this trace is E_{switch} . If instead of using the first derivative we divide the reactivation trace by the averaged fast trace we get the fraction of active enzyme, shown in panel C in black. Displaying the data this way allows the analogy to FT-IR or EPR titration data to be more clearly expressed.

The fraction of active enzyme can be fit with a sigmoidal function,

$$I = I_{min} + \frac{I_{total}}{e^{\frac{nF(E-E_{mid})}{RT}} + 1} \quad (3.3)$$

where $I_{min} = 0.07$, $I_{total} = 0.7$, $E_{mid} = 0.162\text{V}$, and $n = 0.95$

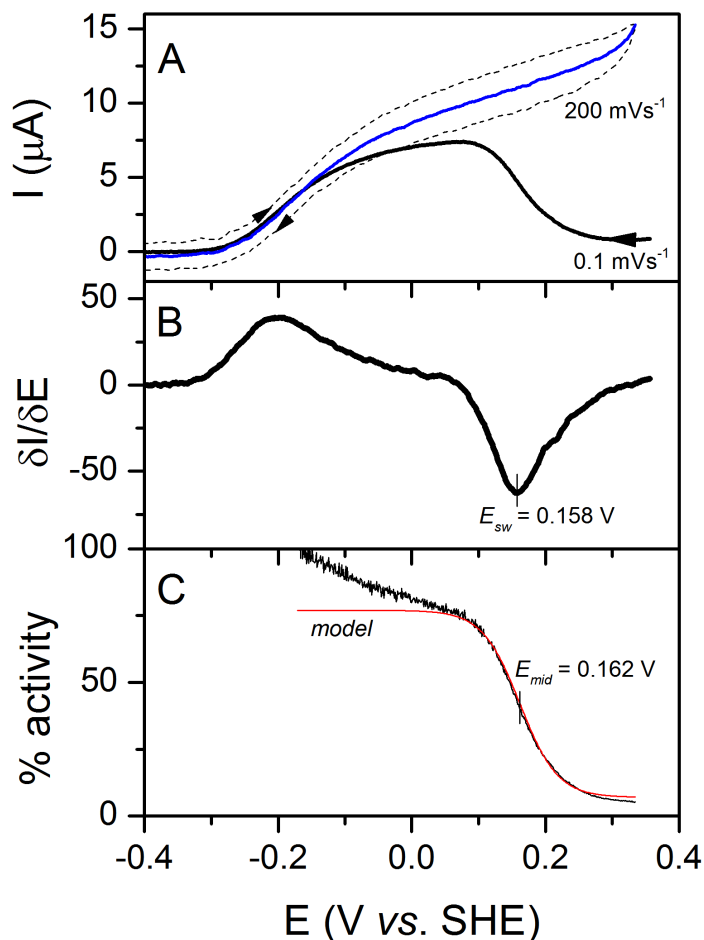


Figure 3.16: A novel method of visualising the E_{switch} experiment, providing evidence that E_{switch} is the midpoint potential of a one-electron reduction of the Ready state. A) A 0.1 mVs^{-1} linear scan was carried out following inactivation at high potential; a 200 mVs^{-1} scan was carried out immediately afterward. Both scans were carried out at pH 7, 37°C , $100\% \text{ H}_2$, $\omega = 2000 \text{ rpm}$. B) The first derivative of the slow scan reveals an E_{switch} of 0.158 V . C) Dividing the slow linear scan by the average of the fast scan allows the data to be visualised as % active enzyme. Fitting this curve with a Nernst curve gives a mid-point potential for the reactivation of 0.162 V .

The sigmoid shown in red, panel C has a mid-point potential of 0.162 V vs. SHE . The traditional definition of E_{switch} , that is, the minimum of the first-derivative of the reactivation scan, shown in panel B differs by only 5 mV , at 0.157 V vs. SHE . This experiment serves as a useful demonstration that the reactivation trace, although convoluted by the effect of potential on total turnover rate, does still demonstrate a conventional reduction step, with $n \simeq 1$. As with other techniques, an accurate assessment of the mid-point potential relies on the system being at equilibrium when measurements are taken; in the case of a PFE scan, this entails the use of a very slow scan rate. Thus, although

the scan rate dependence of E_{switch} has been suggested as evidence that this is not a parameter with thermodynamic significance [207], in fact this is merely a reminder that thermodynamic information must come from a system at equilibrium.

Closer scrutiny of the proportion of active enzyme shown in Figure 3.16C suggests that, upon anaerobic inactivation, more than one inactive species is formed. The fraction of enzyme recovered in the first wave, roughly 75%, corresponds to Ni-B, whereas the different potential for reactivation of the remaining fraction of enzyme points to a second state. When inactivation and reactivation processes are treated kinetically [208] and the rates are used to create a model of a voltammogram for *A. aeolicus* Hase I (Figure 5 of ref. [208]), the model most deviates from experimental data in this same region. Thus, it seems that a second oxidised inactive species may be formed for both *E. coli* Hyd-1 and *A. aeolicus* Hase I. FT-IR studies of Hase I [200] and *R. eutropha* MBH [80] indicate that these enzymes form a small proportion of a second EPR-visible readily activated oxidised state whose structural nature is unknown. This state has been tentatively assigned as Ni-X.

3.2.4 Hyd-1 exhibits a very low Michaelis constant, K_M , for H_2 oxidation

The affinity of O_2 -tolerant [NiFe] hydrogenases for H_2 , measured using the Michaelis-Menten constant, K_M , tends to be lower than that observed for standard hydrogenases. Defined as the substrate concentration at which the turnover rate is one-half the maximum (ie. substrate-saturated) rate, this parameter can also be expressed as a function of steady-state rates of substrate association, dissociation and catalysis at the active site:

$$K_M = \frac{k_{dissociation} + k_{catalysis}}{k_{association}} \quad (3.4)$$

For redox processes, the value of $k_{catalysis}$ is dependent upon potential; thus, we would expect the value of K_M to vary with potential, a fact that is all too frequently ignored in the literature, where values of K_M are quoted without a specified potential, or are compared between experiments carried out at vastly different potentials. Figure 3.17 summarises two methods for determining K_M by PFE, the first (panel A) is the standard chronoamperometry-based method widely used by our group as well as others. The second method (panel B) is one I have developed. This method, based upon cyclic voltammetry scans at varying substrate concentrations, takes roughly the same amount of time as the chronoamperometry method but presents two distinct advantages. First, this method allows the K_M to be calculated across the relevant range of potentials in a single experiment, allowing us to examine the potential-dependence of this variable and to compare to experiments on other systems, regardless of the potentials at which those experiments were conducted. Second, this method allows the experimenter to visually assess each voltammogram for mass-transport limitation at low substrate concentrations, something which is immediately apparent from a voltammogram but is impossible to assess from a chronoamperogram.

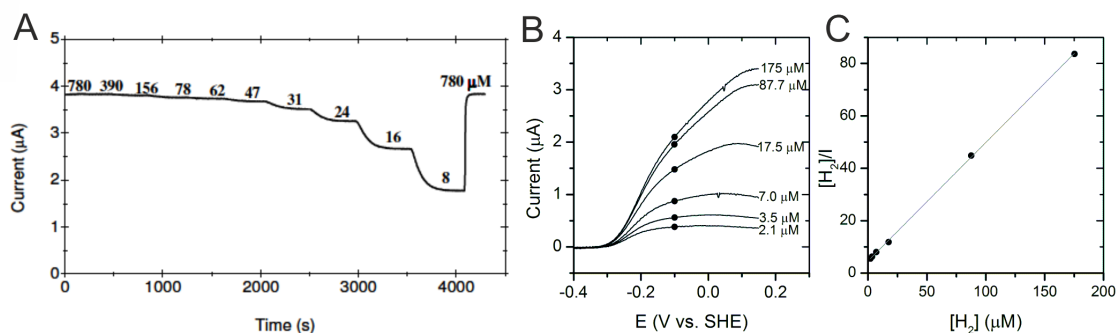


Figure 3.17: A comparison of methods for determining K_M A) Chronoamperometric method, reproduced from [209]. Carried out at 30 °C, pH 6 under a gas flow of 1000 sccm, $\omega = 5000$ rpm, electrode potential -0.175 V vs. SHE B) Cyclic voltammetry-based method, carried out at 45 °C, pH 6, scan rate 1 mVs⁻¹, $\omega = 5500$ rpm. At any given potential, the currents recorded at different [H₂] are used to calculate the K_M at that potential. The data at -0.1 V are demonstrated by ● as an example C) Hanes analysis of data selected from B.

Using the cyclic voltammetry method described above, the $K_M^{H_2}$ of Hyd-1 was assessed as a function of potential, and the effects of pH and temperature were also assessed. These data are summarised in Figure 3.18.

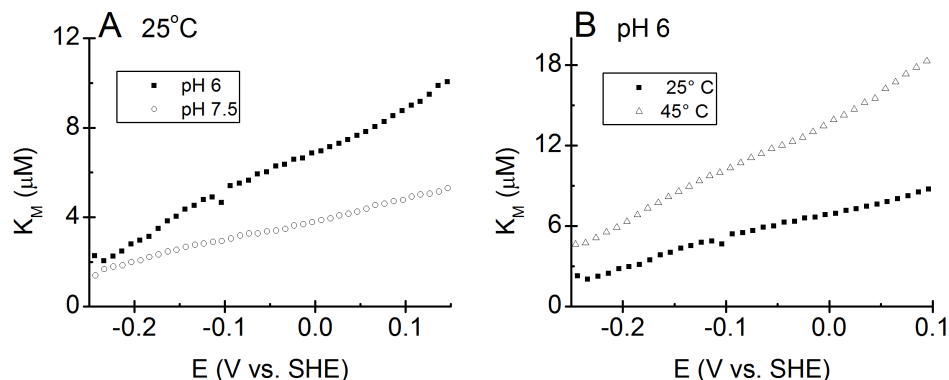


Figure 3.18: The potential-dependence of $K_M^{H_2}$ for Hyd-1. All experiments were conducted under a gas flow of 1000 sccm at a scan rate of 1 mVs^{-1} , $\omega = 5500 \text{ rpm}$. Film loss was monitored throughout the experiment and currents were corrected accordingly.

The $K_M^{H_2}$ of Hyd-1 increases as a function of potential, an effect of the increasing value of $k_{catalysis}$ with increasing driving force. The effect of pH, assessed between pH 6 and 7.5, is minimal at low driving force and grows more important as potential is increased. In other words, at low driving force, additional H^+ has no effect on the $K_M^{H_2}$, as electron transfer likely limits $k_{catalysis}$, rather than it being limited by a chemical step such as product release. The effect of temperature on $K_M^{H_2}$ seems to be roughly consistent across potentials, with approximately double the value at 45 °C as at 25 °C.

3.2.5 Hyd-1 demonstrates a high tolerance to inhibition by CO

Carbon monoxide (CO) is a textbook competitive inhibitor of hydrogenase enzymes [210, 211, 85, 212, 86]. Being of similar size to O_2 , CO provides a good model compound to test for access of O_2 to the active site. CO is widely believed to bind at the same site in [NiFe] hydrogenases as H_2 and O_2 , a belief which is supported by the fact that CO protects against inactivation by O_2 and can aid recovery of O_2 -inhibited states [213, 174], presumably by displacing the hydroxo- or peroxo- ligand at the active site [174]. The

extent of inhibition by CO is often described as an inhibition constant, which tends to be in the 1-10 μM range for ‘standard’ [NiFe]-hydrogenases [44]. O_2 -tolerant enzymes show minimal inhibition by CO [179, 44, 86]. To date, it is unclear why O_2 -tolerance should be correlated with low sensitivity to CO inhibition.

Given a $K_M^{\text{H}_2}$ of $13 \pm 2 \mu\text{M}$ under these conditions, we can calculate a K_I^{CO} of $90 \pm 6 \mu\text{M}$ for Hyd-1.

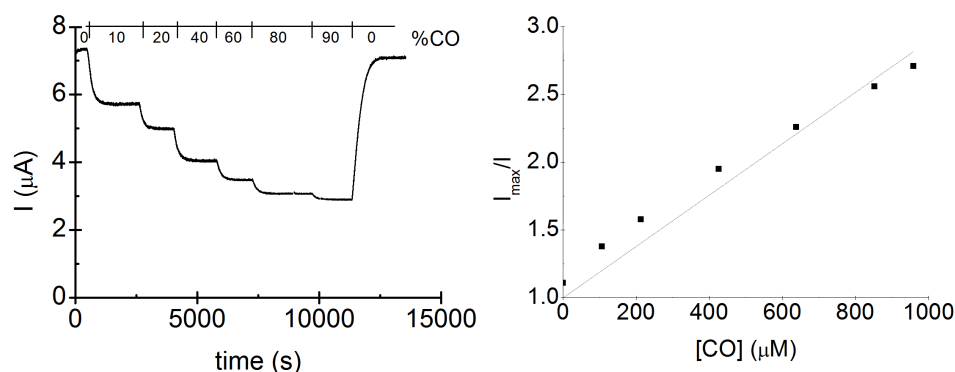


Figure 3.19: The effect of increasing concentrations of CO on H_2 -oxidation current by Hyd-1, pH 5.5, 30 $^\circ\text{C}$, 10% H_2 and -0.1 V vs. SHE.

3.3 Perspectives

This chapter has demonstrated that the overpotential requirement for Hyd-1 and the associated lack of reductive activity are likely related to changes at a redox centre that is not itself involved in catalysis. We suggest that, at low potential, interfacial electron transfer becomes very slow, limiting the ability of Hyd-1 to catalyse H_2 production at low overpotential, and H^+ reduction at any overpotential, when experiments are carried out near neutral pH. For these experiments, the onset potential depends on the availability of protons but not of H_2 (Fig. 3.9), supporting the hypothesis that the rate-limiting step, predicted to be interfacial electron transfer, is occurring at a site whose potential shifts upon protonation. The histidine-ligated distal cluster is a candidate for this centre. For the homologous enzyme from *A. aeolicus*, this histidine is predicted to have a pK_a of 7.1 [214], and the pH-dependence of the redox transition for histidine-

ligated clusters in other proteins has previously been described [185]. The important role of the distal cluster in determining catalytic bias has previously been demonstrated by Dementin et al who found that mutation of the His ligand of the distal cluster of *D. fructosovorans* [NiFe] hydrogenase decreased H₂ oxidation activity significantly, while the H₂ production capability was only mildly affected. H₂ production activity could be restored to an extent by the addition of imidazole [215]. In the mutant lacking histidine, titration of the protein film with imidazole increased the overall current for H₂ oxidation but not for H₂ production; however the onset potential of H₂ production was shifted to higher potential (less overpotential requirement) with increasing imidazole. Results presented here, in combination with the mentioned experiments in *D. fructosovorans*, lend support to a model developed by Hexter *et al.* [59], which states that the potential of an ‘electrochemical control centre’, the cluster at which electrons enter or leave the enzyme, affect the bias toward activity in one direction over another. It is also worth considering the possibility that a haem group of the cytochrome *b* serves as the point of electron entry and exit to and from the enzyme. Haem groups are well known to exhibit a pH-dependent redox potential [205]. A better characterisation of both the distal cluster and the cytochrome subunit will be necessary to determine which of these centres is responsible for the observed bias and overpotential.

It is worth noting here that the literature reflects a widely-held belief that overpotential requirement and bias are separate properties; for example, Abou Hamdan and colleagues [216] suggest that the rate of H₂ oxidation is limited by electron transfer while the rate of H₂ production is limited by H₂ efflux from the active site. This belief is not reflective of our understanding that a catalyst changes the activation energy of a reaction without altering the position of equilibrium, *i.e.*, that the ratio of forward to reverse rates is set exclusively by the driving force.

It is possible that the demonstration of H₂ production by Hyd-1 has some physiological relevance, and recent studies have shown that *E. coli* strains knocked out for

Hyd-2 and Hyd-3 activity exhibit some H_2 production at low pH during fermentation on glycerol [217, 107]. It may be worth speculating that, in the case of extreme acid stress, and assuming availability of reducing equivalents, the emergence of H_2 production activity would serve as a means of eliminating protons from the medium, and that Hyd-1 would be capable of mediating this H_2 production activity. Perhaps more interesting is the updated comparison that this work allows between Hyd-1 and Hyd-2. Numerous studies show that Hyd-2 is maximally expressed at mildly basic pH (~ 7.5), whereas Hyd-1 is maximally expressed at acidic pH (~ 5.5) [218]. Comparing the two enzymes under their respective conditions of maximum expression, their properties are much more similar than can be seen by comparing them under identical conditions: the overpotential requirement is reduced to nearly zero and H_2 production activity is low but present. There is no evidence that the other O_2 -tolerant hydrogenases, which also show an overpotential requirement and a lack of H_2 production activity, are expressed under especially acidic conditions; therefore it seems unlikely that these properties have evolved to mediate optimal activity at low pH.

In the context of the results presented in this chapter, we wish to consider the question: ‘Is it possible to have an O_2 -tolerant hydrogenase that produces H_2 effectively?’ Given the seemingly essential role played by high-potential redox cofactors in mediating O_2 tolerance, and in light of the fact that the potentials of these clusters seem to limit effective electron transfer below a given potential, it may be that the mechanism of O_2 -tolerance seen for the MBHs including Hyd-1 and enzymes from *Ralstonia* species is incompatible with the ability to produce H_2 efficiently, at least near neutral pH. This topic will be further addressed in Chapter 6.

A small amount of attention was paid in this chapter to the other properties that are correlated with O_2 -tolerance in $[\text{NiFe}]$ hydrogenases, namely a high E_{switch} potential and a resistance to inhibition by CO. We wish only to reiterate that E_{switch} is a thermodynamic parameter corresponding to the midpoint potential of a one-electron reduction

of the oxidatively inactivated Ni-B state. Because of the effect of this reactivation on the ability of an enzyme to maintain a given fraction of active enzyme under exposure to O_2 , this parameter has relevance to O_2 tolerance as would be suggested by its strict correlation with O_2 -tolerance among the [NiFe] MBHs.

Future work to characterise the potentials of the distal cluster and cytochrome haems would allow a better assessment of whether these centres are indeed responsible for limiting catalysis at low potential. It will also be important to determine whether the potential of this centre does indeed contribute to O_2 tolerance directly or is rather correlated with O_2 -tolerance due to a coevolution of the potentials of all clusters for maximum rates of electron transfer. Differences at the active site suggested by the high tolerance to CO remain poorly understood, and further characterisation of the states of the active site will play an important role in understanding how the catalytic core is tuned for resistance to O_2 .

Chapter 4

Controlled electron transfer through
covalent linkage of *E. coli* Hyd-2 to
surfaces and molecules by
genetically-inserted cysteine
residues

4.1 Introduction

The protein film electrochemistry techniques applied in Chapter 3 are able to reveal many aspects of the potential-dependent behaviour of redox enzymes. However, some information regarding electron transfer cannot be obtained by PFE. The current passed to each enzyme molecule is controlled indirectly by the application of a potential at the electrode, and cannot be monitored or controlled on a per-molecule basis since the number of enzyme molecules in electronic contact with the surface is unknown. Furthermore, the non-specific attachment of enzyme molecules to the electrode surface result in a dispersion of interfacial electron transfer distances [219], leading to uncertainty in this step. This chapter presents experiments designed to complement PFE techniques by circumventing the problems of nonspecific interaction and ‘uncountable’ electron transfer.

For these experiments, Hydrogenase-2 of *E. coli* was chosen, due to the fact that it is easily purified and that shows reversible catalysis at neutral pH. Hyd-2 seems to be a ‘standard’ hydrogenase whose properties closely match those seen for enzymes from *Desulfovibrio* species [44]. These experiments follow a model set forth for the study of other redox-dependent proteins [220, 221]. By site-directed mutagenesis, we have inserted convenient amino acid ‘handles’ to allow two different types of experiment: attachment to electrode surfaces for well-defined and uniform electron transfer dynamics, and attachment of molecular photosensitizer molecules for light-stimulated electron donation into the FeS cluster chain.

4.1.1 Properties of Ru-based photosensitizers

Since the early days of coordination chemistry, the intense, vibrant colouration of most transition metal complexes arising from Metal-to-ligand charge transfer (MLCT) and Ligand-to-metal charge transfer (LMCT) reactions has served both as a diagnostic fea-

ture of complexes and as part of the allure of the field itself. Characteristic MLCT absorption bands correspond to the excitation of an electron from a molecular orbital (MO) with primarily metal character into a low-lying unoccupied MO with mostly ligand character, most commonly π^* orbitals [57]. Most of these transitions are very short-lived, with charge recombination occurring on the order of ns, but complexes of Os^{2+} and Ru^{2+} bearing aromatic diimines show lifetimes on the order of μs [222, 223], long enough that the complex becomes a useful photochemical redox reagent which can formally be considered as $\text{M}^{3+}\text{L}_2\text{L}^-$, where M stands for the metal and L for the ligand. The most commonly used aromatic ligands are 2,2'-bipyridine (bpy) and 1,10-phenanthroline (phen).

The electron residing on the ligand can be transferred to an external electron sink, such as a semiconductor electrode in dye-sensitized solar cells [224] or to a redox cofactor of a protein. In the presence of a sacrificial reductant (whose role is to reduce the metal), two competing pathways for this electron transfer exist, and their ratios are determined to a large extent by the concentrations of the various system components. If the $\text{M}^{3+}\text{L}_2\text{L}^-$ complex first donates an electron, the driving force for reduction is lower, although still rather strong ($E = -0.86 \text{ V vs. SHE}$ in the case of $[\text{Ru}(\text{bpy})_3]^{2+}$); alternatively, if a soluble sacrificial reductant first reduces the metal to give a $\text{M}^{2+}\text{L}_2\text{L}^-$ complex (referred to as 'reductive quenching'), the driving force is greater ($E = -1.26 \text{ V vs. SHE}$ for $[\text{Ru}(\text{bpy})_3]^{1+}$), and the possibility of charge recombination is reduced as the sacrificial reductant diffuses out of electron transfer distance. Figure 4.1 presents a scheme of reductions and oxidations following photoexcitation of a photosensitizer, using $[\text{Ru}(\text{bpy})_3]^{2+}$ as an example. The electron donor 'A' (for ascorbate) is oxidised to 'A•' (for ascorbyl radical) and the enzyme is reduced from 'E' to 'E-'.

The excitation of the photosensitizer, its quenching and its reoxidation, as well as recombination, can all be monitored by absorption spectrophotometry with nanosecond time resolution in order to monitor the kinetics of electron donation; difference spectra

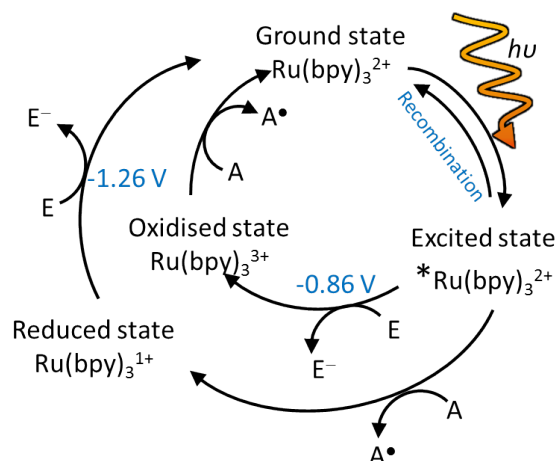


Figure 4.1: A scheme of the pathways available upon photoexcitation of a $[\text{Ru}(\text{bpy})_3]^{2+}$ molecule. ‘A’ represents the sacrificial electron donor, ascorbate. ‘E’ represents the enzyme molecule.

for the excited and quenched (reduced) states, relative to the ground state, are presented in Figure 4.2. Thus, in addition to providing a powerful reductant, the photosensitizer molecule also provides a spectroscopic readout of the state of the system. For experiments in which photosensitizers are used for reduction of other species such as proteins, a high concentration of sacrificial reductant (‘quencher’) is used to ensure most of the photosensitizer is converted to the reduced form. Upon reductive quenching, a soluble radical is formed. This radical can recombine with the reduced photosensitizer, but the rates for this process are reduced due to the fact that the radical often diffuses out of electron transfer distance after quenching.

4.1.2 Electron transfer studies in redox proteins

Functionalisation of one of the ligands of a Ru-based photosensitizer allows for the complex to be attached to the surface of a protein via ‘click’ chemistry [225] with an exposed amino acid residue. If the photosensitizer is positioned sufficiently near to a reducible centre of the protein, an electron can be transferred to (or ‘injected’ into) the centre, and spectroscopic changes at both the Ru photosensitizer complex and at any coloured redox cofactor can be monitored as a means of assessing the rates of electron

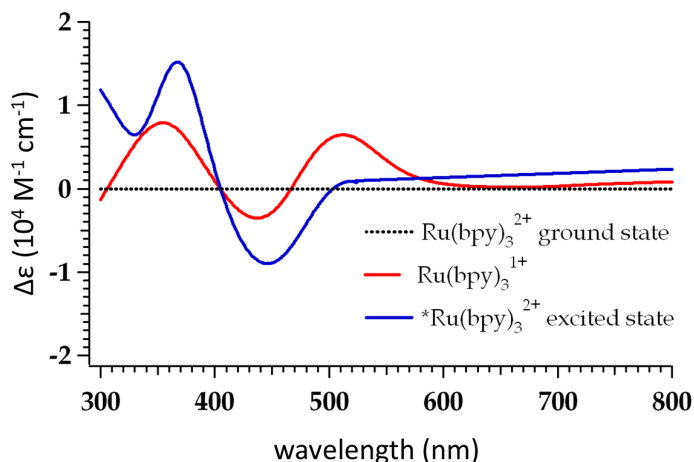


Figure 4.2: Difference spectra for the excited and quenched states of $\text{Ru}(\text{bpy})_3$ relative to the ground state. Data collected by and figure courtesy of D. Streich, Uppsala University, Sweden.

transfer between centres. This approach was pioneered by Harry Gray to study the kinetics of cytochrome *c* reduction [226] as well as electron transfer between *c*-type cytochromes and their redox partners [227, 228, 220]. More recently, attachment of Ru-based photosensitizers to nitrogenase enzyme has allowed, for the first time, activation of nitrogenase for catalysis in the absence of ATP and Iron-protein [221]. The approach has also been used to power reduction of O_2 at a multicopper oxidase [229], as well as fatty acid hydroxylation at a cytochrome P450 [90]. A conceptual representation of the experiment for a specifically-labelled hydrogenase enzyme is presented in Figure 4.3.

Electron transfer to the FeS cluster should be detectable by changes in the lifetime of the reduced photosensitizer species. It is also possible that we may observe reduction of the individual cofactors. Figure 4.4 shows an estimate of the reduced minus oxidised spectra for a Fe_4S_4 cluster and for a synthetic mimic of the [NiFe] active site.

During the course of this work, Zadvornyy *et al.* [232] published an experiment in which they labelled the [NiFe]-hydrogenase from *Thiocapsa roseopersicina* with Ru-based photosensitizers via non-specific NHS-mediated coupling to exposed amino groups. This system did not produce measurable levels of H_2 unless mediator was added to the system, suggesting that low rates for electron transfer from the photosensitizer to the electron

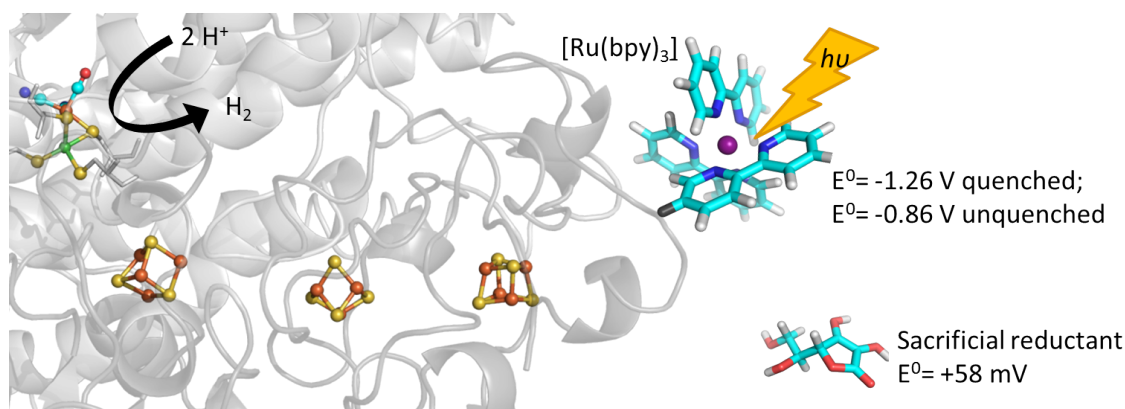


Figure 4.3: A conceptual representation of the photosensitizer labelling experiment. Light-induced metal-to-ligand transfer gives a species with a reduction potential of -0.86 V; in the presence of a sacrificial reductant, such as ascorbate, the Ru³⁺ is reduced to Ru²⁺, yielding a complex with a reduction potential of -1.26 V. The photosensitizer molecule is covalently attached within electron transfer distance of the distal or medial FeS cluster, allowing electron injection into the cluster.

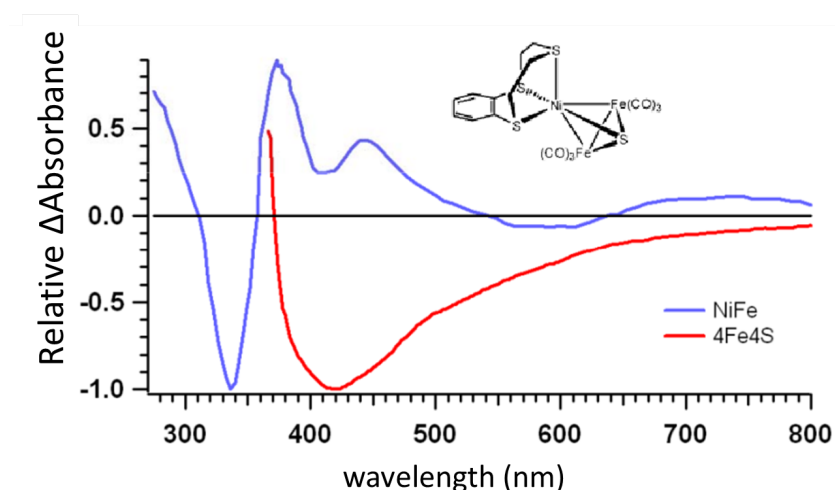


Figure 4.4: Estimated absorbance changes upon reduction of [NiFe] and Fe₄S₄ cluster cofactors. Figure courtesy of D. Streich according to references [230, 231]

relay of the enzyme limited the effectiveness of the system. The approach we have taken aims to attain specific localisation of the photosensitizer on the surface of the molecule in order to more directly ‘inject’ electrons from the photosensitizer to the FeS cluster relay, thus giving information about the kinetics of transfer as well as promoting higher rates of turnover without the need for mediator.

A specifically engineered site of attachment also has precedent as a useful tool for electrochemical experiments. The thiol group of an exposed cysteine residue quickly and spontaneously forms a bond with a gold electrode surface to give a population of enzyme with homogenous interfacial electron transfer distances [233]. It is also possible to create a connection to an electrode by decorating the electrode surface with a click reagent specific to a given amino acid functionality [234]. An exposed cysteine, present near the haem group in the wild-type form of yeast cytochrome *c*, has made this protein a natural case study for the effects of specific adsorption on interfacial electron transfer. These studies have shown that near-complete surface coverage can be obtained using this technique, with fast electron transfer rates. Some critical properties such as haem group reduction potential are seen to differ when measured for specifically attached protein relative to non-specifically adsorbed protein, suggesting that more rapid rates of electron transfer for this system may give a more accurate assessment of its properties [233]. Different orientation of protein molecules on a surface leads to measurable differences in the rate of electron transfer to and from the cluster [235]. Thus, it is possible that mutations designed to allow modification with a photosensitizer molecule may also allow the enzyme to be attached to an electrode surface in a more controlled manner.

4.2 Results and Discussion

4.2.1 Light-driven H_2 production by wild-type Hyd-2

We began by carrying out measurements on wild-type Hyd-2 in solution with free $\text{Ru}(\text{bpy})_3\text{Cl}_2$, in order to assess whether excited photosensitizer complex could donate electrons to Hyd-2. Purified wild-type Hyd-2 in pH 6 100 mM acetate buffer (150 mM NaCl, 1 mM DTT, 0.02% DDM) was mixed with varying concentrations of $\text{Ru}(\text{bpy})_3\text{Cl}_2$ in the presence of 200 mM ascorbate as quencher. The sample was illuminated and H_2 measurements were taken according to Section 2.8. This experiment was carried out by D. Streich, Uppsala University, Sweden.

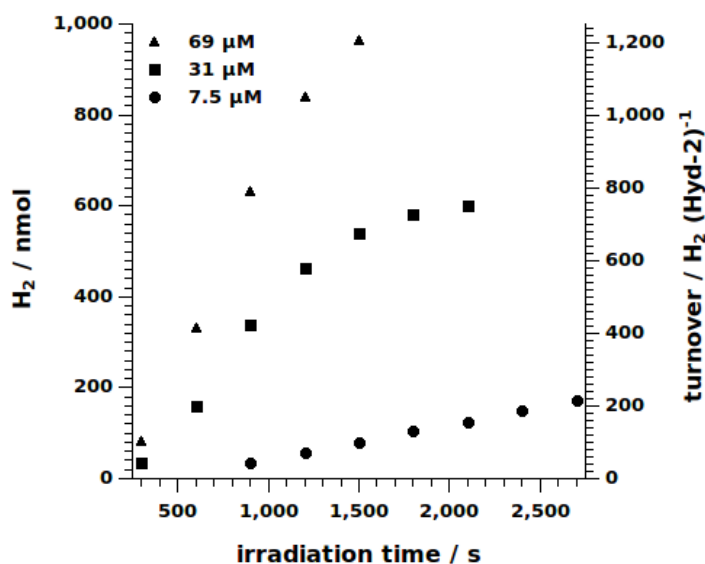


Figure 4.5: Light-driven H_2 production by wild-type Hyd-2 in pH 6.0 0.1 M sodium acetate buffer, 0.15 M NaCl, 1 mM DTT, 0.02% DDM, with varied concentration of $\text{Ru}(\text{bpy})_3\text{Cl}_2$ ($\blacktriangle$) 7.5 μM ; ($\blacksquare$) 31 μM ; ($\bullet$) 69 μM . Irradiation was approximately 80 nmol absorbable photons $\text{cm}^{-2} \text{s}^{-1}$; 200 mM ascorbate, pH 7.5, was added as quencher. Data collected by and figure courtesy of D. Streich, Uppsala University, Sweden.

The observed rate of H_2 production (maximally 1.1 s^{-1}) is quite a respectable rate for such a system, suggesting that electron transfer from the excited $\text{Ru}(\text{bpy})_3\text{Cl}_2$ complex is reasonably good. The dependence on photosensitizer concentration suggests that the limiting factor is likely to be the availability of reduced photosensitizer. The specific H_2 production activity of the enzyme measured by dark assay with reduced MV before

and after the photoproduction experiments shows a higher rate of turnover than that observed for light-dependent H_2 production (approximately 5 s^{-1}), which is unchanged throughout the course of the experiment, supporting the conclusion that the system is not limited by enzyme turnover. This also suggests that the lower rates observed toward the end of the experiment are not likely due to degradation of the hydrogenase, but may be due to accumulation of H_2 in the cuvette headspace or to breakdown of the $\text{Ru}(\text{bpy})_3\text{Cl}_2$ molecule. Changes in the steady-state absorption profile during the course of the experiment support the suggestion that some photosensitizer may be degraded (data not shown).

Quantum yields for this system were on the order of 5% (see Table 4.1), a significant improvement on the values reported for a similar system in which *T. roseopercina* [NiFe] hydrogenase was covalently labelled at exposed lysine residues [232], a system which required addition of soluble mediator to produce H_2 and which reported a quantum efficiency of 0.02%. The rates of H_2 production and quantum yields for different concentrations of $\text{Ru}(\text{bpy})_3\text{Cl}_2$ are given in Table 4.1.

$[\text{Ru}(\text{bpy})_3\text{Cl}_2] (\mu\text{M})$	Turnover rate (s^{-1})	Quantum Yield (%)
7.5	0.095	4.7
31	0.61	6.5
69	1.1	5.7

Table 4.1: The rate and quantum yield for light-driven H_2 production by WT Hyd-2 for varying concentrations of $\text{Ru}(\text{bpy})_3\text{Cl}_2$.

4.2.2 Redox-dependent changes in wild-type Hyd-2 detected by UV-Vis absorption spectroscopy

A photoexcited electron should pass through the FeS clusters of the enzyme in order to reach the active site for H_2 production, and it may be possible to monitor the subsequent reduction steps as the electron passes through the cluster by time-resolved UV-Vis absorption spectroscopy. Fe_4S_4 and Fe_3S_4 clusters both show significant absorption changes

upon reduction ($\Delta\epsilon \approx 2000 \text{ M(Fe)}^{-1} \text{ cm}^{-1}$ [236]) with distinct ΔA maxima (see Figure 4.6, inset). In order to assess these absorption changes, aerobically-isolated Hyd-2 was exposed to a stream of 100% H_2 with stirring and changes in absorption were monitored over time (Figure 4.6).

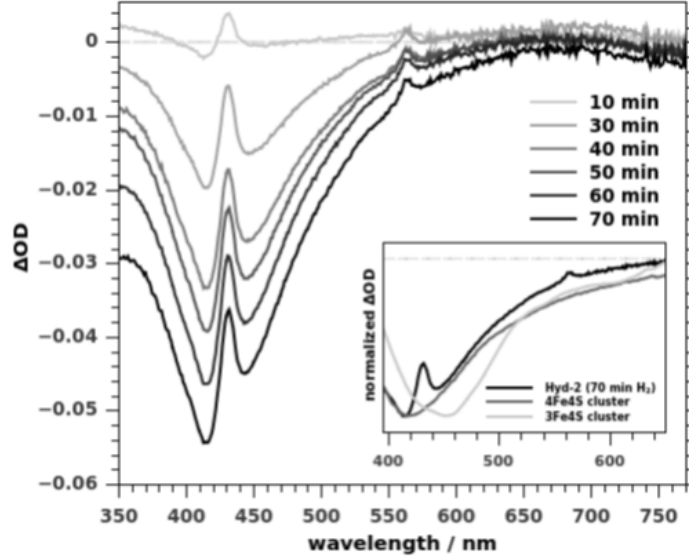


Figure 4.6: Changes in the UV-Visible absorption spectrum for aerobically isolated wild-type Hyd-2, before and after reduction with 100% H_2 , corrected for scattering. Inset: Normalised absorption changes, compared with reported normalized difference spectra for Fe_4S_4 and Fe_3S_4 clusters from other peptides [237, 238]. Experiment carried out by and figure courtesy of D. Streich, Uppsala University, Sweden.

The observed changes in absorbance ($\lambda_{\Delta A_{\text{max}}} \approx 420 \text{ nm}$) correspond well with reduction of Fe_4S_4 clusters, with little or no contribution from Fe_3S_4 clusters. This is most simply explained by the assumption that the Fe_3S_4 cluster is already reduced in the as-isolated sample, owing to its high reduction potential (+65 mV at pH 8 for *D. fructosovorans* [193] and 70 mV at pH 7 for *D. gigas* [192]; data not available for Hyd-2). It is also possible that the absorption changes for the Fe_3S_4 cluster of Hyd-2 are indistinguishable from those for Fe_4S_4 clusters, but this is unlikely. Sharp positive signals are observed at 430 nm and 560 nm and are believed to correspond to the Soret- and Q-bands of low levels of *c*-type cytochrome co-purifying with Hyd-2.

4.2.3 Time-resolved spectroscopy shows electron injection to wild-type Hyd-2

In order to assess the efficiency of electron injection into WT Hyd-2, time-resolved absorption spectroscopy was used to monitor the state of the Ru photosensitizer after excitation with a pulse of laser light. At 510 nm, an absorption maximum for the reduced state, $[\text{Ru}(\text{bpy})_3]^{1+}$, occurs within an isosbestic region for the spectra of the ground state $[\text{Ru}(\text{bpy})_3]^{2+}$ and excited state $^*[\text{Ru}(\text{bpy})_3]^{2+}$, thus monitoring at this wavelength allows the concentration of reduced state $[\text{Ru}(\text{bpy})_3]^{1+}$, $\Delta\epsilon = 6700 \text{ M}^{-1} \text{ cm}^{-1}$ [239]) to be assessed (see Figure 4.2 for difference spectra of $\text{Ru}(\text{bpy})_3$ species; emission from the excited state artificially turns the isosbestic point at 500nm into an isosbestic region from 490nm to 510nm). When a solution of $\text{Ru}(\text{bpy})_3\text{Cl}_2$ with ascorbate as quencher is irradiated as described in Section 2.9, the photo-generated excited state is quenched with an efficiency of 77% (data not shown), producing reduced $[\text{Ru}(\text{bpy})_3]^{1+}$. This species is dissipated by charge recombination with ascorbyl radical and by electron donation to a soluble electron acceptor, such as Hyd-2 enzyme. Figure 4.7 shows the depletion of the $[\text{Ru}(\text{bpy})_3]^{1+}$ state in the presence (red dots) and absence (black dots) of WT Hyd-2.

At short timescales ($< 100 \mu\text{s}$), the high concentrations of $[\text{Ru}(\text{bpy})_3]^{1+}$ and ascorbyl radical (henceforth denoted $\text{A}^\bullet$) cause recombination to dominate ($k_{\text{rec}} = 4 \pm 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$), so that the traces for sample with and without enzyme effectively overlap. At longer timescales, the curves diverge due to falling concentrations of $[\text{Ru}(\text{bpy})_3]^{1+}$ and $\text{A}^\bullet$, allowing electron injection to compete with recombination. The low concentrations of $[\text{Ru}(\text{bpy})_3]^{1+}$ and $\text{A}^\bullet$ on longer timescales allow us to assume that the concentration of oxidised Hyd-2 is effectively unchanged at these timescales. Further, we assume that the increase in the rate of depletion in the presence of Hyd-2 is due exclusively to reduction of Hyd-2 and that the rate of recombination of $[\text{Ru}(\text{bpy})_3]^{1+}$ and $\text{A}^\bullet$ is unchanged

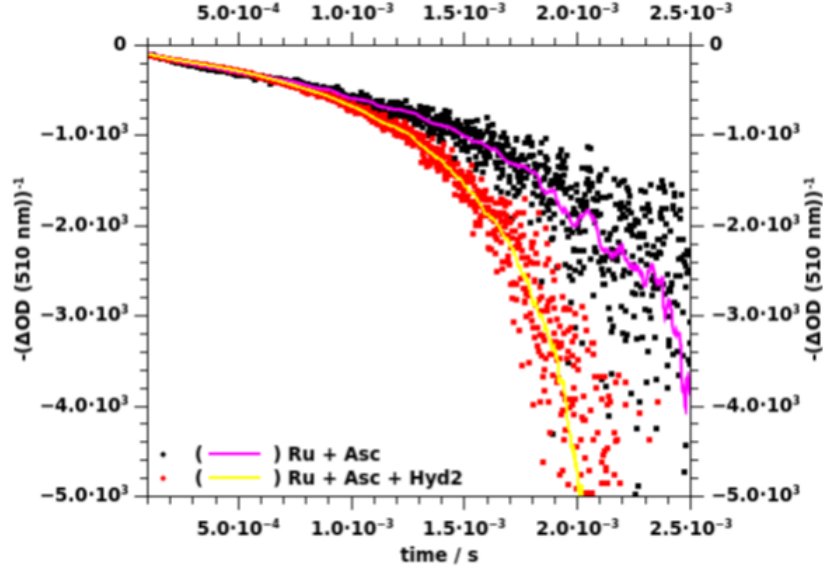


Figure 4.7: The presence of WT Hyd-2 decreases the lifetime of $[\text{Ru}(\text{bpy})_3]^{1+}$ following excitation at 470 nm. Negative inverted time traces of absorption at 510nm of a sample containing 40 μM $\text{Ru}(\text{bpy})_3\text{Cl}_2$ and 200 mM sodium ascorbate in pH 6.0 100 mM acetate buffer, 150 mM NaCl, 1 mM DTT, 0.02% DDM with (red dots) and without (black dots) 13 μM Hyd-2. Experiment carried out by and figure courtesy of D. Streich, Uppsala University, Sweden.

relative to that measured in the absence of Hyd-2 (*i.e.* that depletion of $\text{A}^\bullet$ due to recombination with reduced enzyme is negligible). We can then calculate a probable range of values for the rate constant for the injection of an electron from $[\text{Ru}(\text{bpy})_3]^{1+}$ to enzyme, $4.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} < k_{ei} < 1.3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and a lower limit for the efficiency of electron injection to Hyd-2, η_{ei} , of 3%. Calculating η_{ei} instead as the quantum efficiency for light-driven H_2 production divided by the quench efficiency, ϕ_{H_2}/η_Q , estimates η_{ei} to be $7 \pm 2\%$, on the same order as the efficiency calculated from the kinetic traces. See Section 2.9 for details of the calculations carried out.

The observation of efficient electron injection, as evidenced by the H_2 production efficiency as well as by analysis of the kinetic absorption traces, suggests that we should expect to see redox-dependent changes in Hyd-2 upon electron injection. In addition to absorption signals attributable to the excited and reduced states of the photosensitizer, in the presence but not in the absence of Hyd-2 we observed a sharp negative feature at 470 nm, forming between 1 and 10 μs after the flash and disappearing within 50 μs , and

a sharp positive feature at 530 nm reaching maximum intensity at 50 μ s (Figure 4.8), whose lifetime exceeds 100 μ s and could therefore not be assessed due to low signal-to-noise ratios at longer time periods.

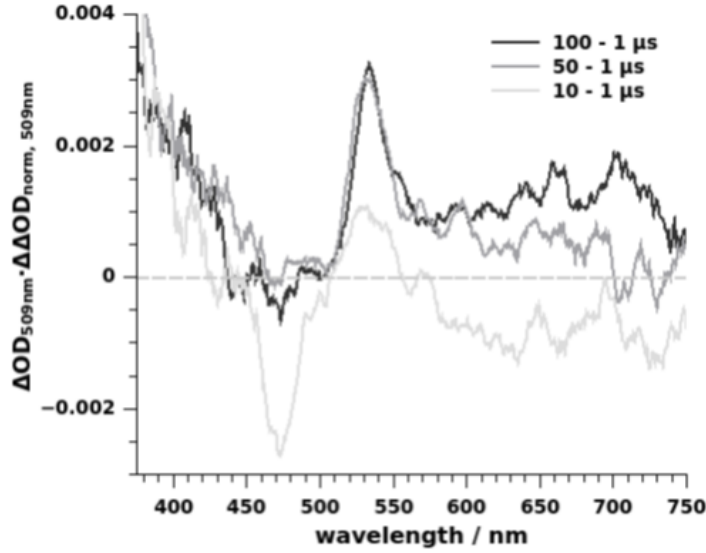


Figure 4.8: Transient absorption signals observed following laser excitation at 470nm of a sample containing 40 μ M Ru(bpy)₃Cl₂, 13 μ M wild-type Hyd-2, 200mM sodium ascorbate in pH 6.0 100mM acetate buffer, 150mM NaCl, 1mM DTT, 0.02% DDM. Transient spectra were normalised to ΔOD_{509nm} and smoothed using sliding window averaging (25 pt. window). The resulting spectra were normalised with respect to the 1 μ s spectrum and rescaled according to ΔOD_{509nm} . Experiment carried out by and figure courtesy of D. Streich, Uppsala University, Sweden.

These signals do not correspond to reduction of FeS clusters or of copurified *c*-type cytochrome (Figure 4.6), nor could they arise from the photosensitizer, the electron donor or any reduced amino acid. Therefore, we suggest that these signals arise as a result of the generation of transient active site species during turnover, and that they arise as a result of a two-step mechanism in which a first intermediate with ΔA_{max} at 470nm is converted to a second intermediate with ΔA_{max} at 530nm. Further data would be required to validate this hypothesis. No signals due to reduction of FeS clusters could be seen, suggesting that electron transfer through the clusters is faster than both electron injection and turnover at the active site.

4.2.4 Selection of residues for mutation

We have chosen to introduce cysteine residues for controlled electron transfer from molecules and surfaces. The thiol group is a versatile and specific target for click chemistry [240]. A maleimide was chosen as the click reagent for quick and specific reaction with reduced thiols. An inserted cysteine is also useful for electrode immobilisation with the use of a gold electrode, owing to the spontaneous formation and relative stability of a gold-thiol bond.

With no available crystal structure for Hyd-2, we constructed several mutants for labelling with the hopes that at least one of these mutants would allow attachment without impairing catalytic function. Mutants were originally designed by alignment of the Hyd-2 sequence with a structure for the [NiFe] hydrogenase from *D. fructosovorans*, but in Figure 4.9 the homologous mutation sites are displayed in *E. coli* Hyd-1, a recently published structure in which the C-terminus of the small subunit is visible, unlike in the structure for *D. fructosovorans*. Two mutants, BJM1 and BJM3, were made in which a single residue near the distal cluster was mutated to a cysteine. A third mutant, termed BJM8, was made in which two residues near the distal cluster were mutated, in the hope that by labelling with two photosensitizer molecules, two electrons could be injected into the FeS relay sequentially. The mutant BJM9 was constructed with two inserted cysteines on the cytoplasmic tail of the small subunit (*i.e.*, C-terminal to the transmembrane helix).

Inspection of the *E. coli* Hyd-1 crystal structure (PDB ID 4GD3 [93]) reveals that the medial Fe_3S_4 cluster is within 7.8 Å of the protein surface; a similar distance is measured for the *D. fructosovorans* hydrogenase, a ‘standard’ hydrogenase with high sequence homology to Hyd-2 [241]. We therefore also constructed a mutant, BJM2, in which this glutamate residue was replaced by a cysteine, with the intention of allowing direct electron transfer to the medial cluster of the enzyme, bypassing the distal cluster. Figure 4.9 presents the homologous residues in Hyd-1 to those chosen for mutation in

Hyd-2. The lower right panel shows a surface representation of the enzyme in which all mutated residues have been coloured.

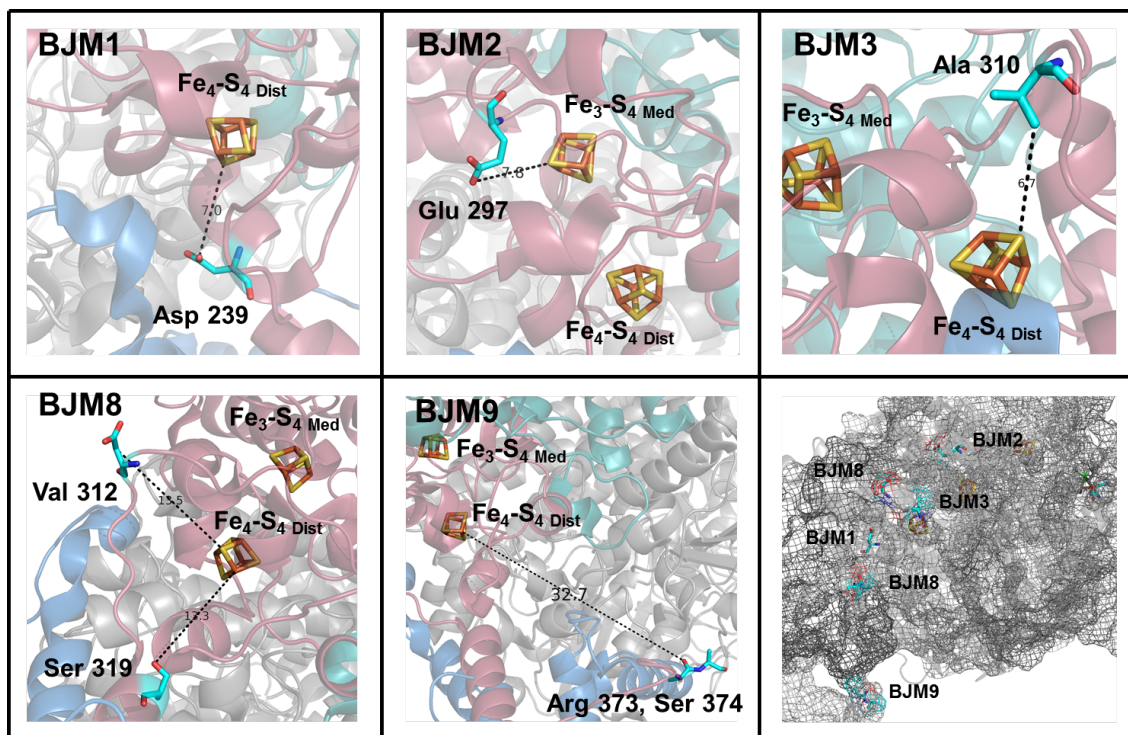


Figure 4.9: Residues chosen for mutation to cysteines. All panels except lower right present individual mutants with one (BJM1, BJM2, BJM3) or two (BJM8, BJM9) inserted cysteine residues, and the edge-to-edge distance from these residues to the nearest FeS cluster, displayed in Å. The small subunit (HyaA) is displayed in red, the large subunit (HyaB) in cyan, the cytochrome (HyaC) in blue, and the adjacent HyaAB subunits are in grey. The lower right panel displays the residues in a surface representation of the enzyme, in which the cytochrome subunit is in dark grey on the left, the small subunit is in light grey in the middle, and the large subunit is in medium grey on the right. All mutations are displayed as homologous residues in the crystal structure of *E. coli* Hyd-1 PDB ID 4GD3 [93], as no structure is available for Hyd-2.

4.2.5 Creation and purification of Cys-bearing mutants

Mutations were either introduced into a copy of the *hybO* gene in pBluescript and ligated as a KpnI/XbaI fragment into the pMAK705 shuttle vector according to sections 2.1.4 and 2.1.5 or the mutation was created directly on the *hybO*-pMAK705 construct according to section 2.1.4. Transformation and chromosomal integration of the mutation were carried out according to sections 2.1.3 and 2.1.5. After successful integration of

the mutated copy of a gene onto the *E. coli* chromosome, the presence of the mutation was confirmed by sequencing of the product of a colony PCR experiment according to Section 2.1.4. Whole-peptide LC-ESI-TOF mass spectrometry was used to detect the expression of mutated protein by changes in the mass of the small subunit, according to Section 2.6. Table 4.2 details the mutations introduced, as well as the expected mass of the mutant small subunit protein.

Mutant name	Mutation introduced into <i>hybO</i>	Expected Mass of HybO (Da)
Wild-type	none	36 861
BJM1	Asp239Cys	36 861
BJM2	Glu297Cys	36 834
BJM3	310Cys (insertion)	36 963
BJM8	Val312Cys Ser219Cys	36 824
BJM9	Arg373Cys Ser374Cys	36 880

Table 4.2: Cysteine insertion mutations introduced for specific labelling of HybO.

All mutant strains produced some His-tagged protein; however, the yield for the BJM1 mutant was low and this protein seemed to be less stable when purified. Later analysis with the new crystal structure of Hyd-1 has also shown that the introduced cysteine is not very well exposed to solvent in Hyd-1, something which could not be seen in the *D. fructosovorans* structure file due to the fact that the C-terminus of that protein was not resolved. For these reasons, work with the BJM1 mutant was abandoned.

4.2.6 LC-ESI-MS confirms that mutants can be labelled

The expression of protein bearing the inserted cysteine residues could be confirmed by LC-ESI-MS according to Section 2.6. Figure 4.10 depicts the output of a typical experiment through different stages of analysis. The raw output is a chromatogram of ion count as a function of elution time. Summing all masses collected for one of these peaks (in this case, the peak at 3.15 minutes corresponding to the small subunit) gives a set of regular peaks normally corresponding to a single peptide with varying degrees of protonation. The MaxEnt tool, included in the BioLynx package of Waters MassLynx

software, uses a maximum entropy algorithm to deconvolute these peaks into a mass for the peptide. For all data in this thesis, only the MaxEnt-derived mass distribution will be shown; however, the method used to obtain the data is critical in understanding the results for noisier spectra: unlike MS characterisation of small molecules, in whole-protein MS, accuracy of the output masses is crucially dependent upon the signal:noise of the raw data.

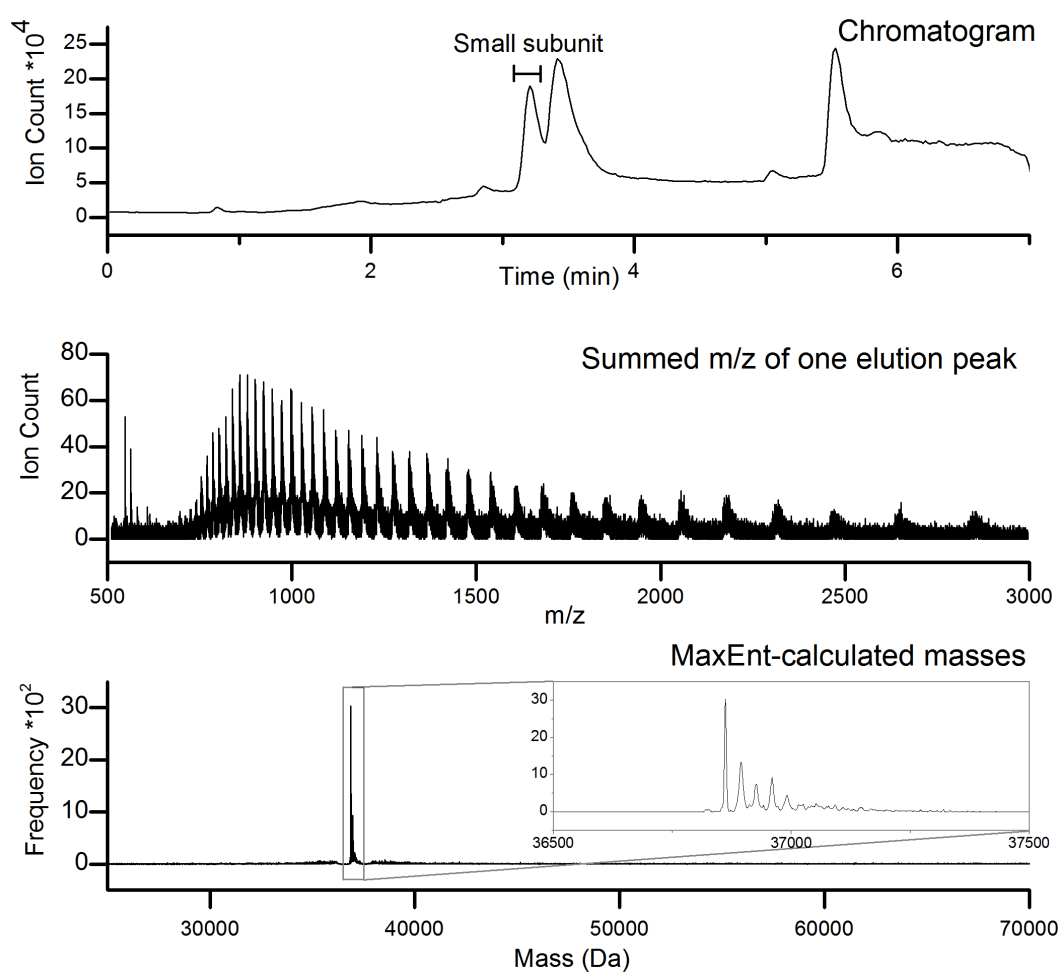


Figure 4.10: The steps of analysis for whole-protein LC-ESI-MS data for Hyd-1. Top: The ion count trace generated by continuous sampling of eluate from a UPLC column. The peak corresponding to the small subunit is indicated at 3.15 minutes; the peak corresponding to the large subunit follows closely at 3.45 minutes. Middle: The m/z spectra summed over the small subunit peak. The repeating peaks are due to the small subunit carrying various numbers of charges. Bottom: The masses calculated by the Maximum Entropy method provided in the MassLynx software package on the basis of the summed spectrum seen in the middle panel. The left-most peak corresponds to the small subunit mass; peaks at intervals of 36.5 ± 2 Da correspond to the small subunit with an increasing number of chloride ions.

The output for whole-protein ESI-MS of the small subunit is shown in Figure 4.11. The average masses of the HybO wildtype and mutant subunits were calculated using the BioLynx protein/peptide editor tool (MassLynx version 4.1; Waters Inc.) and in all cases matched the observed mass within 0.02% (see black traces in Figure 4.11). Thus, the small subunit is expressed carrying the mutation as introduced.

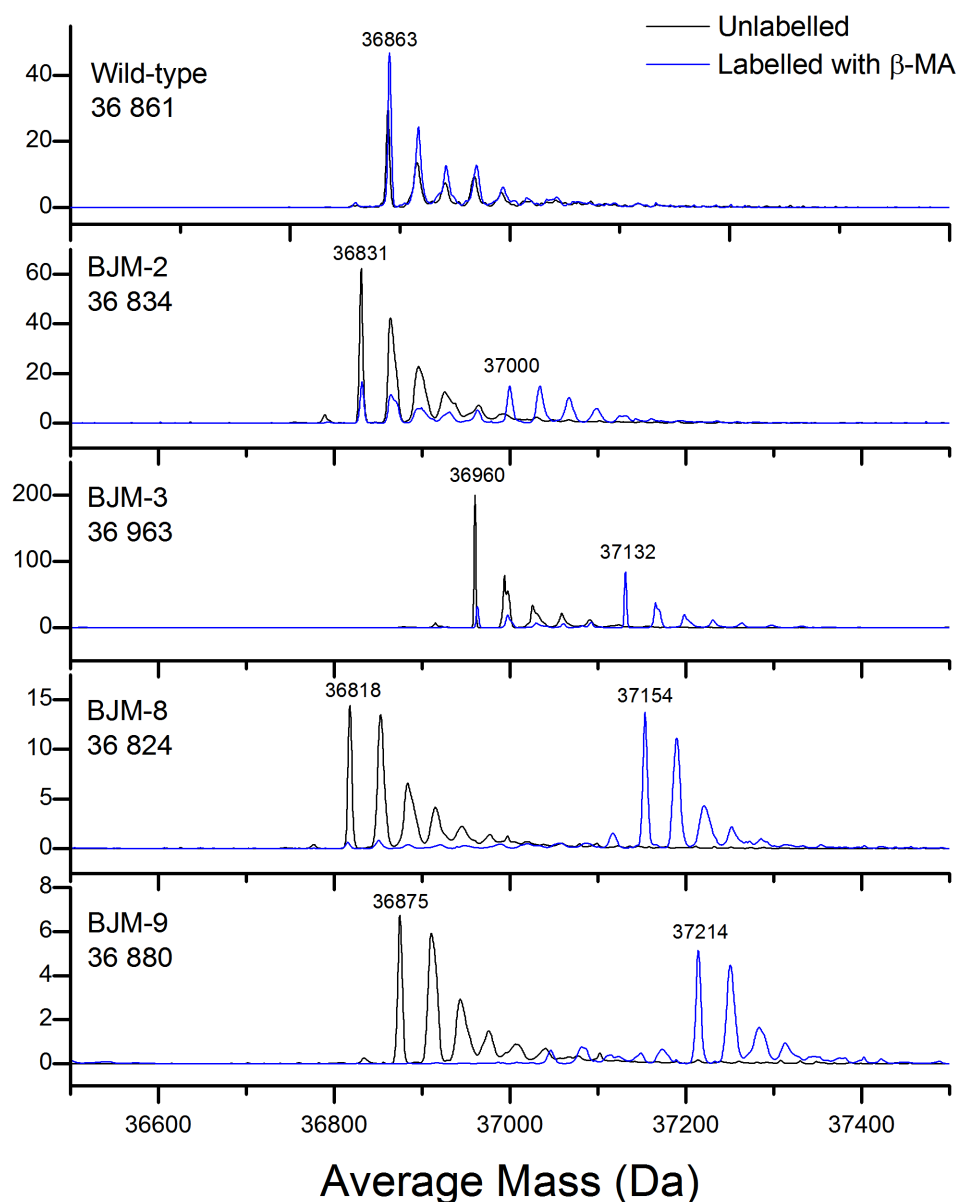


Figure 4.11: MaxEnt-derived LC-ESI-MS spectra of wild-type and mutant Hyd-2 small subunit. Traces for unlabelled protein are shown in black, while those for protein labelled with β -maleoyl alanine (MW = 169 Da) are shown in blue. The predicted mass of the unlabelled subunit is displayed in the upper left corner for each mutant.

The next step was to confirm that the inserted cysteines of HybO were sufficiently well exposed to be labelled and that they had not formed disulphide bonds with other thiols in solution. We also wanted to confirm that no naturally-present cysteine residues could be labelled. Labelling with a maleoyl-photosensitizer molecule gave noisy spectra with low signal intensity, despite the care paid to sample cleanup and preparation. We therefore chose to begin with labelling experiments using a simpler maleimide for labelling. Incubation of the proteins for one hour in the presence of a three- to five-fold excess of β -maleoyl alanine, MW = 169 Da, produced a mass shift of 169 ± 3 Da in single cysteine insertion mutants and a shift of 338 ± 2 Da in double cysteine insertion mutants, while the wild-type enzyme showed no mass shift (see blue traces in Figure 4.11). We were thus able to confirm that the inserted residues are solvent-exposed and reduced, available for labelling, and that labelling is specific to the inserted residues. In many cases, labelling wasn't complete - unlabelled protein in the labelled sample can be seen as a small peak matching the mass for unlabelled enzyme (Figure 4.11). The fraction of protein remaining unlabelled was found to vary with the excess of maleimide used for the labelling reaction; a ten- to fifty- fold excess was therefore used for subsequent labelling and gave nearly complete labelling of all mutants. Peaks at intervals of 36.5 ± 2 Da correspond to chloride adducts of the protein; although efforts were made to fully exchange the buffer to eliminate non-volatile ions, some ions always remained with the protein.

Evaluation of the analysed data for the large subunit (Figure 4.12) is much less interesting, though encouraging. The large subunit is expressed normally in all mutants and no intrinsic cysteine residues are labelled by the β -maleoyl alanine; we can therefore be confident that the only cysteine residues exposed to labelling are those specifically introduced in the mutated small subunit.

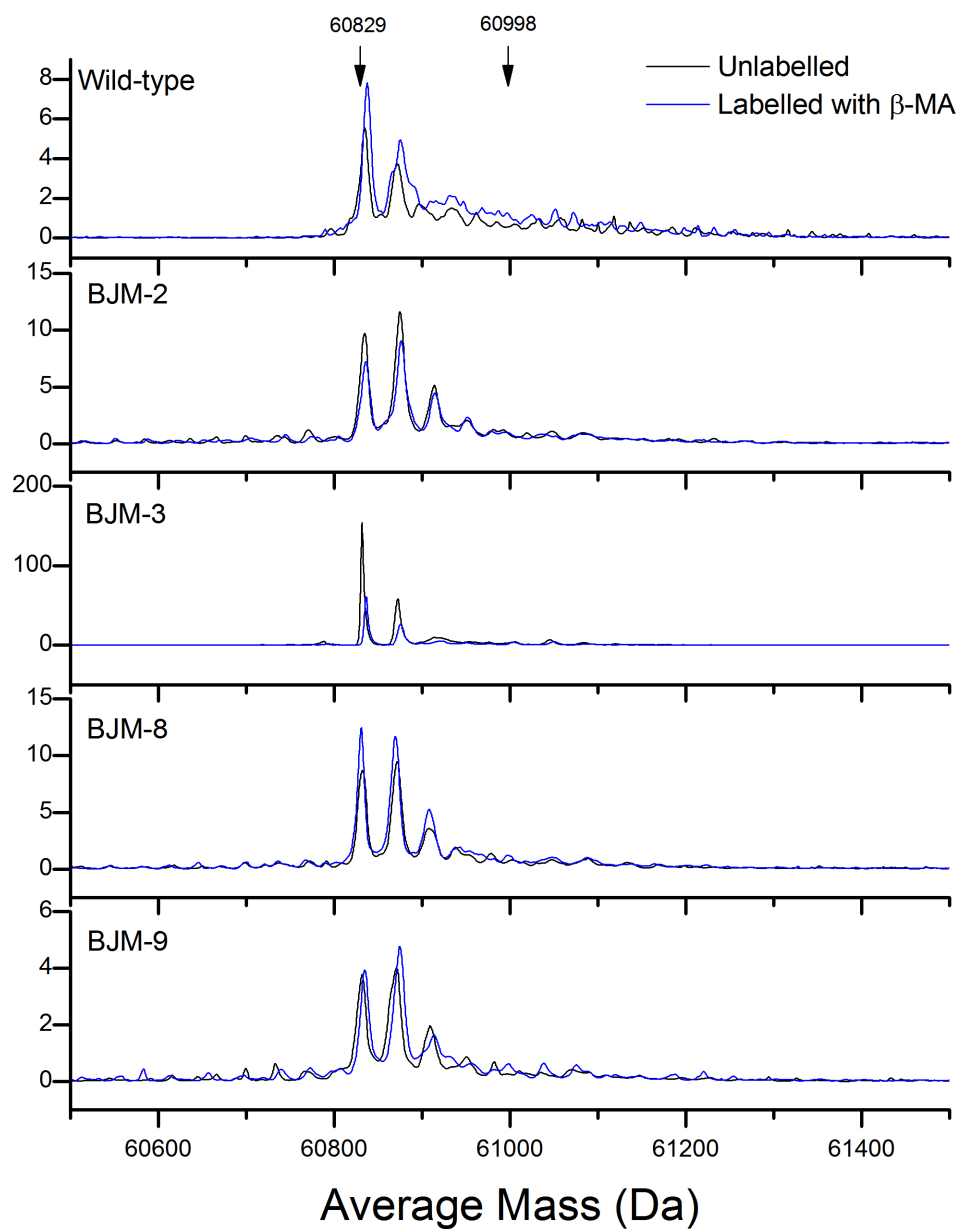


Figure 4.12: LC-ESI-MS of cysteine mutants' large subunit, unlabelled and labelled with β -maleoyl alanine (MW = 169 Da). Traces for unlabelled protein are in black, those for labelled protein are in blue. The arrows indicate the predicted mass of the unlabelled large subunit, and the predicted mass of a large subunit singly-labelled with β -maleoyl alanine.

4.2.7 PFE characterisation of the Cys mutants

In order to understand the behaviour of our labelled systems, we next characterised the mutant proteins under a variety of conditions in order to assess whether the inserted cysteine residues altered the catalytic properties of the enzyme in any significant way. Figure 4.13 shows the behaviour observed with cyclic voltammetry at different pH values, while Figure 4.14 allows evaluation of the effect of changing $\rho(\text{H}_2)$ on catalytic response. All voltammograms were recorded at PGE electrodes with non-specific adsorption of enzyme, as described in section 2.3.

In all cases, inserted mutations have had little to no effect on the properties of the enzymes. Catalysis is reversible, with little to no change in the relative bias toward oxidation or reduction for any of the mutants (Figure 4.13). The relative effect of changes in $\rho(\text{H}_2)$ is approximately the same for all mutants, indicating a similar substrate affinity for all variants. These data allow us to conclude that the mutations have not interfered with the insertion of the distal or medial clusters and that, for all intents and purposes, results obtained for cysteine-bearing mutants are relevant to wild-type Hyd-2.

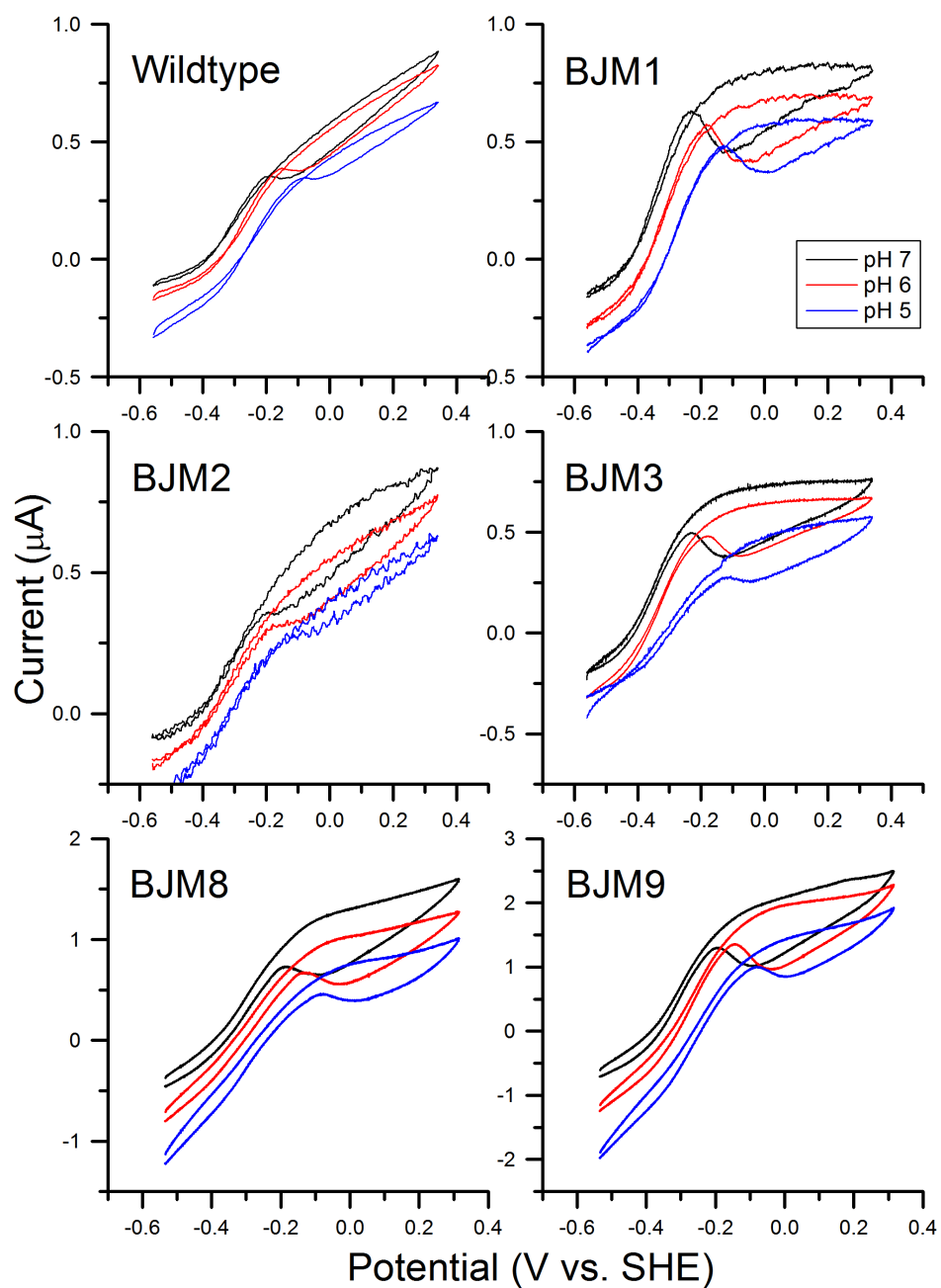


Figure 4.13: PFE of Hyd-2 Cysteine-bearing mutants at pH 5, 6, 7 and 10% H_2 , 37 °C, 5 mVs^{-1} , 500 sccm flow of gas, 3500 rpm.

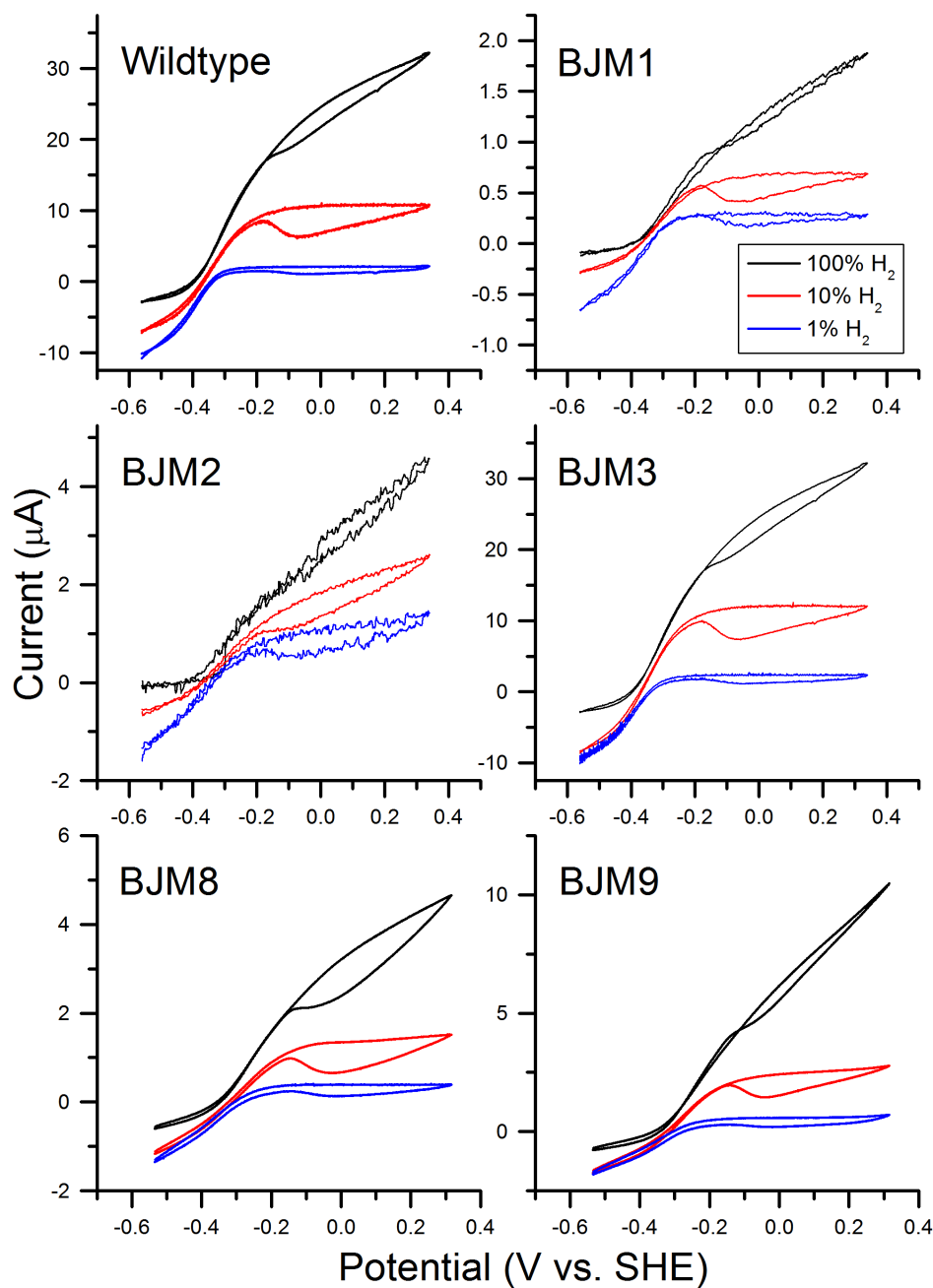


Figure 4.14: PFE of Hyd-2 Cysteine-bearing mutants at pH 6, and 1, 10, and 100% H_2 37 $^{\circ}\text{C}$, 5 mVs^{-1} , 500 sccm flow of gas, 3500 rpm. The total current under 100% H_2 was monitored before and after scans under changing conditions, and scans were corrected for film loss.

4.2.8 Labelling mutants with photosensitizer

In order to specifically label the inserted cysteine residues, $[\text{Ru}(4,4'\text{-dimethyl-bipyridine})_2(5\text{-maleimido-phenanthroline})]^{2+}$ ($[\text{Ru}(\text{dm-bpy})_2(\text{MI-phen})]^{2+}$) (MW = 744.81) was synthesized by D. Streich (Figure 4.15).

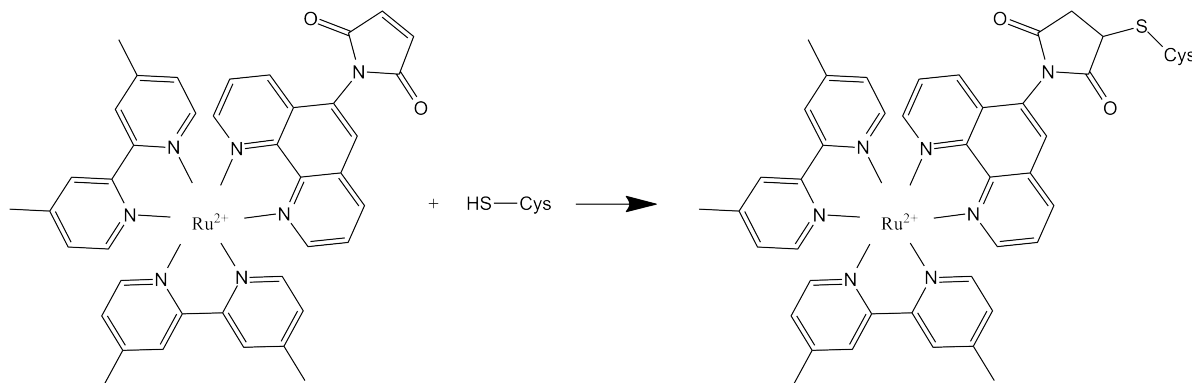


Figure 4.15: ‘Click-chemistry’ approach to labelling exposed cysteine residues with maleimide derivatives. The reaction occurs spontaneously on the timescale of minutes to hours at room temperature; no catalyst is required [225]. The photosensitizer used is $[\text{Ru}(4,4'\text{-dimethyl-bipyridine})_2(5\text{-maleimido-phenanthroline})]^{2+}$.

The data obtained for LC-ESI-MS characterisation of $[[\text{Ru}(4,4'\text{-dimethyl-bipyridine})_2(5\text{-maleimido-phenanthroline})]^{2+}$ -labelled protein is of much lower quality than those obtained for β -maleoyl alanine-labelled protein, with inaccurate masses obtained, resulting from a low signal:noise ratio for these data. Unlike for small mass measurement by MS, in which signal:noise and accuracy are not related, for whole-protein MS, a low signal:noise results in ambiguous mass assignment due to the extra step of data processing to obtain masses from raw m/z values. For a summary of the method used see Figure 4.10. Nonetheless, we do clearly see a shift in the most dominant mass peak by a mass approximately matching that of the complex and, in the case of the BJM-9 mutant in which two cysteines have been inserted, the shift is roughly doubled in magnitude. These data, in the context of the higher-resolution data obtained with the β -maleoyl alanine, give us confidence that labelling is occurring.

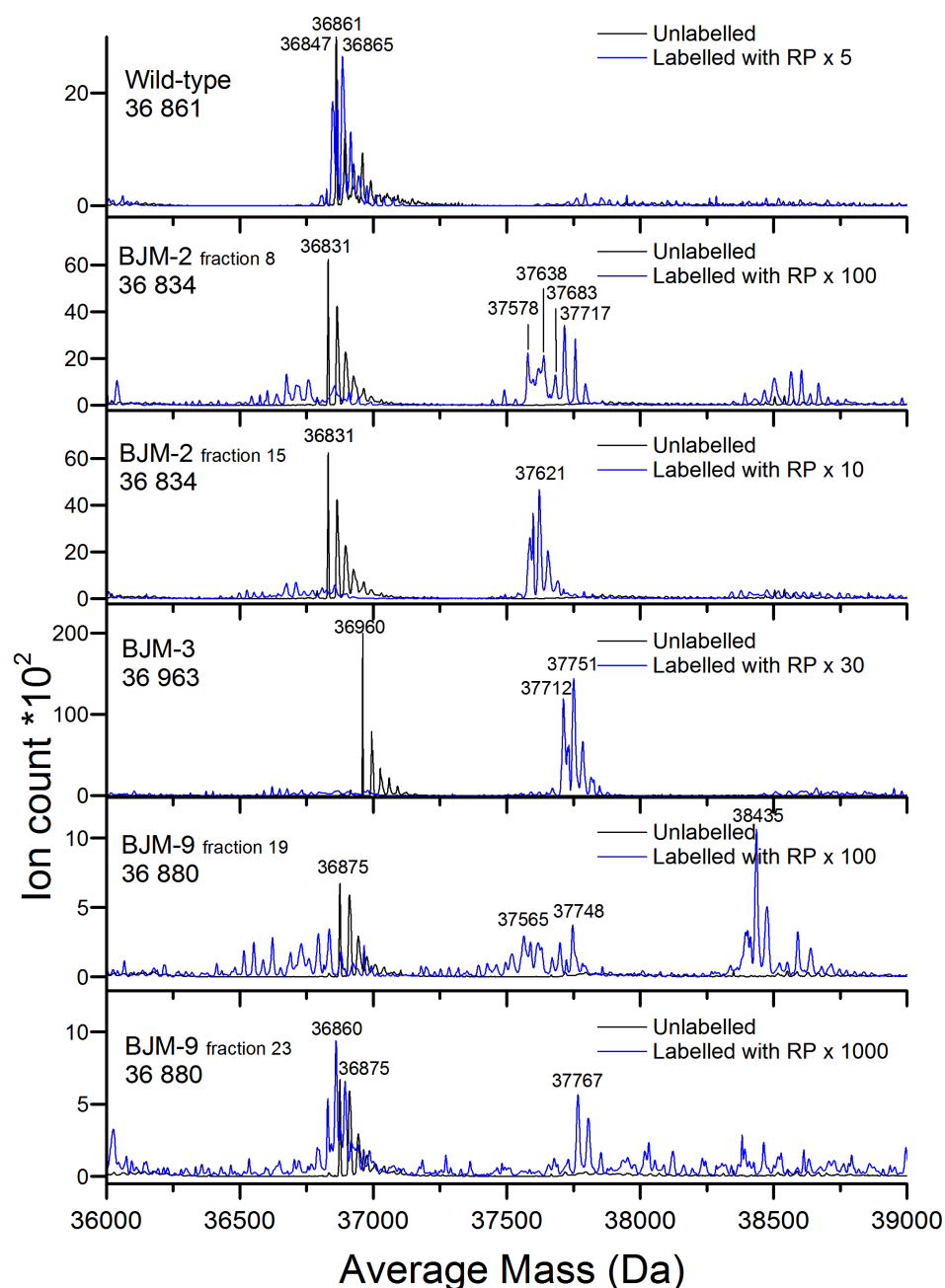


Figure 4.16: LC-ESI-MS analysis of unlabelled and $[[\text{Ru}(4,4'\text{-dimethyl-bipyridine})_2(5\text{-maleimido-phenanthroline})]^{2+}]^{2+}$ -labelled Hyd-2 and mutants. In all cases, unlabelled protein is shown in black, and labelled in blue. In some cases, spectra had to be scaled to be viewed on the same axis; the scaling factor is given in the legend. The predicted masses of the unlabelled subunit are shown in the top left corner for each mutant. RP = ruthenium photosensitizer, MW = 744.81.

4.2.9 Experiments with specifically labelled Cys-bearing mutants

Hyd-2 mutants labelled and purified according to Section 2.7 are active in solution assays at rates comparable with unlabelled enzyme ($\approx 5 \text{ s}^{-1}$; assays carried out by D. Streich before and after labelling), suggesting that the labelling process does not interfere with activity of the enzyme. MS analysis shows the enzyme to be specifically labelled with the photosensitizer (Figure 4.16).

Light-driven H_2 production experiments with samples of specifically-labelled enzyme, surprisingly, showed barely detectable levels of H_2 evolution, and a strong suppression of the formation of reduced state from the excited state (*i.e.* of quenching efficiency). More than 65% of the photogenerated $^*[\text{Ru}(\text{dm-bpy})_2(\text{MI-phen})]^{2+}$ disappears rapidly without yielding any long-lived products; the efficiency of quenching to the reduced $[\text{Ru}(\text{dm-bpy})_2(\text{MI-phen})]^{1+}$ state is only 12.4% for labelled BJM-2 and 16.7% for labelled BJM-3 (with 200mM ascorbate as sacrificial reductant in each case).

It is not clear why this should be the case. The quenching efficiency of the $^*[\text{Ru}(\text{dm-bpy})_2(\text{MI-phen})]^{2+}$ in the absence of protein is 65% with 200mM ascorbate. Reduced access of ascorbate to excited photosensitizer due to crowding by the protein cannot account for the drastic shortening of the excited state lifetime. It is possible that the excited state is being lost through energy transfer quenching with nearby FeS clusters.

4.2.10 Specific attachment of Hyd-2 to surfaces via inserted Cys residues

We next wanted to assess whether the inserted cysteine residues could be co-opted as a handle for attachment of enzyme to surfaces such as electrodes. A self-assembled monolayer with exposed maleimide groups was prepared on a gold electrode as described in Section 2.3.5 and cys-labelled Hyd-2 was spotted on and left to react for 30 minutes.

We then carried out CVs under H₂ and Ar, as shown in Figure 4.17.

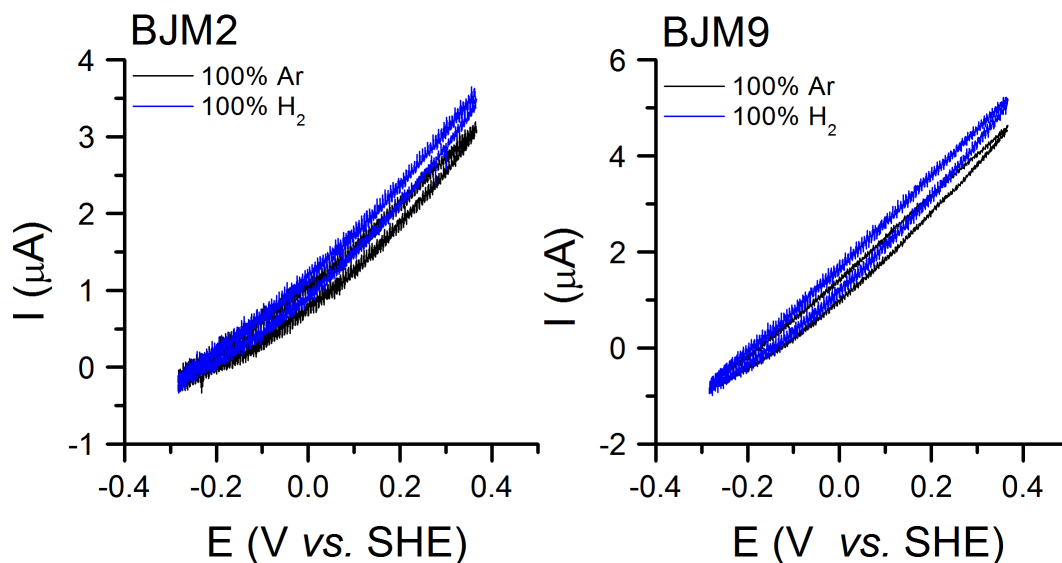


Figure 4.17: Cyclic voltammograms for Hyd-2 mutants BJM-2 and BJM-9 on an Au electrode with a SAM of N-thioethane-maleimide for covalent attachment. Conditions were as follows: pH 7.0 (0.1M phosphate buffer), 20 °C, 5 mVs⁻¹, gas flow = 1000 sccm, 100% H₂ (blue traces) or 100% Ar (black traces), stationary electrode.

The difference between scans recorded under Ar and scans recorded under H₂ are minimal. A very slight increase in current at high potential is observed under H₂ relative to Ar, but there is no defined onset for the activity. It is possible that the enzyme is not attached to the surface; however, the slight gradual increase in current seen at high potentials for the H₂ scan relative to the Ar scan suggests that the most likely explanation is that the enzyme is immobilised but that interfacial electron transfer is extremely inefficient.

Because this was not the principal focus of this project, we did not pursue this avenue further.

4.3 Perspectives

This thesis demonstrates, in several systems, the critical importance that electron transfer plays in determining the catalytic properties of [NiFe] hydrogenases. Protein film electrochemistry experiments can tell us a lot about the properties of a redox system

and much of our understanding of [NiFe] hydrogenases comes from studies using PFE. The disadvantages of PFE for studying redox enzymes include the relatively poor control over the way in which enzyme is attached to the electrode, leading to inhomogeneous rates of interfacial electron transfer, and the impossibility of delivering a known number of electrons to an enzyme molecule. This chapter presents experiments designed to address the shortcomings of PFE in order to generate complementary data for a more complete description of the electrochemical properties of a reversible [NiFe] hydrogenase.

We have successfully inserted cysteine residues near the distal and medial clusters of Hyd-2, and shown that these mutations do not interrupt to any apparent extent the properties of Hyd-2 as a catalyst. We have shown by whole-protein LC-ESI-MS that these inserted residues can be labelled both with a small maleimide compound and with a Ruthenium polypyridine photosensitizer bearing a maleimide group for click chemistry. We have shown that this labelling is specific, with no native cysteines labelled. Experiments with wild-type Hyd-2 in solution with photosensitizer show that electron injection from a reduced photosensitizer molecule to the hydrogenase is efficient, driving H_2 production at a maximum rate of 1.1 s^{-1} and a maximum quantum yield of 6.5%.

No transient signals due to reduction of FeS clusters were visible in this system; this is attributed to the fact that both electron injection and active site turnover are likely to be slower than intramolecular electron transfer along the chain of FeS clusters. It is difficult to imagine a system in which interfacial electron transfer competes with intermolecular electron transfer; indeed, electrochemical experiments suggest that rates of interfacial electron transfer are often limiting near the equilibrium potential, E_{eq} , but as a greater overpotential is applied the rate of electron transfer increases, and the rate of turnover becomes limited by chemical steps at the active site or diffusion of substrate and product to and from the active site [219, 59]. Intramolecular electron transfer between FeS clusters is predicted to outpace catalysis, often by more than an order of magnitude [140]. Therefore it is quite plausible that even for a system in which electron injection

is relatively efficient, signals due to reduction of FeS clusters would not be resolved.

A negative signal at 470nm appears within the first 10 μ s following excitation and seems to be depleted concomitant with the appearance of a positive signal at 530nm on the timescale of 10-50 μ s after excitation. Further experiments with better resolution at these timescales will be necessary in order to establish whether the depletion of the first signal does indeed match the formation of the second. In the absence of other possible explanations for these signals, it seems reasonable to suggest that these are due to formation of catalytic intermediates at the active site. Repeating these experiments at a different pH or under an atmosphere of H₂ or CO, and looking for changes in the lifetime of these intermediates might give insight as to the nature of these species.

One factor that complicates the detection and analysis of signals due to transient redox processes is the heterogeneity of the sample in terms of redox state before excitation. Since two electrons are required for turnover, some enzyme molecules will be undergoing a reduction from the Ni-S state to the Ni-C state, while others will pass from the Ni-C state to the Ni-S state via the two-electron reduced Ni-R leading to H₂ evolution (Figure 1.3). It would therefore be desirable to begin with a sample of enzyme homogeneous in redox state, or at the very least strongly biased toward a given state, which would prevent signals from different enzyme molecules from cancelling one another. However, with an overall quantum yield of 6.5% (that is, 6.5% of photons absorbed contribute to H₂ production), very few enzyme molecules would be reduced by two electrons with two excitation pulses. Furthermore, because the photosensitizer is free in solution and is present in vast excess relative to enzyme, it is possible that two reduced photosensitizer molecules generated by the same excitation pulse would both reduce the same enzyme molecule. We therefore require better control of the electron transfer from photosensitizer to enzyme.

By specific covalent attachment of a [Ru(dm-bpy)₂(MI-phen)]²⁺ to the inserted cysteine residue by click chemistry, we hoped to achieve faster rates and higher numbers of

electrons injected into the Hyd-2 FeS cluster chain. We were surprised to find that photosensitizer attached to Hyd-2 showed decidedly shorter lifetimes for the photo-generated excited state as well as much lower efficiencies of reductive quenching (approximately 15% of photogenerated excited state was quenched to the reduced state). The levels of photogenerated H_2 from this system were barely detectable. Since the hydrogenase activity, assessed by dark solution assay, is unchanged throughout the labelling procedure, we cannot assign the low activity of the system to problems with the enzyme. The short lifetime of the excited state, far shorter than the corresponding lifetime for free complex in the absence of electron donor, suggests that reduced access of electron donor to the excited photosensitizer is not a likely cause. Perhaps the most feasible explanation is that the proximity of the FeS cluster may permit dissipation of the photogenerated excited state through energy transfer to the cluster. We may be able to gain a better understanding of this result using time-correlated single photon counting (TCSPC) experiments with time-resolution below 1 ns.

We are left somewhat puzzled as to the reasons we weren't able to bring this project to full fruition. We have been able to show that each of the necessary steps for specific covalent attachment of a Ru polypyridine photosensitizer near the distal or medial FeS clusters of Hyd-2 has successfully been completed. Interesting results from the WT Hyd-2 system with free photosensitizer point toward a first spectroscopic identification of transient active site species that are nearly impossible to observe by other techniques. Given that our understanding of the [NiFe] catalytic cycle is still primitive, this may prove valuable.

Chapter 5

Purification and Characterisation of Hydrogenase-3 from *Escherichia* *coli*

5.1 Introduction

5.1.1 A History of FHL complex research

In 1875, Popoff reported the first observation of bacterial decomposition of formate to H_2 and CO_2 [242, 243]. This activity was observed in pure cultures of *E. coli* as early as 1901 by Pakes and Jollyman [98] who confirmed that formate was disproportionated to equal parts H_2 and CO_2 and thus concluded that this represented

‘a case of bacterial decomposition of a chemically simple nature, which might serve to elucidate the action of bacteria on organic compounds in general; that this end has so rarely been obtained we are inclined to attribute to the fact that comparatively complex substances, such as the sugars, have hitherto formed the main subjects of investigation’;

in other words, the characterisation of this pathway represents one of the very first fundamental metabolic conversions observed in bacteria. Since the beginning of the 20th century, our knowledge about the formate-hydrogen lyase complex of *E. coli* has grown, with a large number of papers published on the complex. Indeed, much of our understanding of the maturation of hydrogenases comes from studies on this complex [244, 245, 246], and the first description of selenoproteins was for the formate dehydrogenases of *E. coli* [247]. Numerous studies have attempted to engineer the FHL complex or its regulation in order to boost H_2 production from *E. coli* [248, 249, 250]. Despite research efforts spanning 138 years on the FHL complex, an intact, active FHL complex has never before been isolated. This chapter represents the first successful isolation of the pentameric HycEGFB-FdhF complex and demonstrates formate:hydrogen activity of the complex. PFE is used to characterise the hydrogenase activity, and preliminary characterisation by EPR allows for a first analysis of the complex FeS relay of the enzyme.

5.1.2 The role of Formate-Hydrogen Lyase in mixed acid fermentation

During fermentation on glucose, *E. coli* produces roughly two formate molecules for each molecule of glucose consumed; in other words, $\frac{1}{3}$ of glucose-derived carbon atoms are converted to formate [15]. This formate is derived from pyruvate, catalysed by the pyruvate-formate lyase (PFL) complex, whose expression is triggered under anaerobic and microaerobic conditions by a redox-sensitive mechanism [251]. The transmembrane formate transporter, FocA, is coexpressed with PFL and exports formate [125] during early-stage fermentation. When the pH of the media falls below 6.8, FocA begins to operate in the opposite direction, accumulating formate in the cytoplasm [15]. The *E. coli* genome encodes three formate dehydrogenase enzymes, termed Fdh-O, -N, and -H according to their eventual electron acceptors. Fdh-N, encoded by *fdnGHI*, is expressed under anaerobic conditions in the presence of nitrate [126], allowing anaerobic respiration using nitrate via menaquinone (MK) [133]. The very similar Fdh-O, encoded by *fdoGHI*, is expressed under aerobic and nitrate respiration [127] and also reduces MK. Respiration via Fdh-N and Fdh-O is carried out preferentially to expression of Fdh-H; only when oxygen and nitrate respiration are impossible does formate accumulate in the cytoplasm sufficiently to stimulate production of the transcriptional activator FhlA, which is essential for transcription of the FHL genes. The very low K_M values for formate of the Fdh-N and Fdh-O enzymes [252] act as a second safeguard to ensure that formate is used by Fdh-N and Fdh-O preferentially to Fdh-H. Under standard conditions, the dissociation of formate to CO_2 and H_2 is unfavourable by about 10mV; however in environments such as the gut of animals, where H_2 and CO_2 are constantly taken up by methanogens, lowered concentrations of these gases shift the reaction equilibrium potentials sufficiently to make the full reaction favourable by about 111 to 141 mV, which is calculated to be sufficient to drive pumping of a proton [111]. The presence of the HycC

subunit, homologous to the putative H^+ pumping subunits of Complex I, also serves as evidence for proton pumping in FHL. Cell membrane vesicles containing FHL but not Hyd-1 or Hyd-2 show formate-dependent proton translocation, with an approximate H^+ to formate ratio of 0.8, although the authors suggest this is likely to be a lower limit due to the competing effect of medium acidification by dissociation of carbonic acid [253]. Expression of FHL has been shown to be important to the acid tolerance of *E. coli* under anaerobic conditions [138], and the pH-dependence of FHL expression also points toward a role of Hyd-3 in dissipating excess levels of formate when the medium becomes too acidic.

5.1.3 Regulation of FHL expression in *E. coli*

The expression of FHL in *E. coli* is critically dependent upon the intracellular concentration of formate as well as a low pH [130] and the σ^{54} -factor. The presence of molybdenum also stimulates expression of FHL [254, 129]. The inhibitory effects of alternate electron acceptors such as nitrate and oxygen on FHL expression seem to be mediated via their effect on intracellular formate concentrations [130]. Expression of the *hyp* genes, required for successful maturation of FHL, is stimulated under anaerobic conditions by the transcription factor FNR (regulator of fumarate and nitrate reduction) [255, 256].

5.1.4 The structure of the FHL complex

On the basis of sequence similarity, FHL has been classified into the Group IV membrane-bound energy-converting hydrogenases [30]; the characteristics of this group include membrane colocalisation and a multi-subunit configuration that often directly couples a metabolic oxidation, such as CO or formate oxidation, with H^+ reduction. Sequence homology suggests that the Hyd-3 large subunit, HycE, is likely to contain a [NiFe] site similar to those seen for other hydrogenases. The small subunit, HycG, is predicted to contain a single FeS cluster. Subunits HycF and HycB are predicted to contain two and

four FeS clusters, respectively, and are likely play a role in transferring electrons from the formate dehydrogenase subunit to the hydrogenase. The formate dehydrogenase subunit, FdhF, is the best characterised component in the complex, as it has been separately expressed and purified for study by EPR, XAS and X-ray crystallography [257, 258, 259, 260, 261]. The active site of the FdhF subunit, shown in Figure 5.1, contains a redox-active Mo ligated by four thiolate ligands of two identical molybdopterin guanine dinucleotide (MGD) cofactors [262]. The Mo is also ligated by a free -SH or -OH group and by a selenocysteine ligand that seems to be labilised upon reduction with formate [257]. The FdhF subunit contains a single Fe_4S_4 cluster.

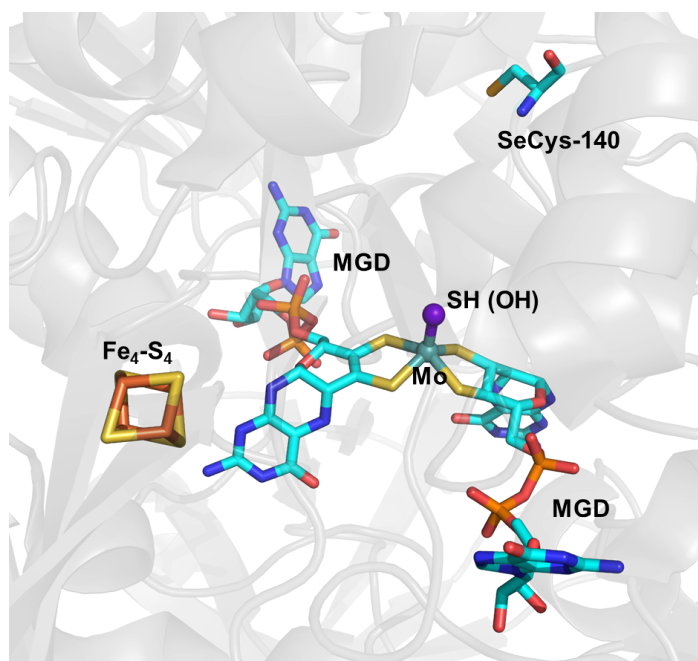
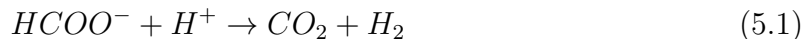


Figure 5.1: The formate-reduced active site of *E. coli* formate dehydrogenase-H, PDB ID 2IV2 [257, 263]. Active site components are coloured by element, cyan = C, yellow = S, blue = N, red = O, orange = Fe/P, purple = unidentified ligand, believed to be SH or OH.

The active cycle of formate dehydrogenases is not fully understood; however, it is clear that the Mo can access the IV,V, and VI oxidation states, and that in the most oxidised state the selenocysteine residue is bound to the Mo(VI) [259, 258]. It is clear that no oxygen transfer from solvent H_2O occurs, *i.e.*, that all atoms of the CO_2 released are derived from oxidised formate, and that the elementary reaction involves the formate

ion and CO_2 , rather than bicarbonate [258], such that the overall reaction catalysed by the FHL is



The minimal complex for FHL activity seems to be HycEGFB-FdhF, but none of these subunits contains a predicted transmembrane helix that would serve to attach the complex to the membrane. HycC and HycD, proteins predicted to contain 14 and 8 transmembrane helices, respectively, serve as membrane anchors for the pentameric complex, and sequence homology suggests that these proteins may also play a role in coupling FHL activity with proton translocation, as discussed in the following section.

5.1.5 Evolutionary links between FHL and other metabolic processes

Section 1.1.3 introduces the many roles that H_2 cycling is thought to have played along the early evolutionary pathway. A body of evidence supporting the evolution of life at a hydrothermal vent points to a deep connection of FHL with early life. At the interface of basic, H_2 -rich hydrothermal emissions with acidic, CO_2 -rich primordial oceans, the H_2 -dependent reduction of CO_2 provides a realistic pathway to the emergence of lifeforms based on reduced carbon. Indeed, the Wood-Ljungdahl pathway, found in acetogenic Bacteria as well as methanogenic Archaea, and therefore believed to be present in the last universal common ancestor, is based on H_2 -dependent CO_2 reduction to formate, and is the only of five CO_2 fixation pathways that does not require ATP hydrolysis [48]. The abiotic formation of formate from H_2 and CO_2 has been detected at hydrothermal vents, lending further support to this theory [264]. Nitschke and colleagues have proposed that a primitive catalyst for this reaction strongly resembles a formate-hydrogen lyase complex running in reverse: a proton gradient at the interface of the hydrothermal emissions

and ocean water could be harnessed to provide driving force for the H_2 -oxidation: CO_2 reduction reaction, producing formate in the process [46].

Sequence homology of Hyd-3 with the Class I membrane-bound hydrogenases is low - the large subunit shows only 14% homology with the [NiFe] hydrogenase from *D. gigas*, while Hyd-1 and Hyd-2 show 40 and 42%, respectively [249]. The Group IV hydrogenases, the so-called energy-converting hydrogenases, which include Hyd-3 of the FHL, comprise hydrogenases found in multimeric complexes linking, for example, CO oxidation with H^+ reduction (*e.g.* complexes from *Rhodospirillum rubrum* and *Carboxydotherrmus hydrogenoformans*) or ferredoxin oxidation with H^+ reduction (*e.g.* complexes from *Methanothermobacter* species). Many group IV hydrogenases have been shown to couple turnover with proton pumping [56]. These complexes seem to be less similar in subunit architecture and sequence to other [NiFe] hydrogenases than they are to Complex I, the proton-pumping NADH:quinone oxidoreductase complex through which reducing equivalents derived from glycolysis and the citric acid cycle enter the electron transport chain for respiration. This homology has been interpreted to suggest that Complex I evolved from an energy-converting hydrogenase [265], adding further relevance to the study of this complex, which may shed light not only on the mechanism of H_2 evolution in *E. coli* and related organisms, but also on the complex that forms a crucial link between cytosolic metabolism and respiration. The homology between FHL and Complex I subunits is denoted by colour in Figure 5.2.

Strong sequence homology between the membrane-bound HycC subunit and the NuoL membrane-bound H^+ -translocating subunit of Complex I suggests that HycC may be responsible for pumping H^+ concomitant with turnover; furthermore, since Complex I possesses several transmembrane domains of this type each believed to pump one electron per turnover, the presence of a single HycC-type domain in FHL has been suggested as indicative that only one H^+ is pumped per catalytic turnover cycle [266]. In contrast, the FHL-2 complex containing Hyd-4 contains three putative H^+ -pumping

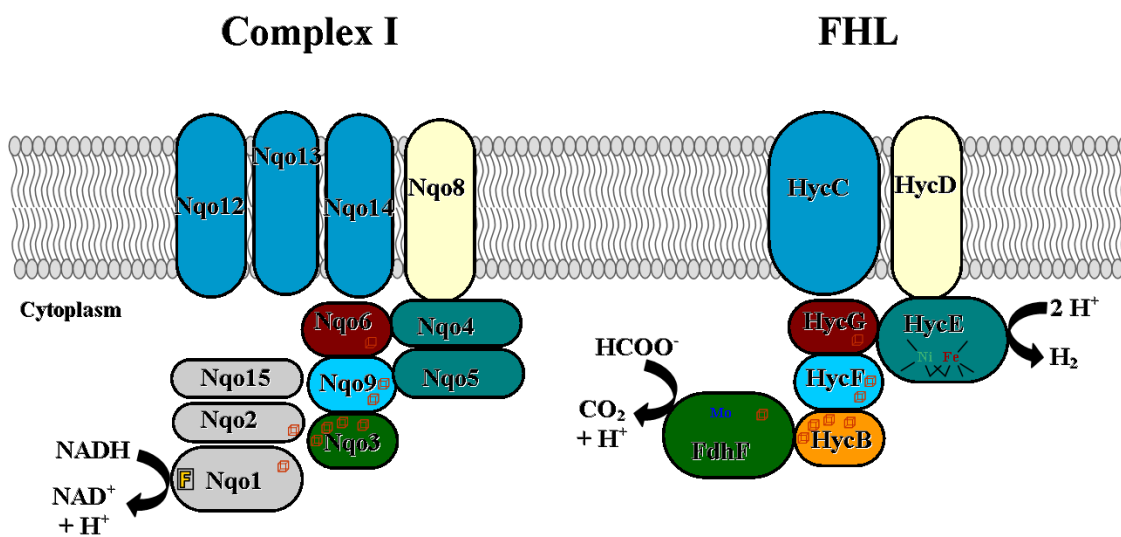


Figure 5.2: Homology between Complex I and the FHL. Complex I from *Thermus thermophilus*. Homologous subunits are depicted in the same colour in both complexes, a flavin cofactor catalyses hydride abstraction from the NADH.

domains, HyfB, D and F, and is therefore suggested to pump more H⁺ per turnover [266], however the expression of FHL-2 exclusively under more basic conditions [108] would lower the driving force for the overall reaction (by ~30mV per unit increase in pH, see equation 5.1), thus calling into question the energetic feasibility of pumping yet more protons with a reduced driving force and by association also challenging the assumption that expression of subunits homologous to NuoL necessarily implies H⁺-pumping.

5.2 Results and Discussion

5.2.1 Isolation of active FHL complex

The purification of active FHL from *E. coli* has been reported by many as difficult, due to the instability of the complex [15]. This enzyme is nonetheless of very considerable interest to both chemists and biologists due to its ability to produce and oxidise H₂ at rapid rates. Indeed, knockout mutants in which the FHL complex is not expressed produce almost no H₂ under most conditions studied [267]. Previously, the formate

dehydrogenase (FdhF) subunit has been overexpressed from a plasmid and purified by affinity chromatography in the absence of the rest of the complex [261]. This subunit was characterised by EPR [259, 258] and XAS spectroscopies [260] and X-ray crystallography [263, 257], as described in section 5.1.4.

Although many efforts have been made to purify hydrogenase components from the complex, these efforts have yielded inactive protein [101, 244, 15] and activity measurements for different enzymes must therefore be conducted on crude cell lysate of strains bearing knockout mutations of different hydrogenases [218]. Attachment of an affinity tag to the N-terminus of the large subunit is reported to give inactive protein [268] while the addition of an affinity tag at the C-terminus is inadvisable, as it is well known that cleavage of the C-terminal portion of the HycE is an indispensable step in protein maturation [244]. Addition of an affinity tag to the small subunit, HycG, results in an unstable complex (J. McDowell, University of Dundee, personal communication). In order to purify FHL by affinity chromatography, it was therefore necessary to use a strain in which the affinity tag, in this case a 10×His sequence, was inserted within the *hycE* sequence to as lie between two predicted alpha-helices of the peptide. This strain, named MG059e1, was created by Frank Sargent (University of Dundee). From this strain, it was possible to harvest active, intact pentameric FHL complex. We developed several purification methods (Section 2.2) yielding complex with differing properties and were able to characterise Hyd-3 by PFE and the FHL complex by CW X-band EPR spectroscopy.

Testing purification methods

Initially, two methods were developed to isolate HycE with associated subunits: the first, similar to the protocol used to isolate Hyd-1 and Hyd-2, and the second, a modification of this protocol in which all steps from harvesting onward were carried out in an anaerobic glovebox ($\rho(\text{O}_2) < 10$ ppm) and in which the cells were lysed by the addition of detergent, since it was not possible to lyse cells using a french press un-

der an anaerobic atmosphere. These protocols are detailed in Sections 2.2.1 and 2.2.2, respectively. Since both the formate dehydrogenase and the hydrogenase of the FHL complex are expected to be O_2 -sensitive [258], it was hoped that this method would yield enzyme complex with higher activity. SDS-PAGE analysis of the isolated enzyme is shown in Figure 5.3. The aerobic method (centre lane; method 1, Table 2.8) purifies

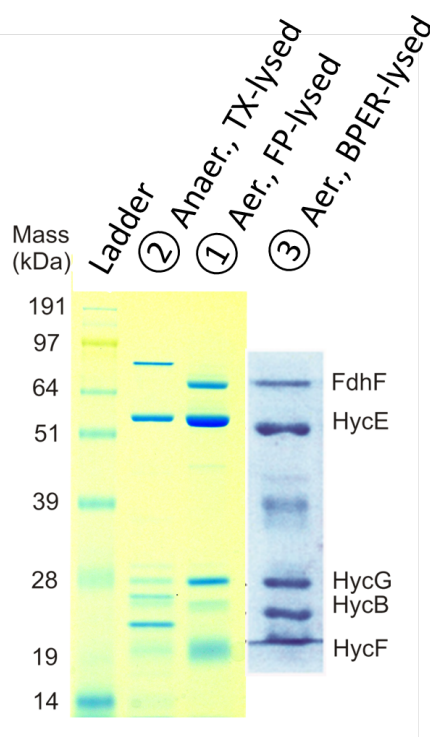


Figure 5.3: SDS-PAGE of FHL complexes isolated with different methods of cell lysis and membrane solubilisation. Lanes are numbered according to the method number in Table 2.8. In the right lane is a gel photo from F. Sargent, University of Dundee.

an enzyme complex containing the five subunits of the soluble HycEGFB-FdhF complex. The bands assigned to the HycB and FdhF subunits are slightly lower in intensity than would be expected for a heteropentameric complex; this is likely due to partial loss of these subunits. The anaerobically purified enzyme (left lane; method 2, Table 2.8) did not show a band corresponding to FdhF. The identity of the band at large molecular weight is unknown, but this is not believed to correspond to any component of the FHL. This method of preparation therefore does not yield intact FHL complex, a conclusion which is supported by metal ion analysis and EPR spectroscopy below. Nonetheless,

the hydrogenase component does purify successfully. A third method, used by our collaborators in Dundee, involves a one-step cell lysis and membrane solubilisation using the proprietary Bacterial Protein Extraction Reagent (B-PER) (Thermo-Pierce), in conjunction with column buffers containing the detergent β -dodecyl maltoside (DDM) in place of Triton X-100. This method employs detergents known to be more successful in the purification of unstable complexes. This protocol allowed purification of the intact pentameric complex (right lane; method 3, Table 2.8). The high cost of B-PER and DDM meant that this protocol could not be used for large scale preparation of enzyme for EPR or XAS analyses, or for formate-driven H_2 production experiments.

Because the B-PER method allowed for anaerobic cell lysis, this protocol could be carried out entirely in the glovebox, similar to the method described in Section 2.2.2. In addition to carrying out various steps of the purification in an anaerobic atmosphere, one purification (Method 6, Table 2.8) also included the addition of ferricyanide as chemical oxidant. Since the oxidised forms of both the formate dehydrogenase and the hydrogenase are inert to further oxidation by O_2 [259], and given that this technique has previously been used successfully to minimise oxidative damage to hydrogenases [269], it was hoped that chemical oxidation would prevent oxidative damage and that purified enzyme would, following reductive reactivation, show a higher activity. Modifications to the preparation method are summarised in Table 2.8.

In order to evaluate the effectiveness of these protocols, hydrogenase activity was assayed in solution using viologen mediators, according to Section 2.2.5. The results are somewhat surprising, indicating that the aerobic protocol with french press lysis gives much higher levels of hydrogenase activity than any other preparation. The ferricyanide oxidation protocol purifies enzyme complex with almost no discernible activity whatsoever, perhaps due to degradation of exposed metal clusters in the presence of ferricyanide, while the other three methods give nearly the same result: an approximately ten-fold reduction in activity. For all methods tested, H_2 oxidation activity is much

higher than H^+ reduction activity. Figure 5.4 gives a summary of the results obtained.

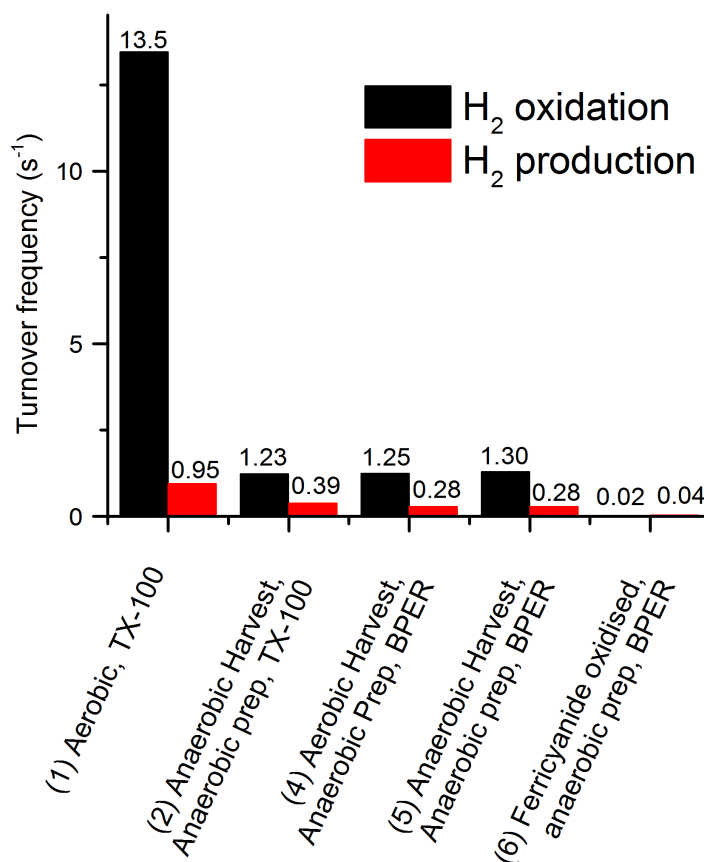


Figure 5.4: The hydrogenase activity measured for various methods of enzyme purification tested. Preparation methods are labelled according to the method number specified in Table 2.8. All assays were carried out at pH 6.5, 20 °C.

Thus it seems that, despite the many efforts, an intact FHL complex with a high specific activity is best and most easily obtained using an aerobic purification protocol that allows cells to be lysed using a french press. Unless otherwise stated, further results in this chapter will be for enzyme isolated aerobically from cells lysed by french press.

Aerobically-isolated FHL complex evolves H_2 from formate

In order to demonstrate that the isolated complex is both intact and active, we measured rates of H_2 evolution from formate. FHL complex, isolated aerobically according to section 2.2.1 (method 1), was suspended in pH 7 100 mM MES buffer, purged under a stream of N_2 with 2% CH_4 as calibrant for 15 minutes in order to remove all traces of O_2 and H_2 . After an initial measurement to confirm the absence of H_2 , a solution of

sodium formate, titrated to pH 7, was added to give a final formate concentration of 5 mM, and the solution was incubated at 25 °C. H₂ concentration in the cell headspace was monitored by gas chromatography (section 2.11). Enzyme concentration was determined by a Bradford assay of a sample of the enzyme (Section 2.2.4). After addition of the formate solution, the enzyme solution was seen to precipitate over time. This precipitation is likely the cause of a slowdown in H₂ production activity seen in Figure 5.5.

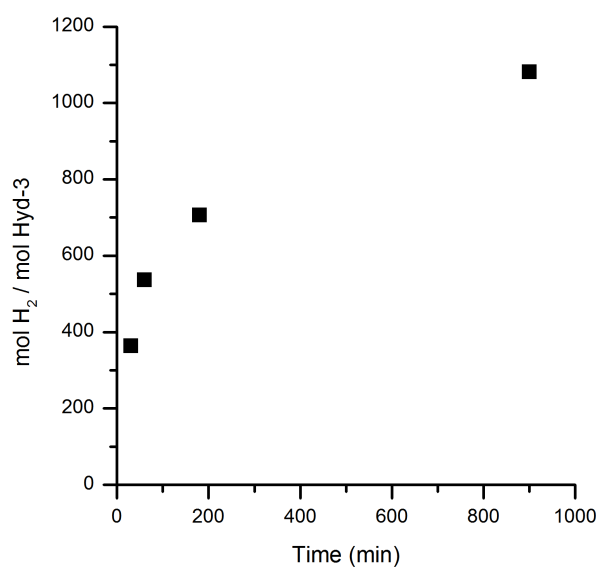


Figure 5.5: Evolution of H₂ from the isolated FHL complex at pH 7, 25 °C, 5 mM formate, monitored by GC.

The maximum turnover rate, observed immediately following addition of formate, was slightly over 12 min^{-1} . Although this rate is not extremely high, we have unequivocally proven that, for the first time, active, intact FHL complex has been isolated from *E. coli*. No H_2 evolution was observed for a similar experiment using the anaerobically-prepared enzyme; this serves as further confirmation that the formate dehydrogenase subunit does not co-purify in this preparation.

Solution-based activity assays of aerobically purified Hyd-3

In order to characterise the activity of the hydrogenase portion of the FHL complex (minimally HycEG), viologen-based assays were carried out for both H_2 production and H_2 oxidation across a range of pH values, according to Section 2.2.5. The turnover frequencies, displayed as a function of pH (Figure 5.6), were much higher than those observed for the formate-to-hydrogen assays. For both H_2 oxidation and H_2 production, the highest rate of turnover was seen at pH 6.5. H_2 oxidation rates were more than ten times greater than H_2 production rates, a fact which is seemingly at odds with the principal role of Hyd-3 as a hydrogen-producing enzyme.

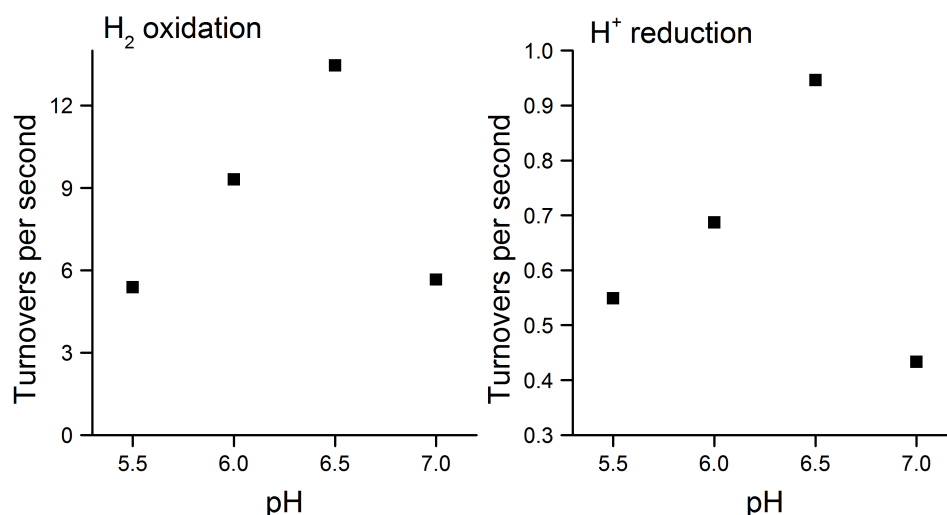


Figure 5.6: Viologen-based assays of hydrogenase activity at varying pH and 20 °C for FHL isolated aerobically.

Metal analysis of aerobically-isolated FHL complex

In order to get a more thorough assessment of the complex in terms of subunit composition and metal content, we sent samples of aerobically-purified (method 1) FHL for analysis by Total Reflection X-ray fluorescence in the lab of Prof. M. Haumann, Freie Universität Berlin. The molar ratios of metals and selenium are presented in Table 5.1.

Element	Fe	Ni	Mo	Se
Moles/mole enzyme	30.25±3	0.98±0.15	0.36±0.07	0.28±0.06
Predicted values	33	1	1	1

Table 5.1: Metal ion content of aerobically-isolated FHL complex (method 1), as mole ratios, determined by TXRF by Prof. M. Haumann, Freie Universität Berlin.

For the predicted subunit architecture shown in Figure 5.2 the expected molar ratios are also shown in the table. The levels of both Mo and Se are well below those expected, however they are within error of one another, suggesting that roughly $\frac{2}{3}$ of the isolated complex is missing the the FdhF subunit. This would contribute to the relatively low rates of formate:H₂ activity observed for the complex. Partial loss of the FdhF subunit also accounts for a lower Fe content, as this subunit contains a Fe₄S₄ cluster.

5.2.2 Electrochemical characterisation of Hyd-3

The traditional approach to setting up a PFE experiment by ‘spotting’ enzyme onto a sanded PGE electrode gave no discernible current for isolated FHL complex, regardless of the method of purification used. The reasons for this are unknown: although the hydrogenase turnover frequencies are not as high as those observed for Hyd-1 and Hyd-2, they should be sufficiently high to record a current at a graphite electrode, if good electronic contact can be established. However, the current observed in a PFE experiment is dependent upon many factors in addition to the overall turnover frequency; in particular the propensity of an enzyme to adsorb to a graphite surface is a critical but poorly understood requirement. Various hydrogenases and other proteins have shown a wide

variability in the strength of adsorption to a PGE electrode [270]. The configuration of adsorption and the exposure of FeS clusters will determine, for adsorbed enzyme, the efficiency of interfacial electron transfer (from electrode to protein and *vice-versa*). Because intact FHL complex, unlike the respiratory MBHs of *E. coli*, represents a ‘closed’ electronic circuit, in which electrons derived from one buried active site are transferred to a second buried active site, it is perhaps not surprising that a classical PFE experiment shows no activity. Our experiments show that some of the complex has lost the FdhF subunit. It is likely that complexes missing this subunit would be more likely to be seen in PFE, due to a more exposed FeS cluster.

In order to increase electrode surface area, we used graphite electrodes modified by deposition of MWCNTs, according to section 2.3.4. After many failed attempts, activity was seen for anaerobically-purified enzyme from TX-100 lysed cells (method 2, Table 2.8). Covalent immobilisation using the pyrene butyric acid-EDC-NHSS method described was tested, but was not found to give significant increases in current, and was therefore eliminated. Adsorption of purified FHL onto the MWCNT electrodes gave small but usable currents. MWCNT-modified electrodes, due to their much larger electroactive surface area, also show much higher capacitance (see Section 2.3.2); it was therefore necessary to carry out CVs at very low scan rate in order have an acceptable signal-to-noise ratio.

A ‘snapshot’ of Hyd-3 activity

Figure 5.7 provides a first assessment of the properties of Hyd-3 in early PFE experiments. It is clear from these voltammograms that Hyd-3 is a reversible catalyst, with minimal overpotential requirement for catalysis in either direction. Lowering the $\rho(\text{H}_2)$ from 100% to 30% results in a significant reduction in the observed H_2 oxidation current with minimal change in the H^+ reduction current; even when H_2 is removed completely, the H^+ reduction activity is not seen to increase drastically. In contrast with other

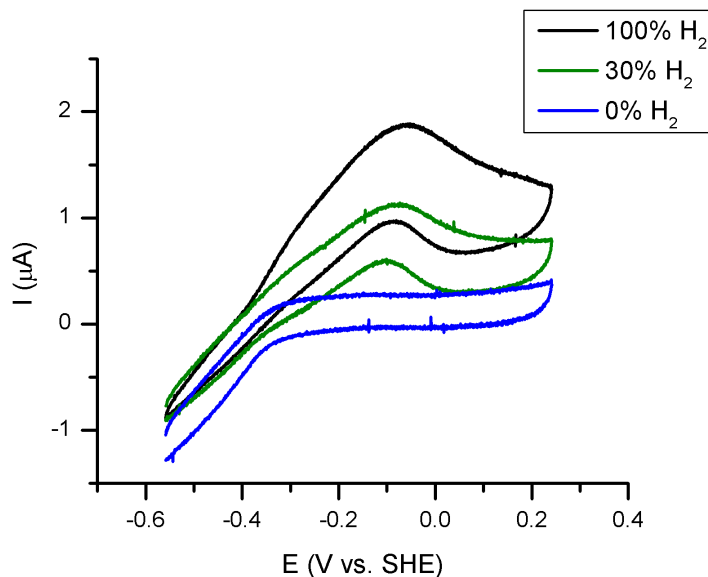


Figure 5.7: A ‘snapshot’ of the activity of Hyd-3 by cyclic voltammetry under varying concentrations of H_2 , at pH 6 and 37°C , 1mVs^{-1} , $\omega = 3000\text{rpm}$, gas flow rate = 1000 sccm . Enzyme was purified anaerobically, with Triton X-100 cell lysis (method 2, Table 2.8).

[NiFe] hydrogenases previously studied, including Hyd-1 and Hyd-2 studied in this thesis, Hyd-3 seems to show a rather low affinity for H_2 (a high $K_M^{\text{H}_2}$) for H_2 oxidation, and a reduced extent of product inhibition during H_2 production (a high $K_I^{\text{H}_2}$).

Although the reversible nature of Hyd-3 (studied by crude cell extract assay) has previously been suggested to indicate a physiological role for Hyd-3 in H_2 oxidation [267], it is difficult to envisage a useful physiological role for this transformation, given that formate is only metabolised once it becomes toxic to the cell. Furthermore, the low affinity for H_2 of Hyd-3 in comparison to Hyd-1 and Hyd-2 suggests that, *in vivo*, H_2 oxidation would occur preferentially via the respiratory hydrogenases. It is therefore likely that the reversibility of Hyd-3 is simply a mark of an efficient catalyst, and is of no physiological relevance. One feature that is immediately obvious from this voltammogram is the ratio of oxidative to reductive catalysis; the rates of these processes seem to be approximately equal at the same overpotential in either direction. This observation is at odds with the ratio of rates observed in viologen-based solution assays in which H_2 oxidation far outpaces H^+ reduction.

Hyd-3 shows a high $K_I^{H_2}$ during H_2 production, and a high $K_M^{H_2}$ during H_2 oxidation, atypical of a [NiFe] hydrogenase

In order to quantify the substrate affinity during H_2 oxidation and the extent of product inhibition during H^+ reduction, detailed chronoamperometry studies were carried out, shown in Figure 5.8. The $K_I^{H_2}$ was found to be 1.5 ± 0.15 mM at pH 6, 37C and -558 mV vs. SHE; to our knowledge this is the highest value ever measured for a [NiFe] hydrogenase.

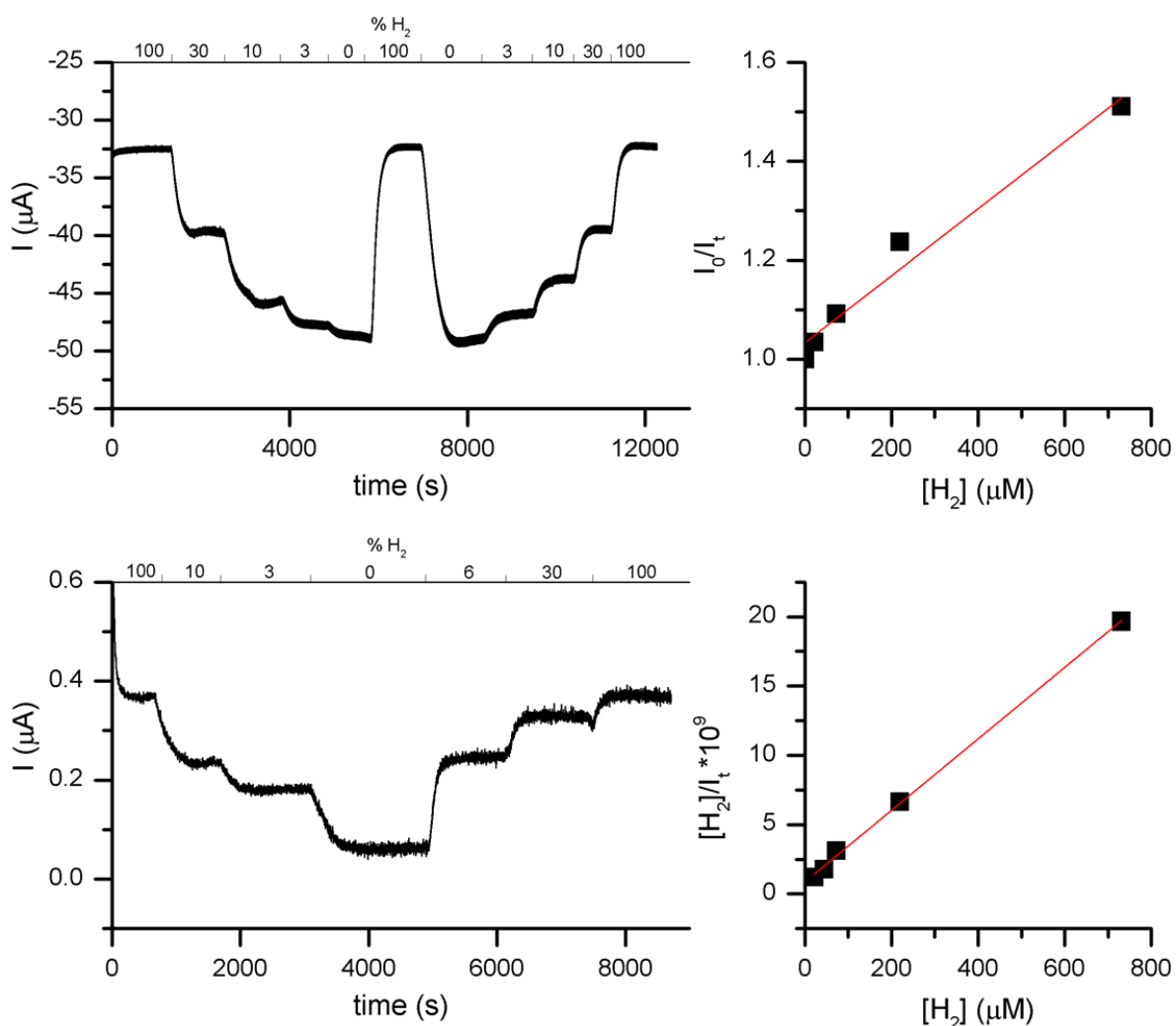


Figure 5.8: Chronoamperometric measurement of $K_I^{H_2}$ (top) and $K_M^{H_2}$ (bottom) of Hyd-3 at pH 6, 37 °C, -0.558 V vs. SHE (top) and -0.108 V vs. SHE (bottom), $\omega = 1000$ rpm, gas flow = 1000 sccm. Enzyme was purified according to method 2, Table 2.8

Hyd-3 displays a $K_M^{H_2}$ of 34 ± 7 μM at pH 6, 37C, -108 mV vs. SHE. Although this value is significantly higher than the $K_M^{H_2}$ of the hydrogen-oxidizing Hyd-1 of *E. coli*, it

is only roughly twice the value for *E. coli* Hyd-2 [44].

These experiments reveal Hyd-3 as unique among [NiFe]-hydrogenases in terms of its ability to maintain a substantial proportion of its H_2 production activity, even under 100% H_2 atmospheres. This property is in line with the physiological role of the enzyme as a H_2 -producer, and is unique among [NiFe] hydrogenases studied thus far. Indeed, the $K_I^{H_2}$ for Hyd-3 is more similar to that observed for [FeFe] hydrogenases than for other [NiFe] hydrogenases (Figure 5.9).

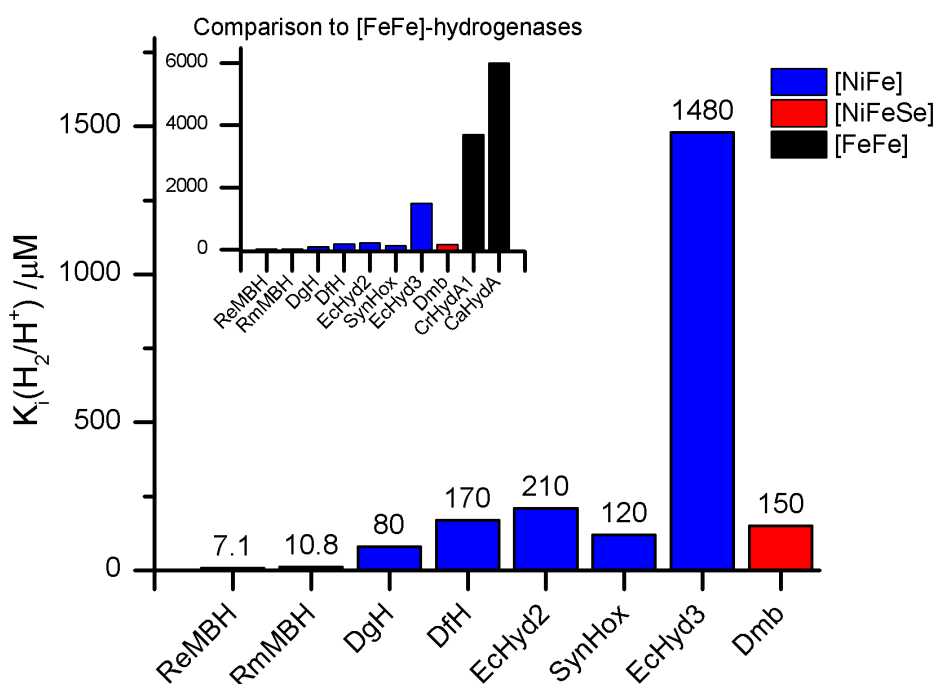


Figure 5.9: A comparison of the $K_I^{H_2}$ values of various hydrogenases. Citations for each measurement, and experimental conditions, are displayed in Table 5.2.

It is important to note that these values are not perfectly comparable, as they are taken from a large number of different studies carried out under different conditions (see Table 5.2); nonetheless, it is apparent that a general difference exists between Hyd-3 and other [NiFe] hydrogenases.

Enzyme	Conditions
<i>R. eutropha</i> MBH	pH 5.5, 30 °C, -450mV <i>vs.</i> SHE [180]
<i>R. metallidurans</i> MBH	pH 5.5, 30 °C, -450mV <i>vs.</i> SHE [180]
<i>D. gigas</i> H	pH 6, 40 °C, -660mV <i>vs.</i> SHE [85]
<i>D. fructosovorans</i> H	pH 6, 40 °C, -660mV <i>vs.</i> SHE [85]
<i>E. coli</i> Hyd-2	pH 6, 30 °C, -600mV <i>vs.</i> SHE [44]
<i>Synechocystis</i> PCC6803 Hox	pH 5.4, 25 °C, -453mV <i>vs.</i> SHE; $K_I^{H_2}$ calculated from data in Figure 9 [270]
<i>E. coli</i> Hyd-3	pH 6, 37 °C, -558mV <i>vs.</i> SHE
<i>Desulfomicrobium baculatum</i> [NiFeSe]	pH 5.5, 30 °C, -450mV <i>vs.</i> SHE [271]
<i>Chlamydomonas reinhardtii</i> HydA1	pH 6, 10 °C, -400mV <i>vs.</i> SHE [271]
<i>Clostridium acetobutylicum</i> HydA	pH 6, 10 °C, -400mV <i>vs.</i> SHE [271]

Table 5.2: Experimental conditions and references for the determination of $K_I^{H_2}$ values presented in Figure 5.9

Hyd-3 shows a reasonable tolerance to inhibition by CO

The reversible inhibitor carbon monoxide is a useful probe of gas access to the active site [272, 273]; in addition, O₂-tolerant [NiFe] hydrogenases show much higher tolerances to inhibition by CO than their O₂-sensitive counterparts. Sensitivity to inhibition by CO is therefore often used as a comparator between different [NiFe] hydrogenases. Figure 5.10 shows chronoamperometry experiments designed to assess the inhibition constants during oxidative and reductive catalysis. The K_I^{CO} for Hyd-3 is $56 \pm 17 \mu\text{M}$ for H⁺ reduction at 37 °C, pH 6.0, -0.537 V and $8.2 \pm 1.8 \mu\text{M}$ for H₂ oxidation at 37 °C, pH 6.0, -0.087 V *vs.* SHE. Hyd-3 is somewhat tolerant to inhibition by CO, with inhibition constants falling somewhere between those measured for Hyd-1 ($52 \mu\text{M}$ at pH 6.0, 30 °C, -0.175 V [44]) Hyd-2 ($1.8 \mu\text{M}$ at pH 6.0, 30 °C, -0.175 V). Because of the much lower $K_M^{H_2}$ of Hyd-1, the apparent effect of a given concentration of CO is considerably larger for Hyd-3 than it is for Hyd-1 or even Hyd-2.

We do not yet understand the significance of CO tolerance as it relates to O₂-tolerance, but a characterisation of Hyd-3, an enzyme very different to previously characterised [NiFe] hydrogenases, may well inform the continuing discussion as to what, if any, relationship exists between the parameters.

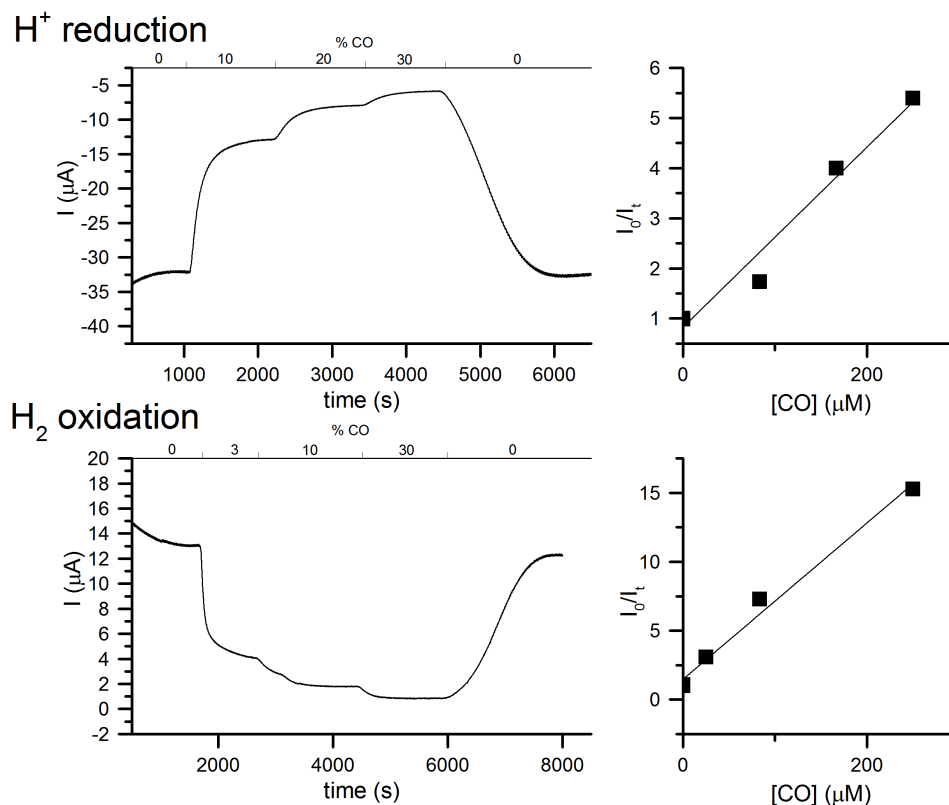


Figure 5.10: Chronoamperometry experiments designed to assess the K_I^{CO} for Hyd-3 during H^+ reduction (top) and H_2 oxidation (bottom). Film loss was estimated according to the steady-state slope at each CO concentration and data were corrected for this according to the procedure described in section 5.2.2. Experimental conditions were as follows: pH 6.0, 37 °C, $\omega = 1000$ rpm, gas flow = 300 sccm 100% Ar (for H^+ reduction) or 10% H_2 in Ar (for H^+ oxidation), $E = -537$ mV *vs.* SHE (reduction), $E = -87$ mV *vs.* SHE (oxidation). K_I^{CO} values were 56 ± 17 μ M for H^+ reduction and 8.2 ± 1.8 μ M for H_2 oxidation. Enzyme was purified according to method 2, Table 2.8.

Hyd-3 shows short-term resistance to low-level O_2 inhibition during H_2 production

The resistance to inhibition by O_2 during H^+ reduction is somewhat difficult to measure, because of the additional signal due to direct O_2 reduction at the electrode. Figure 5.11 demonstrates one technique for assessing the extent of activity under O_2 . First, the current due to H^+ reduction is assessed in the absence of O_2 : it is apparent in this experiment that the ‘film stability’, that is, the stability of current over time, is not particularly good for this film. Introducing O_2 to the system results in a decrease in the H^+ reduction current; at the same time, negative current due to direct O_2 reduction

is observed, and both of these effects are greater as $\rho(\text{O}_2)$ is increased. Eventually, O_2 was flushed from the system and the remaining H^+ reduction activity was near zero. Following this experiment, the enzyme was fully inactivated by soaking the electrode for ten minutes in a saturated solution of NaCl. This method is expected to preserve the electroactive surface area of the electrode, allowing for a blank experiment to be recorded in which the current due to O_2 reduction at the electrode is measured. Subtraction of this blank (grey trace) from the experimental trace (blue trace) allows the H^+ reduction activity to be visualised (black trace).

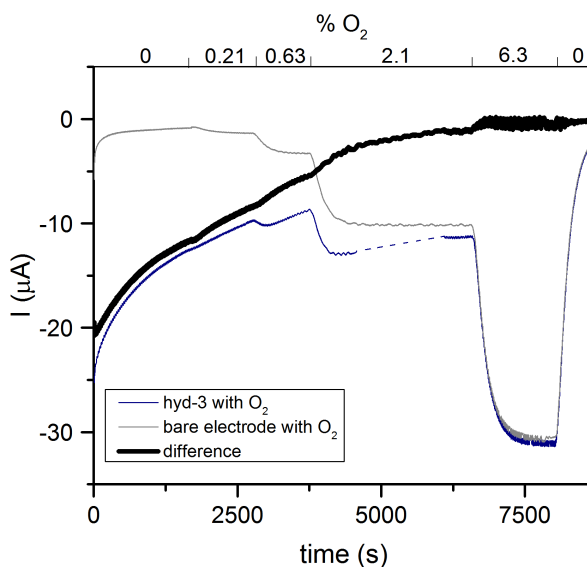


Figure 5.11: The effect of long-term O_2 exposure on Hyd-3 H_2 production activity, at pH 6, 37°C , $\omega = 2000\text{rpm}$, gas flow 1000 sccm Ar/O_2 mix. The black trace is a measure of the H^+ reduction activity over time, measured as the difference between the current at an electrode with active Hyd-3 adsorbed and the current measured under the same conditions for the same electrode after inactivation of the enzyme by soaking in saturated NaCl solution. The dashed line in the experimental trace (blue) is a linear extrapolation of data, due to electrical noise during the experiment that obscured the current in this region. Enzyme was prepped according to method 2, Table 2.8.

Certainly, the activity seen in this scan does not qualify the enzyme as O_2 -tolerant; nonetheless, it seems that, from the perspective of Hyd-3 activity at least, a short exposure to low levels of O_2 in the cell would not overwhelm the FHL.

A characterisation of the (in)stability of Hyd-3 on an electrode

PFE is arguably the best technique for gaining insight into the mechanism and catalytic properties of redox enzymes whose turnover frequencies are high, and who spontaneously establish a stable and intimate electronic connection with the electrode. We understand very little about what makes certain enzymes more likely to adsorb in a configuration that allows lasting electronic communication between the enzyme's active site and the electrode. It seems that, whatever this magic is, Hyd-3 does not have it. We have not observed any activity on unmodified graphite electrodes, despite substantial efforts. MWCNT-modified electrodes provide an increased surface area, and a more porous surface, to increase the chances of success. Even with this method, which has successfully given currents on the order of 1 mA/cm^2 , stable for over a week, when modified with Hyd-1 [167], with Hyd-3 we have obtained currents barely discernible above the capacitance at moderate scan rates, on the order of only approximately $10 \text{ }\mu\text{A/cm}^2$. The activity is also not stable over long periods of time, leading to difficulties with analysis of data for some experiments.

The loss of activity over time has been termed 'film loss' [274] due to the assumption that it is likely to result mostly from desorption of the enzyme from the electrode and/or a change in orientation preventing interfacial electron transfer. This parameter, as seen for other hydrogenases, is dependent to a small extent on destabilising effects such as rapid changes in the potential of the electrode and high temperatures, however during the course of an experiment, the loss in enzyme activity is normally easily described by a single exponential function, insensitive to changes in substrate or inhibitor concentrations or to the potential of the electrode. With such a profile, there is no reason to suspect that film loss for previously studied systems is due to anything other than a physical change at the surface of the electrode of little to no mechanistic interest.

Chronoamperometry experiments carried out with Hyd-3 to assess parameters such as Michaelis-Menten and inhibition constants gave a very different 'film loss' profile, in

which the rate of loss was very rapid under certain conditions but was, in some cases, partially reversible, and in which conditions such as substrate and inhibitor concentrations and electrode potential played a noticeable role. This effect significantly complicated analysis of these data sets; moreover this result suggests that for Hyd-3, processes other than desorption or orientation are occurring, likely chemical changes at the active site.

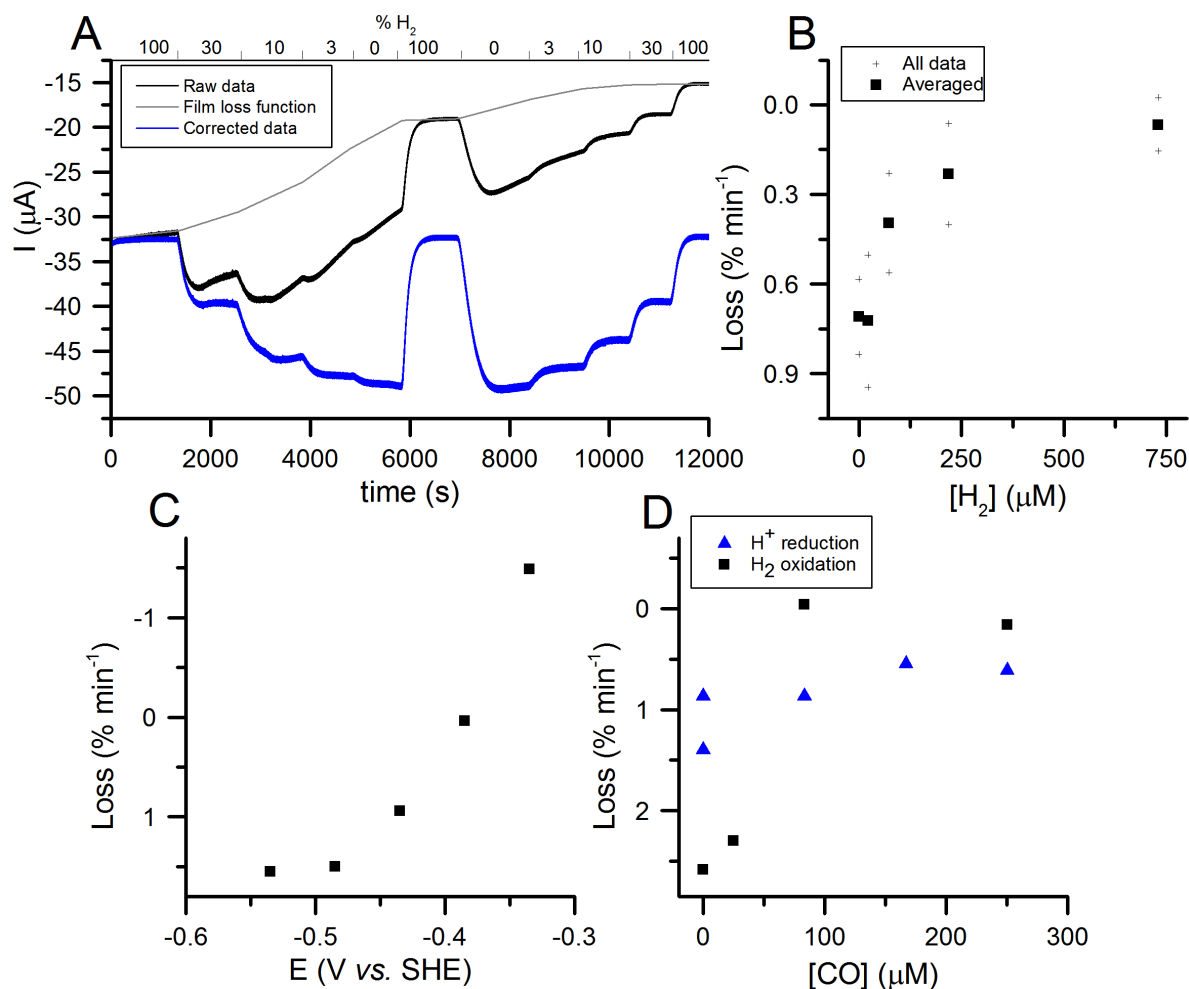


Figure 5.12: Changes in the rate of current loss with substrate and inhibitor concentration, and applied potential. Conditions were as follows: A) and B) pH 6, 37 °C, $E = -0.558$ V vs. SHE $\omega = 1000$ rpm, gas flow = 1000 sccm; C) pH 6, 37 °C, 100% Ar $\omega = 1000$ rpm, gas flow = 300 sccm; D) pH 6.0, 37 °C, $\omega = 1000$ rpm, gas flow = 300 sccm Ar (for H^+ reduction), 10% H_2 , in Ar (for H^+ oxidation) with varying proportions of CO, $E = -537$ mV vs. SHE (reduction), $E = -87$ mV vs. SHE (oxidation). Enzyme was purified according to method 2, Table 2.8.

Figure 5.12A shows the raw data, the film loss correction and the corrected data for the $K_I^{\text{H}_2}$ experiment shown above. Raw data are shown as a black trace, and it is immediately obvious that a very high rate of film loss is observed in the absence of H_2 ,

whereas in high concentrations of H_2 the current is nearly stable. The rate of current loss is faster at the beginning of the experiment, but the trend is consistent across the timescale of the experiment. Such a dependence upon H_2 concentration is not normally observed for film loss, indicating that this may be reflective of a chemical inactivation occurring during catalysis. In order to account for this, it was necessary to construct a ‘film loss trace’, displayed in grey, using the slope of the trace after gas equilibration at each step in gas composition as an indicator of film loss rate. Correcting the raw data according to the film loss trace gives the blue trace, which can then be used for $K_I^{H_2}$ analysis.

The rate of current loss was minimised under high concentrations of H_2 , higher potentials, and high concentrations of CO (Figure 5.12 B, C and D, respectively). For all experiments, the slope of the current trace was taken hundreds of seconds after conditions were changed and was divided by the magnitude of the current to give a relative change. The utmost care was taken to ensure that no O_2 was present in the box atmosphere or in the gases used, furthermore the potential dependence of the rate of current loss (Figure 5.12C) is opposite to what we have seen for O_2 -dependent inactivation; it is therefore unlikely that this effect is due to inactivation by trace O_2 . It is notable that some reversibility was observed during the potential-step experiments (panel C). After exposure to low potentials, moving to potentials just below $E_{eq}^{H^+/H_2}$ results in a slight *increase* in the negative current over time. Given the very low driving force for the FHL reaction, it is unlikely that Hyd-3 is exposed to very negative potentials *in vivo*, although this may or may not be significant. The protective effect of CO was more pronounced during H^+ reduction than during H_2 oxidation (Figure 5.12D).

Given the relative instability of the FHL complex and the associated difficulties in studying the complex by PFE, it is difficult to speculate as to the cause of these trends in current loss, but this characterisation will serve as a useful guide for future studies of the complex, and the dependence of the rate of inactivation on substrate and inhibitor

concentrations and on potential seems to be a unique feature of Hyd-3, and may be indicative of the occurrence of a chemical inactivation process for this enzyme.

Properties of Hyd-3 seen in PFE experiments vary between different preparations

The PFE results presented thus far were all conducted using different aliquots from a single protein purification of anaerobically isolated Hyd-3 from Triton X-100-lysed cells. This purification method did not give intact FHL but rather was lacking the FdhF subunit. The properties of the enzyme were repeatable across experiments, and it is possible that the loss of the formate dehydrogenase subunit allowed electronic contact by exposing an otherwise buried FeS cluster. Different purification protocols, in which gentler cell lysis led to isolation of intact FHL complex, gave a very different set of properties when studied by PFE. Figure 5.13 gives a demonstration of the differences observed between the TX-lysed, anaerobically purified Hyd-3 and the aerobically-purified FHL from French press lysed and B-PER lysed cells.

Whereas the anaerobically-purified, TX-100 lysed enzyme was seen to catalyse both oxidation and reduction at comparable rates, with minimal overpotential requirement in either direction, the B-PER lysed and french press lysed cells used for aerobic purifications gave enzyme that catalysed almost no H^+ reduction, and that required the application of a significant overpotential (~ 0.1 V) for H_2 oxidation. It seems as though the complete FHL complex shows a ratio of oxidative to reductive activity more consistent with rates measured by solution assay, although it is notable that the ratio of oxidative to reductive catalysis was highest for the Triton lysed purification in these solution assays (Figure 5.4). Given the physiological role of the FHL complex in H_2 production, it seems unlikely that Hyd-3 should show little to no reductive activity in its native state.

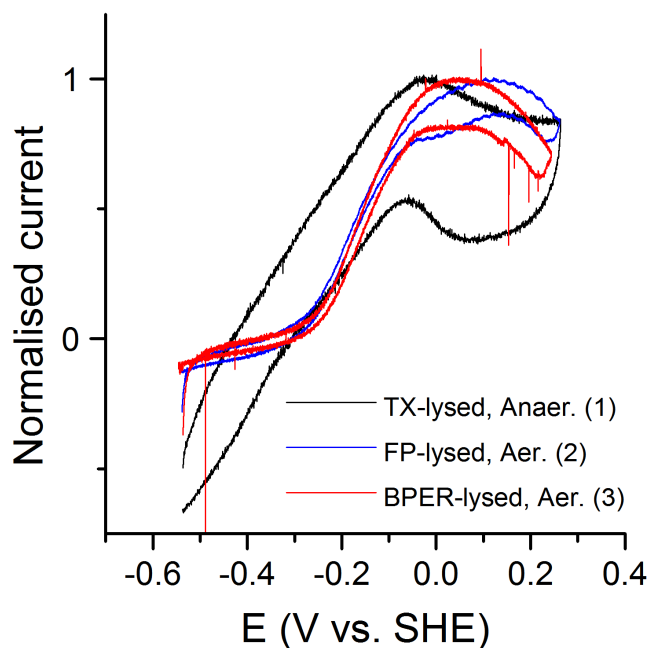


Figure 5.13: Properties of different preparations of FHL as seen in a CV experiment. Black trace: HycEGFB complex purified anaerobically from Triton X-100 lysed cells (method 2); blue trace: FHL complex purified aerobically from french press lysed cells with Triton X-100 membrane solubilisation (method 1); red trace: FHL complex purified aerobically from B-PER lysed cells (method 3). All experiments were carried out at pH 6, 37 °C, $\omega = 1000$ rpm; black trace: 1 mVs^{-1} , gas flow = 300 sccm; blue and red traces 0.2 mVs^{-1} , $\omega = 1000$ rpm, gas flow = 1000 sccm.

In the context of work presented in this thesis on the importance of electron transfer in determining apparent catalytic bias for Hyd-1 (Chapter 3), it seems likely that the redox potential of the cofactor by which electrons enter and leave the enzyme may play a critical role in setting the potential range in which efficient electron transfer, and thus catalysis, is possible. Therefore, we may speculate that the catalytic bias seen for some preparations of intact FHL but not seen for anaerobically-purified Hyd-3 complex may be related to differing points of electron entry for these different complexes.

More work is needed for a full account of the electrochemical properties of this complex, as well as an understanding of the way in which interfacial electron transfer affects properties observed by PFE. Given the long history of researchers who have tried unsuccessfully to purify an intact, active FHL complex, it should not be surprising that some challenges have arisen with regard to the stability of the complex and with repro-

ducibility across different purification conditions. Nevertheless, this work represents a significant contribution to our understanding of the electrochemical properties of Hyd-3, the H₂-producing enzyme of *E. coli*.

5.2.3 Characterisation of FHL by EPR spectroscopy

The pentameric FHL complex is predicted to contain ten individual redox-active cofactors: a Mo-based active site within the formate dehydrogenase subunit, a [NiFe] active site within the Hyd-3 large subunit, and a chain of eight FeS clusters that transfer electrons from the formate dehydrogenase to the hydrogenase. The formate dehydrogenase subunit has been overexpressed and purified, and has previously been studied by in-depth EPR analysis as well as by XAS and X-ray crystallography, and the signals due to Mo(V) are well understood and characterised [259, 263, 260, 258]. The Hyd-3 active site is predicted to be a standard [NiFe] active site, although very little exists in the way of characterisation of the active enzyme, as it has not previously been purified. The eight FeS clusters have not been studied, but the many studies required to understand and assign the FeS cluster signals of Complex I suggest that the full deconvolution of these signals may require several studies combining a variety of advanced EPR techniques.

Preliminary continuous-wave (CW) measurements at X-band frequencies are shown in Figure 5.14. At high potential, a broadened isotropic signal with $g = 2.01$, visible at 15 K but not at 80 K, and resembling a Fe₃S₄ cluster is seen. This signal increases in intensity throughout the course of a redox titration, and is thus likely to result from breakdown of a Fe₄S₄ cluster that is solvent-exposed. Predictions based on sequence homology suggest that the FeS clusters of the FHL complex are likely to all be of the Fe₄S₄-type, which increases the likelihood that this signal is a breakdown product. The signal recorded at 80 K for this high-potential sample is nearly flat, showing no Ni(III) signal characteristic of [NiFe] hydrogenases at high potential.

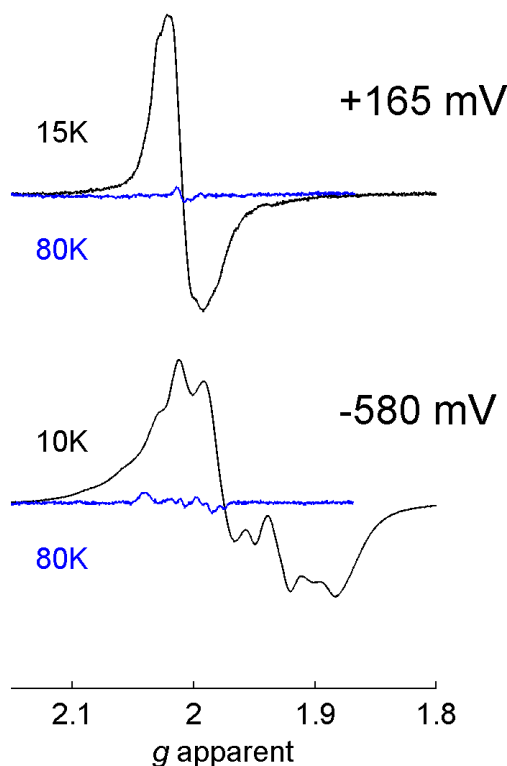


Figure 5.14: Preliminary characterisation of the FHL complex by X-band CW EPR at high and low potential. Top) High-potential sample(+165 mV) at 15 K (black), and 80 K (blue). Bottom) Low-potential sample (H_2 -reduced) at 10 K (black), and 80 K (blue). All spectra were measured under the following conditions: Microwave power (MP) = 2.0 mW; Modulation Amplitude (MA) = 5 G. Protein samples were purified according to the aerobic protocol in which cells are lysed by french press and membranes are solubilised using Triton X-100 (method 1, Table 2.8).

At low potential (Figure 5.14, bottom), a complicated signal is visible at low temperature. The complexity of the signal suggests it arises from a large number of paramagnetic species, likely reduced FeS clusters. Further studies of this signal are presented below.

A flow of H_2 in Ar was used to attain various potentials to survey the reduced signal. The low-potential signal first appears at -295 mV, and is already a rather complicated signal with many peaks at this potential, while at -227 mV no signal is seen (Figure 5.15A). It is likely, therefore, that several of the Fe_4S_4 clusters that contribute to this signal have a midpoint potential between or just below the range -227 mV to -295 mV *vs.* SHE. Reduction to more negative potentials changes the signal observed, mostly in terms of peak magnitude. The signal centred around $g = 2$ for the -295 mV sample is

due to a mediator in solution. Panel B demonstrates the temperature dependence of the signal in a sample reduced to -358 mV *vs.* SHE with a flow of H₂. The signal is effectively relaxed as low as 45 K, providing further evidence that the signal could arise from FeS clusters, which are known to relax relatively quickly. Panels C and D provide a saturation analysis of the different peaks of the signal, recorded for the same sample.

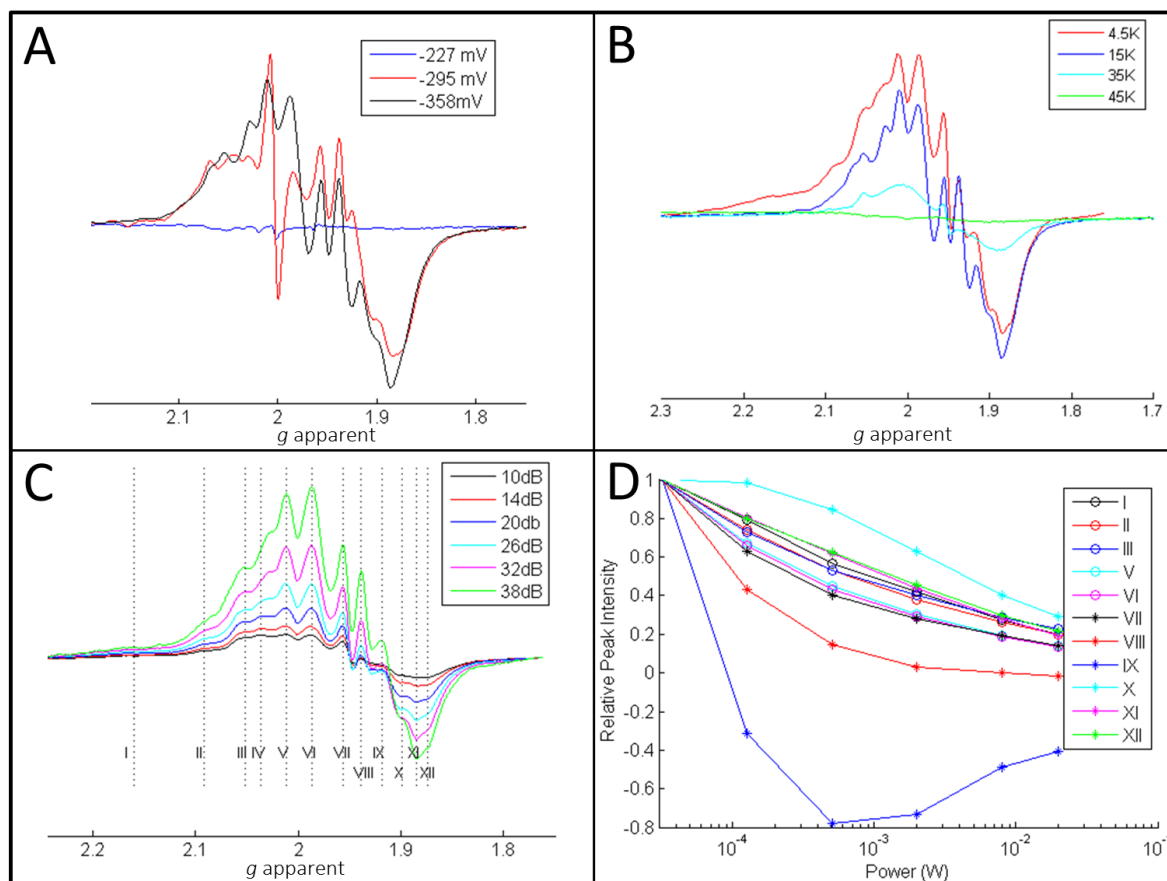


Figure 5.15: Studies of the Fe₄S₄ clusters of the FHL complex. A) Potential dependence of the low-potential signal: 10 K, MP = 2.0 mW, MA = 5 G B) Temperature dependence of the low-potential signal suggests it is due to fast relaxing species, likely Fe₄S₄ clusters, MP = 0.5024 mW, MA = 5 G C) H₂-reduced, -358 mV sample measured at 4.5K and different powers, with relative peak intensity measured at *g*-values indicated by dotted lines, as follows: I. 2.16; II. 2.09; III. 2.05; IV. 2.04; V. 2.01; VI. 1.99; VII. 1.96; VIII. 1.94; IX. 1.92; X. 1.90; XI. 1.89; XII. 1.87. MA = 5 G D) The relative intensity for each peak as a function of microwave power. All measurements were carried out on complex prepared anaerobically by TX-100 cell lysis and brought to a stable potential under a flow of H₂ and Ar, mixed in ratios according to the potential desired. In panel A, the sample at -295 mV contains a cocktail of redox mediators as described in section 2.4; the other samples did not contain mediators. Protein was purified according to method 1, Table 2.8.

As attenuation increases (power decreases), the relative intensity of peaks changes. Because the saturation properties of various centres are likely to differ, this type of analysis allows us to begin assessing which peaks might be due to the same paramagnetic species [275]. The different saturation profiles observed suggest that at least four paramagnetic species contribute to the complex spectrum recorded at -358 mV. The g -value of peak maximum for peak IV shifts with power; this peak has therefore been omitted from the figure in panel D. The saturation profile of peak IX is indicative that at least two signals, one positive and one negative at this g -value, and with different saturation properties, contribute to the observed peak.

Seeing and characterising the active site cofactors of the formate dehydrogenase and hydrogenase subunits proved to be difficult. At high potential and high temperature, where one would normally expect to see Ni(III) signals corresponding to Ni-A and/or Ni-B (Figure 1.3), the spectra for aerobically-purified enzyme contained no discernible signal. For anaerobically-purified, Triton-X lysed complex (lacking FdhF), a small rhombic signal could be seen with g -values of 2.17, 2.12, 2.01. (Figure 5.16A); however, this signal integrated to only 0.06 spins/molecule, and we cannot be confident that it is due to Ni.

These g -values most closely match those for Ni-C in other [NiFe] hydrogenases ($g = 2.20, 2.15, 2.01$ for *D. vulgaris* Miyazaki F [276]), a signal which appears under reducing conditions. Thus, it seems possible, though unlikely, that this signal is attributable to an oxidised form of Ni at the active site. Characterisation of the reversible [NiFe] hydrogenase from *Synechocystis* PCC 6803, an enzyme thought to act reversibly *in vivo*, has so far shown no classical oxidised Ni signals in EPR [176]. It would be very desirable to study this signal further, as currently very little is known regarding the [NiFe] site of this enzyme. It is likely that the very high $K_I^{H_2}$ observed for Hyd-3 (Section 5.2.2) among [NiFe] hydrogenases, a very desirable property for a H_2 -producing catalyst, is related to differences at the active site.

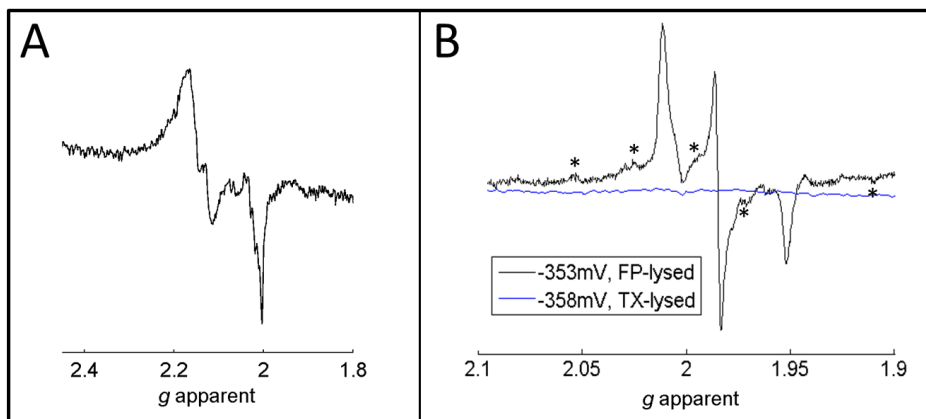


Figure 5.16: Putative active site EPR signals for the FHL complex. A) Signal seen at high-potential (+369 mV *vs.* SHE) for anaerobically-purified enzyme (method 2, Table 2.8 integrating to 0.06 spins/molecule, with g -values of 2.17, 2.12, and 2.01, measured at 60 K, MP = 2.0mW, MA = 5G. Aerobically-prepared enzyme did not exhibit a similar signal. B) Signal seen at mid-potential (-353 mV *vs.* SHE) for aerobically-prepared (method 1) complex (black trace), with g -values of 2.011, 1.985, 1.952. No signal was observed for the anaerobically-purified HycEGFB complex purified by method 2 (blue trace). Measurements were recorded at 60 K, MA = 5 G, Power = 2.0 mW (black trace); 0.5024 mW (blue trace).

A rhombic signal with $g_{average} < 2$ was visible at mid potential for the aerobically-purified (method 1) FHL but not for the TX-100 lysed, anaerobically-purified (method 2) complex lacking FdhF (Figure 5.16B). The signal disappeared both at higher and at lower potential, supporting its assignment as Mo(V). The g values for this signal do not match those reported for overexpressed FdhF purified in the absence of the other components of the FHL complex; in those studies a Mo signal with $g_{average} > 2$ was observed [258], which would not be expected for the case of a less than half-filled d shell in a square pyramidal field. The authors of this paper suggest that the unusual result is attributable to some covalency of the Mo-Se bond (17-27%); they also note that upon exposure to O_2 , a Mo signal with $g_{average} < 2$ was observed. This is likely what we observe here. The isotopic distribution of Mo should lead to hyperfine splitting in a Mo-derived signal, as the ^{95}Mo and ^{97}Mo isotopes, accounting for 25% of naturally occurring molybdenum, both have a nuclear spin of $I = 5/2$. The signals observed by Khangulov et al. [258] did show a low intensity hyperfine splitting, which may be the

cause for the small peaks and shoulders marked with asterisks, but the data collected were not of sufficiently good quality to simulate reliably. The intensity of the small peaks observed here is not in line with the 1:4 intensity ratio that would be expected for hyperfine splitting, although

Given the extensive studies of the formate dehydrogenase subunit, it is perhaps not fruitful to continue characterisation of this subunit in the context of a very large and labile complex, especially given the extreme sensitivity of the formate dehydrogenase to O₂ exposure [258], and the fact that it does not co-purify when cells are lysed with detergent. It is perhaps better to focus, in future studies, on further exploration of the [NiFe] active site, which is not only uncharacterised to date, but also shows signs in PFE experiments of being unique among well-characterised [NiFe] hydrogenases.

Advanced EPR studies of this complex are now being carried out by Dr. M. Rößler.

5.3 Perspectives

The formate-hydrogen lyase complex of *E. coli* encompasses the longest- and arguably most-studied hydrogenase in the world, Hyd-3. A large part of the interest in this complex is due to the very high rates at which H₂ is produced by this complex *in vivo*. Many suggest that an engineered strain of *E. coli*, overexpressing native or recombinant FHL, would be a viable source of biohydrogen from the fermentation of waste products, and some notable progress has been made toward the engineering of such a strain [248, 277]. Despite the many papers published on the topic, the intact, active complex has never before been isolated. This work represents a significant milestone in our understanding of the FHL complex, including isolation of a pentameric FdhF-HycEGFB complex capable of evolving H₂ in the presence of formate. At each stage, the labile nature of the complex along with the O₂ sensitivity of its active site cofactors has proven a challenge.

By comparison with measurements *in vivo*, our activity measurements likely represent

a drastic underestimate of the activity of this complex. One of the advantages of PFE as a technique is that only active enzyme is visible, so that even for a preparation containing a large fraction of inactive enzyme, signals from the active enzyme do not have to ‘compete’ with signals from inactive enzyme, as is the case for most spectroscopic techniques. It is therefore rare to see different properties in PFE according to the method of purification. Since it is relatively uncommon to have alterations at the active site that change the properties of catalysis, but nonetheless leave the catalyst in an active state, we suggest that the most likely explanation for the different properties of differently-prepared FHL and HycEGFB complex is that the varying subunit composition (the loss of FdhF in TX-100 lysed cells) influences adsorption at the electrode surface and/or exposes an otherwise buried FeS cluster and therefore alters the rate and potential dependence of interfacial electron transfer. By comparison with the work presented in Chapter 3 on Hyd-1, we can speculate that the potential of the cluster exposed for electron transfer to and from the electrode sets a potential window within which electron transfer is favourable and may therefore impose a bias on the rates of catalysis observed. If the loss of the FdhF subunit causes a different cluster to be exposed for electron transfer, the corresponding properties of the enzyme as observed by PFE may be altered.

For respiratory enzymes, such as Hyd-1 and Hyd-2, that interact with the membrane-bound quinone pool, the distal cluster or haem group is likely to be making electrical contact with the electrode surface when the enzyme is studied by PFE, and thus measurements taken should replicate to a reasonable extent the behaviour of the enzyme *in vivo*. For a complex such as the FHL, which encompasses both oxidase and reductase, it is more difficult to assess where the electrons should enter or leave the complex to give a true simulation of the activity of the complex *in vivo*. A comparison of the voltammograms obtained for intact FHL complex and HycEGFB complex suggests that electron transfer is more efficient to and from the HycEGFB complex. This is unsurprising given that the FHL complex should represent an insulated circuit that does not exchange

electrons with the surrounding environment in the normal operation of the cell. The properties of the HycEGFB complex observed by PFE also seem to be more correct in the context of the physiological function of the Hyd-3 enzyme, which is the evolution of H₂. We therefore chose to focus on characterising this complex.

The results obtained by PFE reveal a number of interesting properties of the Hyd-3 enzyme. The enzyme shows a low sensitivity to product inhibition during H₂ production, making Hyd-3 more similar to [FeFe] hydrogenases than to other [NiFe] hydrogenases in this regard. Catalysis is fully reversible, with minimal overpotential requirement in either direction. The sensitivity to inhibition by CO, measured as the K_I^{CO} , falls between the reported values for Hyd-1 and Hyd-2. Future work with Hyd-3 should include a more thorough development of the method using MWCNT for enzyme adsorption, to increase reproducibility. If this could be accomplished, the use of MWCNT electrodes might allow characterisation of other proteins formerly resistant to adsorption [278]. Preliminary characterisation by EPR allows us to place an upper limit of -227 mV on the midpoint potential for all Fe₄S₄ clusters of the complex.

An interesting further project in Hyd-3 characterisation would be the study of a set of mutants developed by Maeda and coworkers [249] in which deletion of C-terminal regions of the HycE (hydrogenase large subunit) protein results in increased H₂ production rate up to thirty-fold, with concomitant increases in total H₂ yield from fermentation of up to nine-fold. At the time of the study, the mutated complexes could not be isolated or studied further, so an important opportunity to gain insight into the mechanism of the complex and the factors that limit its turnover was missed. Rarely do mutagenesis studies allow for such drastic improvement in the catalysis of an enzyme for its physiological substrate, and this result has puzzled me for some time now. I would speculate that since HycE and its Complex I homologues Nqo4/5 lie immediately next to the membrane-bound HycD/Nqo8 subunit believed to be involved in translating catalysis to the proton translocation machinery, and since deletion of the whole loop and/or various

parts of it in rather non-specific ways seems to increase the rate of H₂ production, is it possible that the C-terminus of the HycE subunit is involved in translating turnover to the proton pumping machinery, and that its deletion results in a complex that does not translocate protons. If this could be proven, we would have a system in which to further investigate the mechanism of proton pumping in the FHL, a study that would be of broad relevance due to the homology between FHL and Complex I.

Chapter 6

Perspectives and Future Directions

The ability of some [NiFe] hydrogenases to continuously oxidise H_2 in the presence of O_2 is truly a remarkable characteristic, and one with significant implications for technology ranging from microbial H_2 production to biomimetic catalyst design. In early studies of these enzymes, the emphasis was placed upon the search for differences at the active site [279] or in the hydrophobic channels allowing gas access to the active site [280, 241], and the certainty that these structural factors would underpin O_2 tolerance in some cases led to erroneous conclusions [281, 241]. The model of O_2 -tolerance put forward by Cracknell and coworkers [182] predicts that the rapid availability of electrons is critical to the mechanism of O_2 tolerance. In this context, the FeS cluster chain, previously thought of as simply a molecular ‘wire’ tasked with conducting electrons to and from the active site, begins to take on a new level of importance. With detailed electrochemical, mutational and spectroscopic analyses presented elsewhere [94, 95, 214, 96], we have gained an appreciation for the extent to which FeS clusters determine and tune the properties of a redox catalyst by ‘storing’ reducing power for rapid reduction of O_2 reacting at the active site.

This work led to a second question because, although mutations of the proximal and distal clusters did lead to drastic changes in O_2 -tolerance, many of the catalytic properties associated with O_2 -tolerance, such as a strong catalytic bias toward H_2 oxidation over H^+ reduction, an overpotential requirement for H_2 oxidation, a high midpoint potential for the reactivation of the ‘Ready’ oxidised Ni-B state (E_{switch}) and a high tolerance to inhibition by CO, were unaffected by the proximal and medial cluster mutations. It is likely that tolerance to O_2 is dependent upon several structural features, and that the rapid supply of electrons to the active site by the proximal and medial clusters is not in itself sufficient to explain the stability in O_2 . In this context, we sought to develop a more comprehensive understanding of these properties, as well as the structural and mechanistic differences between O_2 -tolerant and O_2 -sensitive enzymes that could explain the different properties observed. We believed that the catalytic bias and the overpoten-

tial requirement were both manifestations of a single property: a lowering of the rate of catalysis due to low rates of electron transfer, gas diffusion or active site turnover at low potential. The insensitivity of onset potential to the concentration of H_2 suggested that active site transitions were not the cause. We hypothesized that by drastically altering the solution pH we could bring the potential of the H^+/H_2 equilibrium above the potential limiting catalysis, and begin to see reversible catalysis, with minimal overpotential in either direction, for the first time in an O_2 -tolerant enzyme. The resulting experiment showed that overpotential requirement and bias are related properties in hydrogenases, in contrast to other work suggesting that different factors limit catalysis in the oxidative *vs.* the reductive direction [216]. Furthermore, the shape of voltammograms observed for H^+ reduction at low pH support the suggestion that electron transfer, rather than a change at the active site or a redox-dependent change in the diffusion of gas, is responsible for limiting activity at low potential. Thus, differences in the catalytic properties of O_2 -tolerant and O_2 -sensitive enzymes are likely due to differences in the potential of the cofactor at which electrons enter and leave the enzyme: in the case of Hyd-1, either the distal FeS cluster or one of the haems of the b-type cytochrome.

What role could this property play in mediating O_2 -tolerance? The redox cofactors of O_2 -tolerant enzymes seem, in general, to be shifted to higher potential with respect to the cofactors of O_2 -sensitive enzymes, and it is certainly true that high-potential clusters are more likely to be reduced, thus increasing the likelihood of having enough electrons to fully reduce attacking O_2 to H_2O . Whether the distal cluster must itself have a high midpoint potential in order to directly facilitate O_2 reduction to ‘Ready’ states, or whether the higher potential is required for efficient electron transfer with the medial Fe_3S_4 cluster whose unusually high potential has been shown to be directly important to the formation of exclusively ‘Ready’ states is yet to be seen.

In light of the work herein, we can perhaps reassess the schematic presented in Figure 2.5 and adapted from reference [159] for the case of O_2 -tolerant MBHs. This

early schematic depicted a metalloenzyme on the surface of an electrode as a set of resistors, shown here in Figure 6.1A. We know now that a novel Fe_4S_3 cluster able to undergo two redox transitions over a small potential range acts as a storage mechanism for an additional electron that is rapidly delivered to the active site upon reaction of O_2 ; the function of the cluster can be seen in analogy as a capacitor connected in series which is charged by the reducing potential under H_2 cycling and discharged upon reaction of O_2 .

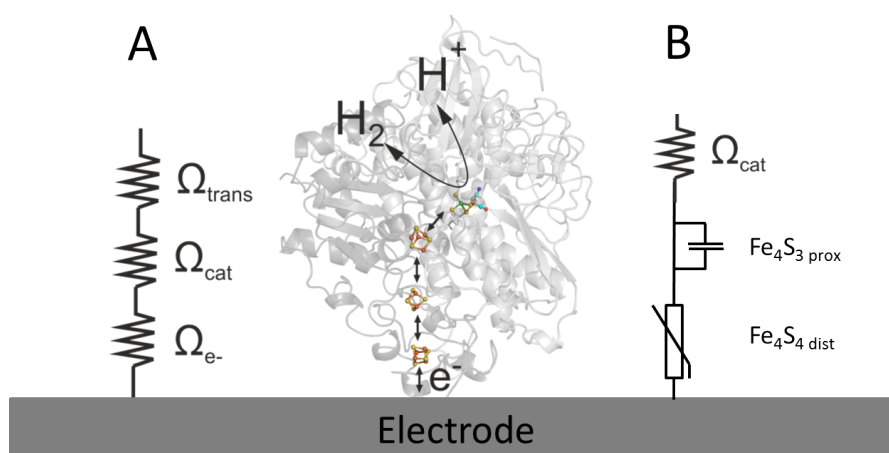


Figure 6.1: Thinking about an O_2 -tolerant $[\text{NiFe}]$ hydrogenase as an electrical circuit. The medial Fe_3S_4 cluster also plays a role in facilitating O_2 tolerance, but has been omitted for simplicity.

The point of electron entry to the enzyme, either the distal Fe_4S_4 cluster or a cytochrome haem, has a potential that limits efficient interfacial electron transfer below a certain potential. Perhaps the best analogy in the world of electronic devices would be a variable resistor. The analogy is not perfect, since a variable resistor responds with an increased current to an overpotential in either direction, but the analogy is perhaps still worth consideration. For lack of a better analogy, the active site will continue to be represented by a resistor. Finally, it seems as though the limitation due to diffusion through the protein is not as significant as was once thought [91, 272] and is not well described as a resistor; it has therefore been excluded from the present analogy. Therefore, a modified electrical circuit diagram, seen in Figure 6.1B, serves as a thought-provoking

demonstration of the roles played by the FeS clusters of an O₂-tolerant hydrogenase; it is apparent that a chain of FeS clusters is far more than simply a ‘molecular wire’.

In the context of this work, it is worthwhile asking the question: ‘Is it possible for a hydrogenase to be both O₂-tolerant and a good H₂ producer?’. The differences between Hyd-1, an O₂-tolerant enzyme, and Hyd-3, a good H₂ producer, are several, but the difference in FeS cluster potential is notable. The highest potential cluster in Hyd-1, the medial Fe₃S₄ cluster, has a midpoint potential of +190 mV *vs.* SHE [76], whereas the highest potential cluster in Hyd-3 (with the exception of the Fe₃S₄ signal believed to be a breakdown product) is between -227 and -295 mV *vs.* SHE: a difference of over 415 mV. In the context of the work in Chapter 3 showing that the very different catalytic properties of Hyd-1 and Hyd-2 are most likely due to a difference of only approximately 85 mV at the entry point for electrons, we may speculate that a difference of greater than 415 mV between our prototypical O₂-tolerant and H₂-producing enzymes is significant indeed. A general upshifting in cofactor potentials for O₂-tolerant *vs.* O₂-sensitive enzymes limits activity at low potential and may therefore be incompatible with high rates of proton reduction. Although we have shown that H₂ production is not entirely prevented by the high cluster potentials in Hyd-1, it is nonetheless true that the potential window within which catalysis is most efficient will be higher in enzymes with upshifted active site transitions, a factor which may impact upon the ability of O₂-tolerant enzymes to produce H₂ efficiently and at high rates and may thus impeded efforts to develop and O₂-stable H₂ producing catalyst.

A novel solution is represented by the soluble hydrogenase from *R. eutropha*. Although electrochemical evidence for long-term resistance to O₂ inhibition is lacking for this enzyme, solution-based assays and physiological data suggest that this enzyme is likely to be tolerant to O₂. The enzyme, which forms a complex with an NADH dehydrogenase (diaphorase) moiety, seems to mostly play a role as an uptake hydrogenase physiologically, but the hydrogenase subunit does show reversible catalysis in PFE ex-

periments [278]. In addition to the FMN cofactor found in the diaphorase subunit, the complex contains an FMN cofactor in the small subunit of the hydrogenase, HoxY, and the FeS cluster proximal to the active site is a standard Fe_4S_4 cluster without the ability to undergo a second redox transition seen for O_2 -tolerant MBHs. The FMN cofactor is therefore proposed to serve as a source of electrons for reactivation upon reaction of O_2 [278]. In this case, the electron-conducting function played by the FeS clusters of the complex is separated from the reductive rescue function played by the FMN, perhaps circumventing the clash between the need for high potential clusters for O_2 tolerance and the need for low potential clusters for fast H_2 production.

The discussion has been largely focussed upon the properties of FeS clusters, but it is likely that properties of the active site also contribute to differences in O_2 tolerance; the high E_{switch} and low $K_M^{\text{H}_2}$ associated with all O_2 tolerant MBHs characterised to date are likely related to differences at the active site. Recent work by other groups has questioned the assignment of E_{switch} as the thermodynamic potential of the couple and has suggested that the thermodynamics of the process are unimportant in determining O_2 tolerance [207]. Here we present a simple new way of visualising the E_{switch} experiment that provides solid evidence that the parameter E_{switch} corresponds to the midpoint potential of a one-electron reduction of oxidatively inactivated Ni-B. A high redox potential of the Ni-B transition is accompanied by a high potential for the other redox transitions at the [NiFe] site implicated in the catalytic cycle.

We also provide a more in-depth analysis of the Michaelis-Menten constant for H_2 oxidation for Hyd-1. This analysis acknowledges and explores the importance of potential in determining the K_M for an oxidoreductase, showing that the measured value can vary by as much as a factor of five over a 400 mV range. A strong correlation exists between O_2 -tolerance, a low value of $K_M^{\text{H}_2}$ during H_2 oxidation and a low value of $K_I^{\text{H}_2}$ during H^+ reduction. In contrast to Hyd-1, the H_2 producing Hyd-3 shows an extremely high value of $K_I^{\text{H}_2}$ (is product inhibited during proton reduction to a much lesser extent)

and also has a high $K_M^{H_2}$. These properties are in line with the physiological functions of the enzymes. Again in the context of active site properties, the question arises as to whether the ability to produce H_2 at high rates and the ability to withstand prolonged exposure to O_2 are fundamentally opposed. The low $K_M^{H_2}$ of Hyd-1 allows H_2 to compete more favourable with O_2 for binding at the active site, a property that impacts upon the steady-state fraction of active enzyme. But a low $K_M^{H_2}$ for H_2 oxidation is related to a low $K_I^{H_2}$ during H_2 production as both are dependent upon the dissociation constant of H_2 at the active site. The ability of Hyd-3 to produce H_2 during fermentation, when dissolved H_2 concentrations can reach several millimolar [282], is dependent on a resistance to product inhibition, embodied by its high $K_I^{H_2}$. Therefore it seems that the structure and properties of the active site may be tuned for either O_2 -tolerance or high rates of H_2 production, but not both.

The significance of a resistance to inhibition by CO is poorly understood, but the strong correlation between O_2 -tolerance and a high K_I^{CO} suggest that this inhibitor may also hold a clue as to active site differences that mediate O_2 -tolerance.

Spectroscopic and electrochemical investigations of Hyd-3 are at an early stage, hindered by the lability of the complex as well as the apparent O_2 sensitivity of its cofactors. It is intriguing that both the *R. eutropha* soluble hydrogenase and Hyd-3, both of which catalyse efficient H_2 production, have shown little to no indication of classical Ni-A or Ni-B signals normally seen for [NiFe] hydrogenases at high potential. With the available data, it is impossible to say whether this is due to a difference in the nature of the active site or to an increased stability to degradation or modification of the cofactor upon purification, although the activity of the isolated enzymes suggests that degradation, if it has occurred, is minor. An electrochemical characterisation of the formate dehydrogenase subunit would be a worthwhile next step in this project. The low rates of formate: H_2 activity and the putative Mo(V) EPR signals with g -values different from those published for anaerobically-isolated FdhF in the past, suggest that more efforts are

needed to develop an efficient and affordable purification protocol that can be carried out anaerobically, although this may be a task for a lab with more specialised facilities suited to anaerobic protein purification. If sufficiently active complex could be isolated, a particularly interesting question to ask would be: is it possible to force the FHL complex to work backwards, fixing CO_2 in the presence of H_2 to produce formate?

The development of methods to study electron transfer in redox enzymes will be essential to a more complete assessment of the ways in which electron transfer modifies the behaviour of the enzyme as a whole. We have engineered the reversible enzyme Hyd-2 by adding cysteine residues near the distal and medial clusters. This technique allows the protein to be specifically labelled with Ru-based photosensitizer molecules. The precedent for this study, the work of Harry Gray and colleagues [226] suggests that this method should allow photoexcited electrons to be injected into the enzyme to drive reductive catalysis. We have been able to show success at each step of creating and characterising this system, but the final product did not show electron injection at any considerable rate. Work with the free photosensitizer complex and wild-type Hyd-2 gave reasonably efficient electron transfer with production of H_2 at rates exceeding 1s^{-1} . Transient absorption signals observed for this system may be due to active site changes.

As chemists, we are challenged to understand the ways in which proteins, with a repertoire of 21 amino acids, tune the properties of simple transition metal complexes, elevating them to some of the most efficient, rapid, and selective catalysts known. And we are beginning to understand that, for the oxidoreductase enzymes, this tuning occurs not only on the level of the catalyst itself, but also on the level of the electron transfer cofactors, namely the FeS clusters, that serve to ‘wire’ these complexes into the cellular circuitry.

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