

PRINCIPLES OF ELECTROCATALYSIS  
BY HYDROGEN ACTIVATING  
METALLOENZYMES



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St. John's College

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A thesis submitted to the Board of the Faculty of Physical  
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Hydrogenases catalyse the interconversion of  $\text{H}_2$  and  $\text{H}^+$ . Protein Film Electrochemistry (PFE), a technique in which a redox enzyme is adsorbed directly onto an electrode, enables a detailed description of the catalytic function of these metalloenzymes to be obtained. Unlike small-molecule electrocatalysts, the hydrogenase active site is surrounded by a protein structure ensuring that it is relatively unperturbed by the electrode surface. In this thesis, PFE is used alongside mathematical modelling to explain differences between [NiFe]- and [FeFe]-hydrogenases, highlighting some important considerations for efficient, reversible electrocatalysis.

This thesis probes the unusual reaction between [NiFe]-hydrogenases and cyanide. Through a detailed study utilising PFE, Electron Paramagnetic Resonance (EPR) and Attenuated Total Reflection Infrared spectroelectrochemistry (ATR-IR), it is demonstrated that cyanide promotes the formation of the inactive Ni-B state. Preferred formation of the Ni-B state over more slowly reactivating Unready states is considered an important characteristic of the  $\text{O}_2$ -tolerant class of [NiFe]-hydrogenases.

The nature of the Ni-L state, commonly thought to be an artefact formed when a [NiFe]-hydrogenase is exposed to visible light, is probed *via* EPR and ATR-IR. In this thesis, the Ni-L state is shown to occur in samples of Hydrogenase-1 from *Escherichia coli* that have not been exposed to visible light, calling into question the true nature of this state.

Finally, this thesis details the first study in which PFE is used to investigate the spontaneous incorporation of a synthetic active site mimic complex into apo-hydrogenase. Incorporation into apo-hydrogenase from *Chlamydomonas reinhardtii* and *Clostridium pasteurianum* is discussed, in both cases resulting in fully functional [FeFe]-hydrogenase, electrochemically indistinguishable from the native enzyme.





# Collaborations

Several collaborators have contributed to the success of this work. Samples of *CaHydA*, *CrHydA1* and *CpI* were prepared by Danuta Krawietz and Julian Esselborn working within the research group of Prof. Thomas Happe at the Ruhr-Universität in Bochum, Germany. Julian Esselborn kindly also provided samples of apo-*CrHydA1*, apo-*CpI* and  $[\text{Fe}]^{\text{MIM}}$  used in Chapter 7. Samples of *EcHyd1* and *EcHyd2* were purified with the kind help of Elena Nomerotskaia working within the Armstrong Group, University of Oxford.

The Protein Film Electrochemistry experiments described in Section 3.3, Chapter 3 on *CaHydA*, *EcHyd1* and *EcHyd2* were completed by Felix Grey under my supervision.

The mathematical model described in Chapter 3 was developed with the help of Prof. Victor Climent working at the Institute of Electrochemistry, University of Alicante, Spain. This model was extended in Chapter 4 with the help of Dr. Thomas Esterle working within the Armstrong Group.

All Attenuated Total Reflectance Infrared spectroelectrochemistry experiments described in Chapter 5 were undertaken and analysed by Min-Wen Chung working under the supervision of Prof. Kylie Vincent, University of Oxford. Dr. Philip Ash, working also within the group of Prof. Kylie Vincent, carried out and analysed all Attenuated Total Reflectance Infrared spectroelectrochemistry experiments described in Chapter 6.

Finally, Julian Esselborn provided useful discussions that aided the design and analysis of the experiments described in Chapter 7.



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# Publications

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# Symbols and Abbreviations

|                     |                                                                    |
|---------------------|--------------------------------------------------------------------|
| $\text{\AA}$        | Angstrom                                                           |
| A                   | Ampere                                                             |
| $A$                 | geometric surface area of an electrode                             |
| $\mathbf{A}$        | hyperfine coupling ‘constant’                                      |
| ATP                 | adenosine triphosphate                                             |
| ATR                 | Attenuated Total Reflectance                                       |
| ATR-IR              | Attenuated Total Reflectance Infrared Spectroelectrochemistry      |
| $A_v\text{MBH}$     | <i>Allochromatium vinosum</i> membrane bound hydrogenase           |
| $\mathbf{B}$        | applied magnetic field                                             |
| $C$                 | capacitance                                                        |
| $Ca\text{HydA}$     | <i>Clostridium acetobutylicum</i> Hydrogenase A                    |
| CODH                | carbon monoxide dehydrogenase                                      |
| $Cr\text{HydA1}$    | <i>Chlamydomonas reinhardtii</i> Hydrogenase A1                    |
| $Cp\text{I}$        | <i>Clostridium pasteurianum</i> Hydrogenase I                      |
| CW                  | continuous wave                                                    |
| $D$                 | diffusion coefficient of the substrate                             |
| $d_0$               | distance above closest approach of the electron donor and acceptor |
| DFT                 | Density Functional Theory                                          |
| $E$                 | electrode potential                                                |
| $E_{\text{eqm}}$    | equilibrium potential of the substrate                             |
| $E_{\text{Ox/R}}^0$ | reduction potential of the electrochemical control centre          |
| EPR                 | Electron Paramagnetic Resonance                                    |

|                       |                                                                      |
|-----------------------|----------------------------------------------------------------------|
| $E_{\text{switch}}$   | potential at which two potential-dependent states interconvert       |
| $EcHyd1$              | <i>Escherichia coli</i> Hydrogenase I                                |
| $EcHyd2$              | <i>Escherichia coli</i> Hydrogenase II                               |
| $f$                   | revolutions per second                                               |
| $F$                   | the Faraday constant = 96,485 C mol <sup>-1</sup>                    |
| Fe <sub>d</sub>       | distal Fe                                                            |
| Fe <sub>p</sub>       | proximal Fe                                                          |
| FTIR                  | Fourier Transform Infrared Spectroscopy                              |
| $g_e$                 | the value of $g$ for a free electron <i>in vacuo</i> = 2.00232       |
| $h$                   | the Planck constant = 6.626 x 10 <sup>-34</sup> J s                  |
| HEPES                 | 4-[2-hydroxyethyl]-1-piperazine- <i>N</i> -2-ethane-sulfonic acid    |
| HYSCORE               | Hyperfine Sublevel Correlation spectroscopy                          |
| $i$                   | current                                                              |
| <b><math>I</math></b> | nuclear spin                                                         |
| $i_{\text{MT}}$       | mass transport limiting current                                      |
| $i_{\text{obs}}$      | observed current                                                     |
| $j_{\text{lim}}$      | limiting current                                                     |
| K                     | Kelvin                                                               |
| $k$                   | rate constant                                                        |
| $k_B$                 | the Boltzmann constant = 1.381 x 10 <sup>-23</sup> J K <sup>-1</sup> |
| $k_{\text{cat}}$      | catalytic rate                                                       |
| $K_M$                 | Michaelis constant, $K_M = (k_{-1} + k_2)/k_1$                       |
| Ox                    | oxidised form of the electrochemical control centre                  |
| $n$                   | number of electrons                                                  |
| NADPH                 | nicotinamide adenine dinucleotide phosphate                          |
| PCR                   | Polymerase Chain Reaction                                            |
| PFE                   | Protein Film Electrochemistry                                        |
| PGE                   | pyrolytic graphite edge-plane                                        |
| $q$                   | charge on a capacitor                                                |



|                     |                                                          |
|---------------------|----------------------------------------------------------|
| $R$                 | gas constant = $8.314 \text{ J K}^{-1} \text{ mol}^{-1}$ |
| RDE                 | rotating disk electrode                                  |
| Red                 | reduced form of the electrochemical control centre       |
| rpm                 | revolutions per minute                                   |
| $R_s$               | solution resistance                                      |
| sccm                | standard cubic centimeters per minute                    |
| SCE                 | saturated calomel electrode                              |
| SHE                 | standard hydrogen electrode                              |
| $S$                 | electron spin                                            |
| $t$                 | time                                                     |
| $T$                 | temperature                                              |
| $\nu$               | kinematic viscosity of the solvent                       |
| $v$                 | rate of reaction                                         |
| V                   | volt                                                     |
| <i>vs.</i>          | <i>versus</i>                                            |
| $\alpha$            | transfer coefficient                                     |
| $\beta$             | medium-dependent decay constant                          |
| $\beta d_0$         | effective tunnelling factor                              |
| $\Gamma_m$          | total electroactive coverage of enzyme on the electrode  |
| $\Gamma_I$          | coverage of enzyme in State I                            |
| $\Gamma_{II}$       | coverage of enzyme in State II                           |
| $\Delta H^\ddagger$ | activation enthalpy                                      |
| $\mu_B$             | Bohr magneton = $9.274 \times 10^{-24} \text{ J T}^{-1}$ |
| $\nu$               | scan rate                                                |
| $\nu$               | frequency                                                |
| $\nu_J$             | stoichiometric coefficient of J                          |
| $\tau$              | decay constant                                           |
| $\Phi$              | electric potential                                       |
| $\omega$            | rotation rate (revolutions per minute)                   |

$\omega$  angular velocity ( $\text{s}^{-1}$ )

$^{\circ}\text{C}$  degree Celsius

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# Chapter 1

## Introduction



## 1.1 Enzymes as Electrocatalysts

The study of electrocatalysis as carried out by enzymes was first pioneered by I. V. Berezin and S. D. Varfolomeev, chemists working within the Soviet Union in the late 1970's. At an International Symposium on the Frontiers of Bioorganic Chemistry and Molecular Biology held in 1978, these two scientists gave a paper entitled 'Bioelectrocatalysis as a New Phenomenon'<sup>1</sup> which described a 'theory for constructing electrocatalysts based on immobilized enzymes'. This seminal work provided the basis for many more decades of work in this field including the work presented in this thesis.

Highly specific and highly active enzymes provide us with catalysts, perfected through evolution, able to carry out the most challenging chemical transformations, often reversibly.<sup>2</sup> Modern crystallographic techniques mean that even with an enzyme diameter of over 100 Å, the catalytic site can be structurally well defined and the position of all non-hydrogen atoms that make up the ordered domains of surrounding protein, can be known. This surrounding protein shell means that enzymes can be attached to an electrode surface by a combination of numerous weak interactions between the electrode surface and the protein, whilst the catalytic site, which is more often than not buried deep within the protein, is structurally unaffected by the presence of the electrode. This varies significantly from the two more commonly studied types of electrocatalysts; metal surface electrocatalysts, such as Pt metals and alloys and conducting metal oxides and sulfides, and molecular electrocatalysts, such as metal complexes. In the case of metal surface electrocatalysts there may be many different surface sites presents, for example steps, kinks, troughs and corners, and it may be uncertain at which of these sites catalysis occurs.<sup>3</sup> On the other hand, crystallography and spectroscopy techniques mean that molecular electrocatalysts may have much better defined structures in the free state, that is, in the absence of catalysis, but the small size of these molecules means that upon immobilisation onto an electrode surface the structure of the catalytic site is likely to undergo considerable change.

The relatively large size of an enzyme structure necessitates that redox enzymes must be composed of specialised parts each performing a specific task. An electron relay is needed to shuttle electrons between the catalytic site and the protein surface and a proton transfer pathway must be available to ensure that protons can be removed from, or supplied to, the catalytic site when needed to ensure synchronous proton-electron transfer. The substrate must be able to access the catalytic site and the product must be able to leave, often requiring designated hydrophobic channels. Additionally, the catalytic site must contain substrate binding sites and be optimally positioned in space for catalysis to occur. These various parts may be well separated in space, even contained within separate subunits of an enzyme, and act independently of each other.

Modern techniques mean that different parts of an enzyme can be investigated individually and an understanding of the important aspects which result in efficient catalysis can be obtained. Mutagenesis studies allow for the possibility of altering the protein structure, for example close to an electron transfer relay<sup>4</sup> or a gas channel,<sup>5,6</sup> without affecting the catalytic site itself. In this way we are able to compare molecules which have exactly the same catalytic site but subtly different surroundings.

In the past small ‘model’ compounds based upon a known enzyme catalytic site, have often been studied with the view that their relative structural simplicity will aid the understanding of the catalytic reactions which take place within an enzyme. An alternative strategy, one which is taken in this thesis, is that the study of the enzymes themselves, exploiting their unique properties and structure, will enable a better understanding of the most important components of electrocatalysis.

## 1.2 Hydrogen in Biology and the Role of Hydrogenases

There are many bacterial processes which produce  $H_2$ : fermentative bacteria release  $H_2$  as a waste product from the fermentation of organic matter; nitrogen fixing microorganisms release  $H_2$  as a side product of the reduction of nitrogen into ammonia; and photosynthetic bacteria, cyanobacteria and green algae use solar energy to convert  $H_2O$ , reduced sulfur compounds and organic compounds into  $H_2$ .<sup>7</sup> However, we do not see  $H_2$  accumulation in the atmosphere. Instead there is interspecies hydrogen transfer; as  $H_2$  is produced by one organism, it is used up by another.<sup>8</sup>

Many organisms can generate energy by the oxidation of  $H_2$ . The protons and electrons generated from this can then be used to reduce oxygen to water or, in the case of bacteria, maybe an alternative oxidising compound such as sulfate. Protons produced by the oxidation of  $H_2$  lead to the formation of a proton gradient across cell membranes, a form of stored energy.<sup>8</sup> Adenosine triphosphate (ATP), the chemical energy intermediate in many life processes, can then be produced by ATP synthase which is driven by the proton gradient.

The key enzyme involved in biological hydrogen cycling is hydrogenase, the enzyme which catalyses the reversible oxidation of  $H_2$  to protons. The first hydrogenase to be isolated was from *Desulfovibrio desulfuricans* in 1954 by Sadana *et al.*<sup>9</sup> and it is these enzymes which enable many bacteria to use  $H_2$  as an energy source.

Hydrogenases are found in a wide variety of organisms, from numerous environments, including both aerobes and anaerobes, prokaryotic and eukaryotic photosynthetic organisms, knallgas bacteria, methanogens, sulfate reducers,  $N_2$  fixers, fermentative organisms, hyperthermophiles and anaerobic fungi among others.<sup>8</sup> They can be found in various locations within the cell and both cytoplasmic ‘soluble’ hydrogenases and periplasmic ‘membrane-bound’ hydrogenases have been isolated. Although many hydrogenases are capable of reversible catalysis, *in vivo* they tend to operate only in one direction: [NiFe]-hydrogenases (Section 1.4) generally catalyse

H<sub>2</sub> oxidation whilst [FeFe]-hydrogenases (Section 1.5) generally catalyse H<sub>2</sub> evolution.

## 1.3 Classification of Hydrogenases

Hydrogenases are classed as either [Fe]-,<sup>10</sup> [NiFe]-<sup>11</sup> or [FeFe]-hydrogenases,<sup>11,12</sup> according to the metal atoms within their active site. [Fe]-hydrogenases, found only in methanogenic Archaea, do not catalyse the reversible oxidation of H<sub>2</sub>, instead carrying out the reversible reduction of methenyl-H<sub>4</sub>MPT<sup>+</sup> to methylene-H<sub>4</sub>MPT (H<sub>4</sub>MPT = tetrahydromethanopterin).<sup>13,14</sup> This class of hydrogenase will not be discussed further in this thesis. An outcome of convergent evolution, all three classes of hydrogenase contain a low-spin iron ligated by thiolates(s) and CO.

## 1.4 The [NiFe]-Hydrogenases

### 1.4.1 The Protein Structure

The [NiFe]-hydrogenases can be grouped into three subsets: O<sub>2</sub>-sensitive [NiFe]-hydrogenases (or ‘standard’ [NiFe]-hydrogenases), O<sub>2</sub>-tolerant [NiFe]-hydrogenases and [NiFeSe]-hydrogenases. Crystal structures have been determined for a number of hydrogenases within each of these subsets and distinct differences between hydrogenases in different subsets have been observed.

[NiFe]-hydrogenases, including those in the [NiFeSe]-hydrogenase subset, typically consist of a heterodimer made up of large and a small subunit. The large subunit contains the active site which is ‘wired’ to the protein surface by an [FeS]-cluster relay chain which is housed within the small subunit (Figure 1.1A). Different [NiFe]-hydrogenases have been isolated as heterodimers, dimers of heterodimers and larger oligomeric states, but the physiological significance of this still remains uncertain. The O<sub>2</sub>-tolerant [NiFe]-hydrogenases, Hydrogenase-1 from *Escherichia coli*<sup>15,16</sup> (Figure 1.1B) and the membrane bound hydrogenase from *Hydrogenovibrio mari-*

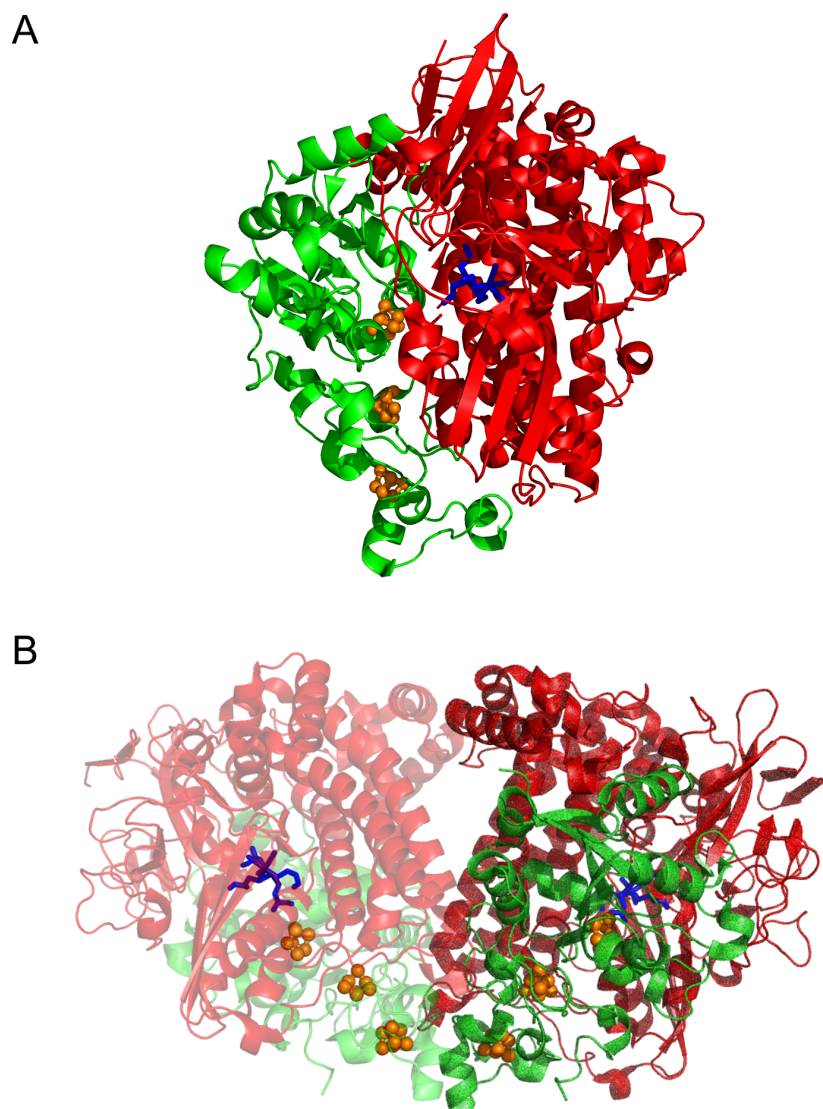
*nus*,<sup>17</sup> have both been isolated as a dimer of heterodimers and there is some evidence that this may be related to the O<sub>2</sub> resistance of these enzymes.<sup>16–18</sup>

### 1.4.2 The Active Site

The active site (catalytic centre) of all [NiFe]-hydrogenases consist of a Ni and an Fe atom connected by two bridging sulfur-cysteine residues. In the O<sub>2</sub>-sensitive and O<sub>2</sub>-tolerant [NiFe]-hydrogenases the Ni atom is ligated to a further two sulfur-cysteine residues as depicted in Figure 1.2. In the [NiFeSe]-hydrogenases one of these terminal sulfur-cysteine residues, indicated by an asterisk in Figure 1.2, is replaced by a seleno-cysteine.<sup>19</sup> In all cases, these four amino acid residues anchor the active site in place within the protein.<sup>20</sup> The Fe atom is coordinated to three inorganic ligands, identified through infrared spectroscopy as two CN<sup>−</sup> and one CO.<sup>21–23</sup> These are highly unusual biological ligands and their presence at the active site is significant (*vide infra*).

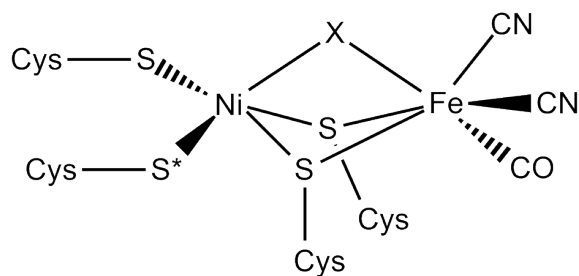
The Ni atom is redox active, adopting a Ni<sup>1+</sup>, Ni<sup>2+</sup> or Ni<sup>3+</sup> state, depending upon the state of the enzyme (see Section 1.6).<sup>24</sup> The Fe atom remains in a low spin Fe<sup>2+</sup> at all times, as favoured by the strong field CN<sup>−</sup> and CO ligands.

The 2+ oxidation state of the Fe atom and its coordination to the five ligands outlined in the text above results in this metal having a total valence electron count of 16. This means that the Fe atom is susceptible to attack, as predicted by the ‘18-electron rule’, which expresses the tendency of a transition metal atom to use all its valence orbitals as fully as possible in metal-ligand bonding.<sup>25</sup> An additional ligand is usually present, as indicated by ‘X’ in Figure 1.2. During catalysis it is thought that this ligand takes the form of a hydride. In the oxidised, inactive forms of the enzyme this site is occupied by an oxygen based ligand.<sup>26</sup> The various identities of ‘X’ are discussed in more detail Sections 1.6 and 1.7.



**Figure 1.1:** (A) Representation of the X-ray crystal structure of the  $O_2$ -sensitive [NiFe]-hydrogenase heterodimer from *Desulfovibrio fructosovorans*, PDB code 1YQ9, showing the large subunit (red) which houses the buried active site (blue) and the small subunit (green) that contains an FeS relay (orange). (B) Representation of the X-ray crystal structure of the  $O_2$ -tolerant [NiFe]-hydrogenase-1 from *Escherichia coli* (*EcHsd1*), PDB code 3UQY. This hydrogenase is made up of a dimer of heterodimers, each heterodimer consisting of a large and a small subunit, containing the active site and an FeS relay respectively (coloured as in panel A with one heterodimer represented in a higher transparency for clarity). In both panels subunits are shown in a ribbon representation whilst the FeS clusters and active site are shown in a ‘ball and stick’ and ‘stick’ representation respectively.



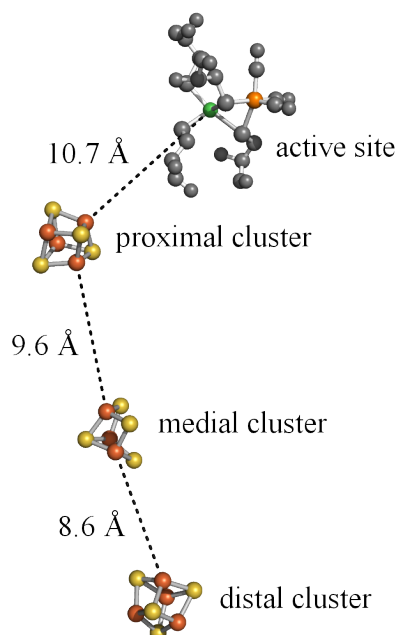


**Figure 1.2:** A representation of the [NiFe]-hydrogenase active site. The bridging ligand ‘X’ can have a number of identities, as discussed in Sections 1.6 and 1.7.

### 1.4.3 Electron Transfer Through the Protein: The [FeS]-Cluster Relay Chain

In all [NiFe]-hydrogenases, the [FeS]-cluster relay chain is made up of three clusters, separated by about 10 Å, allowing efficient quantum-mechanical electron tunnelling through the chain (Figure 1.3). Importantly, the distance between the clusters is smaller than 14 Å, the distance at which electron-transfer through the protein may start to become rate limiting.<sup>27</sup> It has been calculated that rates of electron transfer over the  $\sim 10$  Å distance between FeS clusters in the [NiFe]-hydrogenase from *Desulfovibrio gigas* occurs at a rate in the range of  $2 \times 10^7 \text{ s}^{-1}$ , a value some three or four orders of magnitude greater than the overall catalytic rate,  $k_{\text{cat}}$ .<sup>27</sup> Extensive interaction between the large and small subunits,<sup>28</sup> and the positioning of the active site close to the centre of the heterodimer, means that electrons can be transferred efficiently through the protein matrix between the active site and molecular surface. Sequential one-electron transfer through the [FeS]-cluster relay chain to and from the active site allows the enzyme to accumulate the electrons required to carry out the two-electron reduction of  $\text{H}^+$  into  $\text{H}_2$  and the two-electron oxidation of  $\text{H}_2$  into  $\text{H}^+$ .

The exact composition of the various clusters which make up the relay chain differ between the three subsets: In  $\text{O}_2$ -sensitive [NiFe]-hydrogenases, the relay chain consists of two [4Fe4S]-clusters positioned either side of a [3Fe4S]-cluster,<sup>28</sup> (the ‘medial’ cluster) whilst in [NiFeSe]-hydrogenases the relay chain is made up of three [4Fe4S]-clusters.<sup>19,29,30</sup> The relay chain in  $\text{O}_2$ -tolerant [NiFe]-hydrogenases includes a



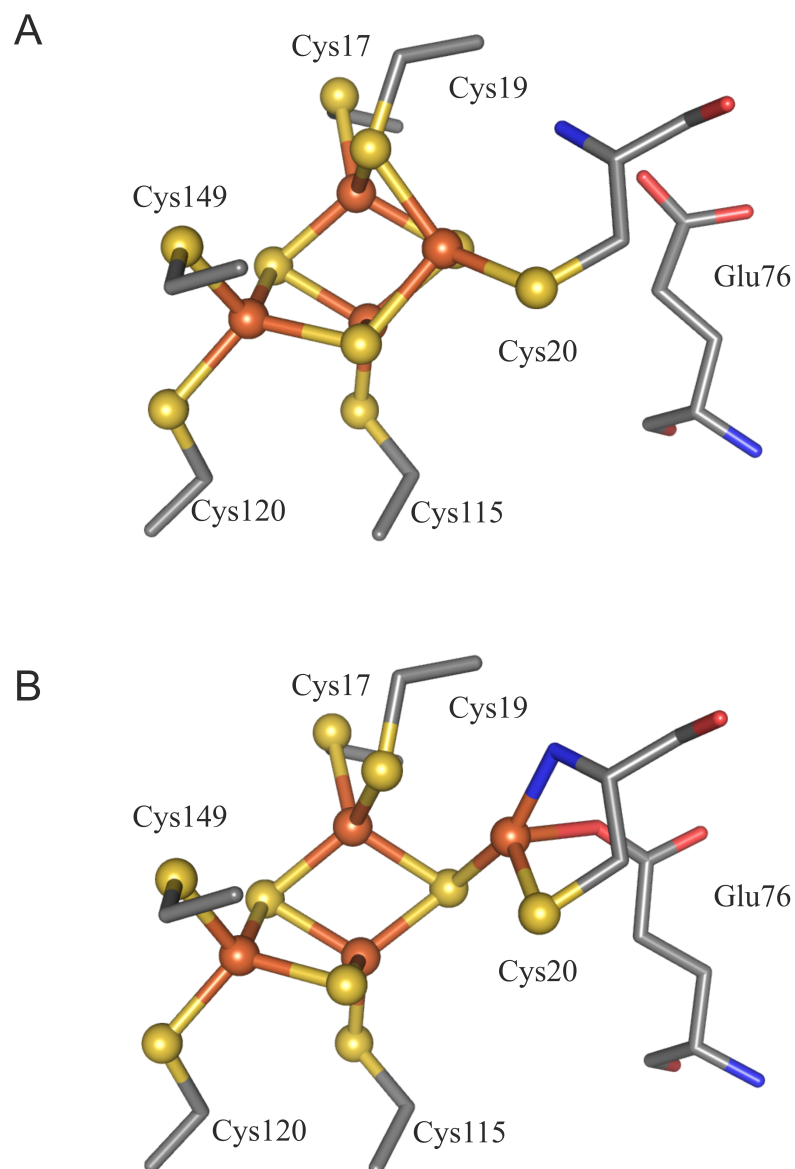
**Figure 1.3:** Representation of the X-ray structure of the [NiFe]-hydrogenase from *Desulfovibrio fructosovorans*, PDB code 1YQ9, showing only the active site and the proximal, medial and distal [FeS]-clusters and the distances between them. Colours represent the following atoms: Ni green, Fe orange, S yellow, N, O and C grey.

novel [4Fe3S]-cluster,<sup>15,17,31</sup> as yet not detected in any other systems, which replaces the [4Fe4S]-cluster located in the position closest (proximal) to the active site in O<sub>2</sub>-sensitive [NiFe]-hydrogenases. This unusual cluster is held in place within the protein structure through ligation to four cysteines conserved in all [NiFe]-hydrogenases as well as by ligation to two additional cysteines found exclusively in O<sub>2</sub>-tolerant hydrogenases.<sup>32,33</sup> The ‘missing’ sulfide in the [4Fe3S]-cluster, when compared to the standard [4Fe4S] structure, is occupied by the thiolate sulfur of one of these additional cysteine ligands (labelled as Cys19 in Figure 1.4). This results in the cluster having a distorted, ‘open’ conformation which permits redox-dependent structural changes to take place (Figures 1.4A and 1.4B).<sup>17</sup> In the oxidised form, the amide-N bound to a cysteine residue directly ligating one of the Fe ions (Cys20 in *EcHsd1* as shown in Figure 1.4) coordinates to the same Fe atom. This is only possible after the deprotonation of the N atom and it is speculated that a nearby glutamic acid residue (Glu76 in Figure 1.4) acts as the base.<sup>15</sup> Furthermore, the electrostatic interaction between the Fe ion and this glutamic acid residue modulates

the approach of the labile Fe ion to the amide-N of the cysteine residue.<sup>15</sup> The resulting stabilisation afforded by the formation of a Fe-N(amide) bond allows the [4Fe3S]-cluster to undergo a second electron transfer, i.e. undergoing two redox transitions ( $3+/2+$  and  $2+/1+$ , also denoted  $5+/4+$  and  $4+/3+$ ), within a small potential range of approximately 150 mV at high potential.<sup>33,34</sup> This ability to release two electrons and one proton, all of which can be transferred to the active site, plays a crucial role in protecting the active site from oxidative stress<sup>32,33,35</sup> by rapidly supplying two of the four electrons required to fully reduce  $O_2$  to  $H_2O$ .<sup>18,36</sup> Apart from the unusual proximal cluster found in  $O_2$ -tolerant [NiFe]-hydrogenases, the other [FeS]-clusters making up the relay chain remain as for the  $O_2$ -sensitive [NiFe]-hydrogenases.

#### 1.4.4 Gas Transport Through the Protein

The distance between the active site and the surface of the protein (approximately 30 Å) means that hydrophobic gas channels are needed along which hydrogen (and other neutral molecules) can be transported to and from the active site. A Xe binding study, in which X-ray diffraction was performed on the  $O_2$ -sensitive [NiFe]-hydrogenase from *Desulfovibrio fructosovorans* under a 9 bar Xe atmosphere, showed the presence of 10 Xe atoms all located in positions previously speculated to be possible gas channels.<sup>37</sup> Molecular dynamics simulations of Xe and  $H_2$  diffusion through the protein were then performed using the experimentally-determined positions of the Xe sites as starting points. In these simulations, Xe atoms were not able to reach the active site whereas  $H_2$  was able to get as close as 1.4 Å and 1.8 Å to the Ni and Fe atoms of the active site respectively.<sup>37</sup> Further studies with a 1 Å probe showed that the gas channel seems to end at the vacant coordination site of the Ni atom. It was also suggested that these gas channels may not only have a role in allowing  $H_2$  to travel to the active site, but they may also act to trap  $H_2$  within the protein, increasing the concentration of substrate within the enzyme relative to the surrounding environment.



**Figure 1.4:** (A) Representation of the proximal FeS cluster from the O<sub>2</sub>-tolerant [NiFe]-hydrogenase-1 from *Escherichia coli* (EcHyd1) in the H<sub>2</sub> reduced,  $[4\text{Fe}_3\text{S}]^{+1}$ , conformation (PDB code 3UQY). Colours represent the following atoms: Fe orange, S yellow, N blue, O red and C grey. (B) Representation of the proximal FeS cluster from EcHyd1 in the ferricyanide oxidised,  $[4\text{Fe}_3\text{S}]^{+3}$ , conformation (PDB code 3USC). Atoms coloured as in (A).

### 1.4.5 Proton Transport Through the Protein

An efficient proton-transfer pathway is required to transport protons between the active site and protein surface. As of yet, little research has been done investigating possible pathways, with crystal structures and a small number of site-directed mutagenesis studies being the only experimental information obtained so far.<sup>31,38,39</sup>

Protons can be transported through a protein structure by hydrogen bonding to water molecules or amino acid groups which are able to provide donor and acceptor groups with suitable  $pK_a$  values. This is often referred to as the Grotthuss mechanism.<sup>40</sup> One of the most likely amino acid residues involved in proton transfer in [NiFe]-hydrogenases is a fully conserved glutamic acid residue located within hydrogen-bonding distance of one of the terminal cysteine ligands located on the active site Ni atom. There is evidence that when crystallised both the glutamic acid and cysteine can be isolated in different protonation states.<sup>39</sup> Mutation of this glutamic acid residue to a glutamine in the [NiFe]-hydrogenase from *Desulfovibrio fructosovorans* did not affect the EPR spectroscopic characteristics of the active site Ni signals but it completely abolished all catalytic activity and H/D exchange.<sup>38</sup> Conversion of *para*-H<sub>2</sub> to *ortho*-H<sub>2</sub> was not affected however, indicating that heterolytic cleavage/recombination of H<sub>2</sub> was still possible but further proton transfer was not.

The crystal structure of the O<sub>2</sub>-tolerant membrane-bound hydrogenase from *Ralstonia eutropha* H16 has identified water-filled cavities which connect the active site to the solvent surrounding the enzyme. These are absent in the crystal structures of O<sub>2</sub>-sensitive [NiFe]-hydrogenases and although their main task may be to translocate water molecules formed during the reduction of O<sub>2</sub> at the active site,<sup>18,31,36</sup> this is probably also accompanied by proton transfer.<sup>31</sup>

Moving further away from the active site many proton transfer pathways become possible<sup>39</sup> and more experimental work is needed to define the correct one. It is likely that away from the active site there are a number of pathways taken by protons during catalysis<sup>38</sup> and that these pathways may be different between different species

and between O<sub>2</sub>-tolerant and O<sub>2</sub>-sensitive hydrogenases.<sup>35</sup>

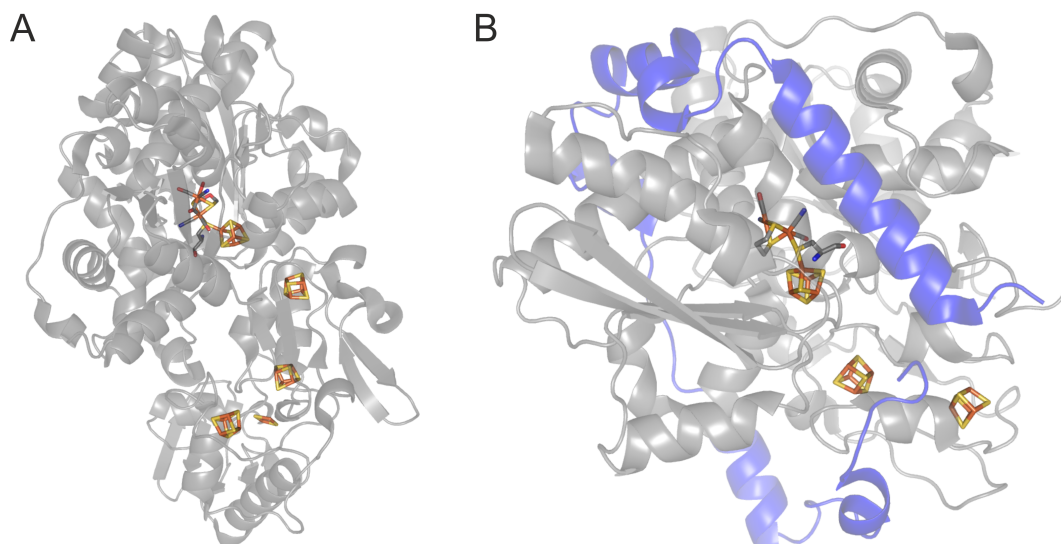
## 1.5 The [FeFe]-Hydrogenases

### 1.5.1 The Protein Structure

Unlike the [NiFe]-hydrogenases, the [FeFe]-hydrogenases differ in the number of subunits and the number and composition of [FeS]-clusters. Crystal structures of the [FeFe]-hydrogenase from two organisms have been obtained. The first structure obtained was that of the cytoplasmic, monomeric [FeFe]-hydrogenase I from *Clostridium pasteurianum*.<sup>41,42</sup> Represented in Figure 1.5A, this enzyme consists of the active site, referred to in [FeFe]-hydrogenases as the ‘H-cluster’, as well as three [4Fe4S]-clusters and a single [2Fe2S]-cluster. The second structure, shown in Figure 1.5B, is that of the periplasmic, heterodimeric [FeFe]-hydrogenase HydAB from *Desulfovibrio desulfuricans*.<sup>43</sup> This enzyme is made up of a large subunit, housing the active site and two [4Fe4S]-clusters, and a small subunit, which wraps around the large subunit.

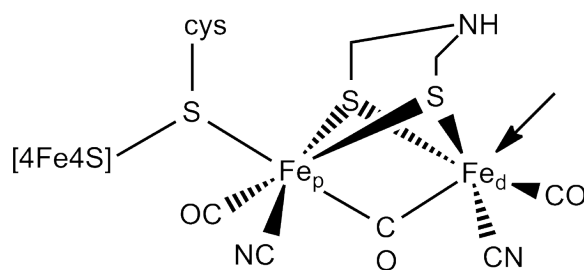
### 1.5.2 The Active Site

The active site of the [FeFe]-hydrogenases is referred to as the ‘H-cluster’ and is highly conserved across enzymes from different organisms. Depicted in Figure 1.6, it consists of a [4Fe4S]-cluster which is connected to a binuclear Fe cluster *via* the sulfur atom of a cysteine residue.<sup>12</sup> Not only does this cysteine residue hold these two sub-clusters together, but it also holds the H-cluster in place within the protein structure. Bridging the two Fe atoms which make up the binuclear Fe cluster, referred to as the proximal Fe (Fe<sub>p</sub>) and the distal Fe (Fe<sub>d</sub>) due to their position with respect to the [4Fe4S]-cluster, is an unusual SCH<sub>2</sub>NCH<sub>2</sub>S dithiolate ligand. Until recently it was unclear as to whether the N atom in this dithiolate ligand was instead an O or C. The first experimental evidence that the bridgehead was in fact a N atom was from a <sup>14</sup>N HYSCORE investigation in which three <sup>14</sup>N signals



**Figure 1.5:** (A) Ribbon representation of the X-ray crystal structure of the [FeFe]-hydrogenase I from *Clostridium pasteurianum*, PDB code 3C8Y. The H-cluster and the FeS clusters are represented as sticks. (B) Corresponding structure for the [FeFe]-hydrogenase HydAB from *Desulfovibrio desulfuricans*, PDB code 1HFE. The large subunit is shown in grey and the small subunit is shown in blue. In both panels colours represent the following atoms: Fe orange, S yellow, N blue, O red and C grey.

were observed, one of which could be assigned to an N atom at the bridgehead position.<sup>44</sup> Recent maturation studies have been completed in which active site complexes containing a CH<sub>2</sub>, NH and O bridgehead species were successfully inserted into the apo-protein as demonstrated by EPR and FTIR spectroscopy.<sup>45,46</sup> However, only addition of the complex containing a NH bridgehead led to active enzyme, unequivocally demonstrating that N is the bridgehead atom. More information about this study can be found in Chapter 7.



**Figure 1.6:** A representation of the [FeFe]-hydrogenase active site, known as the ‘H-cluster’. Fe<sub>p</sub> and Fe<sub>d</sub> refer to the proximal and distal Fe atoms respectively. The arrow indicates the vacant binding site at the Fe<sub>d</sub> atom.

Similarly to the active site of the [NiFe]-hydrogenases, the H-cluster contains

the biologically unusual CO and  $\text{CN}^-$  ligands, with the  $\text{Fe}_p$  and  $\text{Fe}_d$  both being coordinated to one CO and one  $\text{CN}^-$  ligand.<sup>41</sup> Another CO ligand is present and is found either bridging the  $\text{Fe}_p$  and  $\text{Fe}_d$  atoms or is bound terminally to  $\text{Fe}_d$  depending upon the redox state of the enzyme. There is a vacant binding site at the  $\text{Fe}_d$  atom, indicated by an arrow in Figure 1.6, that is vulnerable to attack.

Both Fe atoms of the binuclear cluster are redox active, cycling between the +1 and +2 oxidation states.<sup>47</sup> Electrons get transferred to the  $\text{Fe}_p$  and  $\text{Fe}_d$  atoms *via* the adjoining [4Fe4S]-cluster and this can be found in a +1 or +2 oxidation state also. A more detailed description of the redox behaviour of the H-cluster can be found in Sections 1.8 and 1.9.

### 1.5.3 Electron Transfer Through the Protein: The Presence of [FeS]-Clusters

Similarly to the [NiFe]-hydrogenases, [FeS]-clusters provide an electron relay system allowing efficient transfer of electrons between the active site and protein surface. In the crystal structures obtained to date, the [FeS]-clusters have been separated by less than 10 Å<sup>41,43</sup> ensuring that electron transfer through the protein will not limit the rate of catalysis.<sup>27</sup>

Like the [FeFe]-hydrogenase I from *Clostridium pasteurianum*, of which it shares 34% sequence identity, the [FeFe]-hydrogenase HydA1 from *Chlamydomonas reinhardtii* (*CrHydA1*) is a monomeric enzyme (determined by only a single band being detected by SDS gel electrophoresis).<sup>48</sup> However, *CrHydA1* is significantly smaller (47.5 kDa *versus* 66.1 kDa)<sup>42,48</sup> and no auxiliary [FeS]-clusters are necessary to aid electron transfer between the protein surface and the [4Fe4S] sub-cluster of the active site, either *in vivo*, where electron transfer is coupled to a ferredoxin partner,<sup>11</sup> or *in vitro*, as displayed by the high activity of *CrHydA1* measured through spectroscopic assays<sup>49</sup> or electrochemistry.<sup>50</sup>



### 1.5.4 Gas Transport Through the Protein

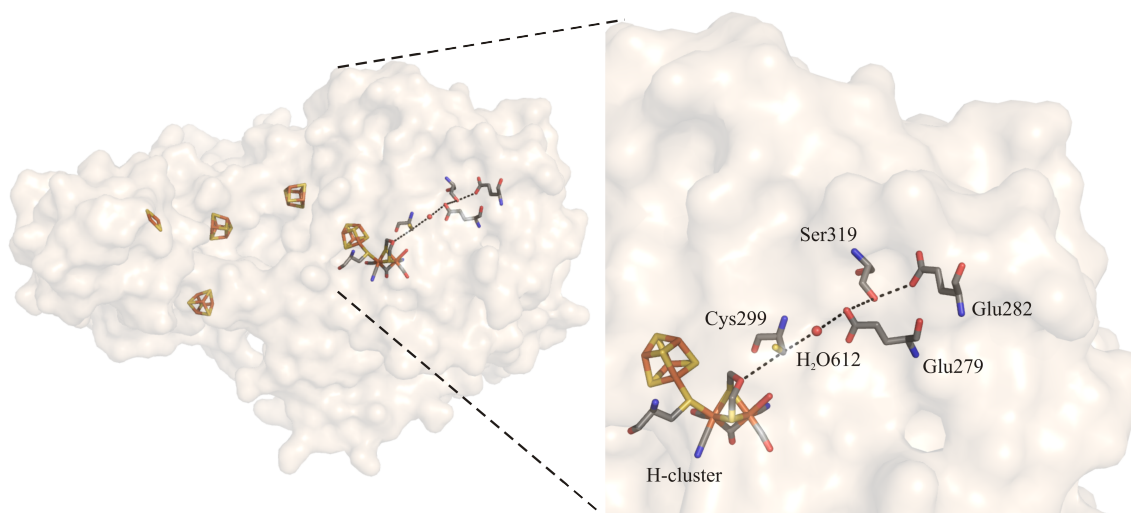
As for the [NiFe]-hydrogenases, there is some evidence of hydrophobic gas channels in [FeFe]-hydrogenases connecting the active site to the protein surface. Molecular dynamics simulations on the [FeFe]-hydrogenase from *Desulfovibrio desulfuricans* found a continuous cavity running from the protein surface to the Fe<sub>d</sub> atom of the H-cluster.<sup>43</sup> This channel was only apparent when a 0.75 Å probe was used and given that the radius of H<sub>2</sub> is about 1 Å, this indicates that some internal protein flexibility is required to allow substrate diffusion to the active site. Further molecular dynamics simulations have been carried out on Hydrogenase I from *Clostridium pasteurianum* which, using a 1 Å probe, found a continuous hydrophobic gas channel in the same location as that found in *Desulfovibrio desulfuricans*.<sup>51</sup> The possible pathways taken by H<sub>2</sub> and O<sub>2</sub> (a known inhibitor of [FeFe]-hydrogenases) in Hydrogenase I were studied further and two predominant pathways taken by H<sub>2</sub> and O<sub>2</sub> molecules when exiting the protein from the active site were found.<sup>52</sup> However, the transport of O<sub>2</sub> was found to be much more dependent upon the random motions of the protein, whereas many more channels were accessible to H<sub>2</sub> simultaneously.<sup>52</sup> The results confirmed that a permanent channel is not needed to allow sufficient transport of gas molecules between the active site and protein surface and that transient channels arising from the dynamics of the protein structure are sufficient to define predetermined pathways for hydrophobic gas transport.

### 1.5.5 Proton Transport Through the Protein

Due to the smaller size, and so shorter distance between the active site and protein surface, there has been more success shedding light on a viable proton transfer pathway within the [FeFe]-hydrogenases relative to the [NiFe]-hydrogenases.

One study proposes that a strictly conserved cysteine residue located close to the active site may act as a proton donor/acceptor for the interconversion of H<sub>2</sub>.<sup>41</sup> The finding that the bridgehead atom in the active site is in fact N has added weight to this argument as it is possible that this secondary amine, together with

the  $\text{Fe}_d$  atom, may be involved in the heterolytic cleavage of  $\text{H}_2$ .<sup>39</sup> It is proposed that the resulting proton could then be transferred from the N atom to the cysteine residue before being transported out of the enzyme by a proton transport pathway consisting of a water molecule, two glutamic acid residues and a serine residue. As depicted in Figure 1.7, the precise residues proposed for the pathway are Cys-299, Glu-279, Ser-319 and Glu-282, numbered according to the amino acid sequence of *Clostridium pasteurianum*.<sup>41</sup> All residues and water molecules involved in this pathway are conserved in the two [FeFe]-hydrogenases with known crystal structures.<sup>39</sup>



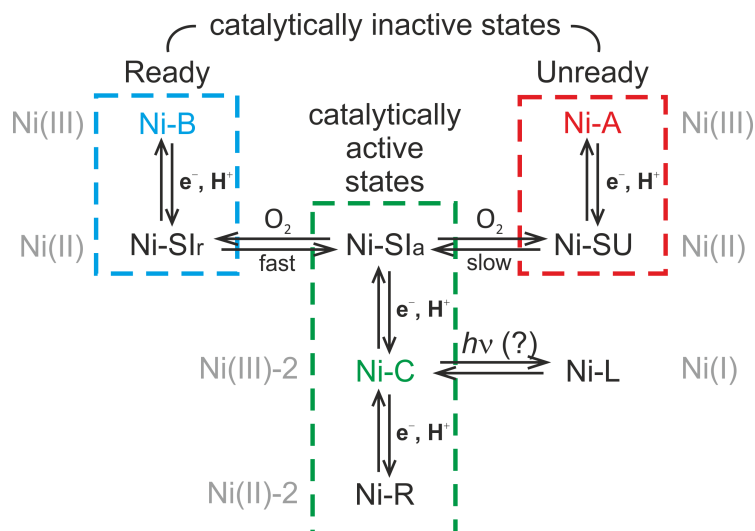
**Figure 1.7:** A representation of the proposed pathway by which protons may be transported between the H-cluster and the protein surface. Residues and the  $\text{H}_2\text{O}$  molecule are numbered according to the amino acid sequence of *Clostridium pasteurianum* (PDB code 3C8Y). Colours represent the following atoms: Fe orange, S yellow, N blue, O red and C grey. The  $\text{H}_2\text{O}$  molecule proposed to be part of the pathway ( $\text{H}_2\text{O612}$  in the crystal structure corresponding to PDB code 3C8Y) is shown as a red sphere.

In a second study the four amino acids involved in this putative pathway were substituted by site-directed mutagenesis in order to limit the  $\text{H}_2$  bonding between them.<sup>53</sup> All mutations resulted in a sharp decrease in hydrogenase activity compared to the native enzyme, with  $\text{H}_2$  evolution activity being more suppressed than  $\text{H}_2$  uptake activity, demonstrating the importance of these residues.

## 1.6 The Redox States of [NiFe]-Hydrogenases

Many studies have been published which investigate the various redox states available at the active site of [NiFe]-hydrogenases. The first studies which reported the redox sensitivity of the Ni atom at the active site were spectroscopic Electron Paramagnetic Resonance (EPR) investigations which observed three sets of signals associated with the Ni atom and these were termed Ni-A, Ni-B and Ni-C.<sup>54–56</sup> These names now refer to defined oxidation states of the entire active site.<sup>24</sup> Redox titrations of these species found that additional EPR-silent states of the active site also exist.<sup>56,57</sup> These can be detected through the use of Fourier Transform Infrared (FTIR) spectroscopy which is able to measure changes to the electron density on the Fe atom (as a result of the changing oxidation state of the Ni as the Fe atom remains in a formal +2 oxidation state at all times) *via* measuring changes to the vibrational frequencies of the bound CO and CN<sup>−</sup> ligands (Figure 1.2).<sup>58</sup> The use of FTIR allowed the first full spectroelectrochemical characterisation of a [NiFe]-hydrogenase to be carried out.<sup>59</sup> The close correlation between potentials of the various redox states as obtained *via* EPR and FTIR led to the conclusion that the Ni and the Fe atom at the active site are strongly electronically coupled.<sup>59</sup> Through the use of these two techniques a rich picture of the various oxidation states available to the active site has been obtained and these are summarised in Scheme 1.1.

The two most oxidised states are referred to as Ni-A and Ni-B.<sup>24</sup> These are inactive states in which the Ni is in the +3 oxidation state and an oxygen-based ligand occupies the position indicated by ‘X’ in Figure 1.2. The identity of this ligand in the Ni-B state is widely accepted to be OH<sup>−</sup><sup>60–62</sup> whereas the nature of the corresponding ligand in the Ni-A state is still unclear. It is now generally agreed that the bridging ligand in both states contains an O atom<sup>61,63</sup> with the most likely candidates for the Ni-A state being a OH<sup>−</sup> or OOH<sup>−</sup> ligand.<sup>24,64,65</sup> In the past Ni-A and Ni-B have also been referred to as *Unready* and *Ready* respectively. These names were indicative of the rate at which these inactive states, made upon exposure to oxygen or, in the case of Ni-B, anaerobically at high potentials, could be reduced



**Scheme 1.1:** Catalytically active and inactive states of [NiFe]-hydrogenases and their interconversions. States depicted in colour refer to those visible by EPR spectroscopy (EPR active states). The formal oxidation state of the Ni atom in each state is shown alongside in grey, with any other charge on the active site also shown.

under a  $\text{H}_2$  atmosphere to form active enzyme, Ni-A reactivating very slowly, over a number of hours, whilst Ni-B could be reactivated over a number of minutes.<sup>66–68</sup> Recently it has been shown that there are many slowly reactivating states which may form upon oxygen exposure,<sup>69</sup> most of which are EPR silent, and so the term *Unready* is now taken to mean any one of these slowly reactivating states. On the other hand, Ni-B and *Ready* are used interchangeably.

One-electron reduction of Ni-A and Ni-B leads to the formation of the EPR silent and catalytically inactive Ni-SU and Ni-SIr states respectively. The Ni-SIr state is in an acid/base equilibrium with another state referred to as Ni-SIa.<sup>70</sup> This state is also EPR silent and Ni-SIr to Ni-SIa conversion has been attributed to the protonation and dissociation of the bridging  $\text{OH}^-$  ligand. Ni-SIa is the most oxidised catalytically active form of the enzyme.

A second one-electron reduction converts the Ni-SIa state into the active Ni-C state. This is a formal  $\text{Ni}^{3+}$  state in which a  $\text{H}^-$  takes the place of the bridging position 'X'. On loss of  $\text{H}^-$  as  $\text{H}^+$ , the Ni-L species, a formal  $\text{Ni}^{1+}$  state, can be formed.<sup>71</sup> The interconversion between the Ni-C and Ni-L states is the subject of ongoing research and the relationship between the states is still unclear. The Ni-

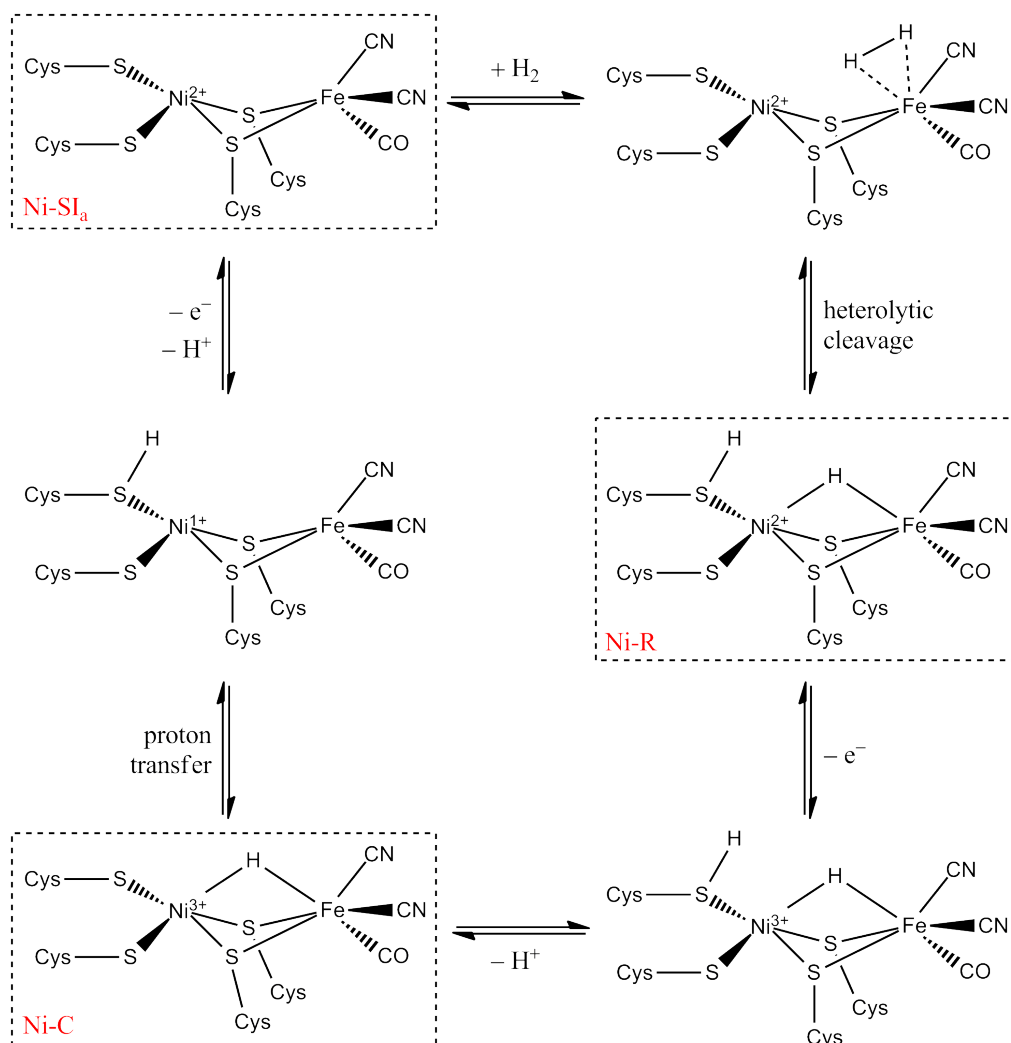
L state was first detected by illumination of the Ni-C state at low temperatures (77 K).<sup>72</sup> The original Ni-C spectrum could be completely regained by warming the sample to 200 K in the dark for 10 minutes. Often more than one Ni-L state is detected by EPR or FTIR, but the difference between them remains unclear.<sup>72,73</sup> The Ni-L state is often thought of as an artifact, only forming in the presence of light at cryogenic temperatures. However, in a number of O<sub>2</sub>-tolerant hydrogenases the Ni-L state is detected in the absence of light.<sup>34,73</sup> The relationship between the Ni-C and Ni-L states in the O<sub>2</sub>-tolerant Hydrogenase-1 from *Escherichia coli* is investigated in Chapter 6.

Further one-electron reduction of the Ni-C state leads to the Ni<sup>2+</sup> EPR silent Ni-R state, the most reduced catalytically active state.

## 1.7 The Catalytic Cycle of [NiFe]-Hydrogenases

Although it is widely agreed that Ni-SIa, Ni-C and Ni-R are all involved in H<sub>2</sub> cycling, there is not yet a widely agreed mechanism for the interconversion of H<sub>2</sub> and H<sup>+</sup> by [NiFe]-hydrogenases. One possible mechanism proposed by Siegbahn *et al.* is represented in Scheme 1.2.<sup>74</sup> This mechanism proceeds *via* H<sub>2</sub> binding to the Fe atom whilst the active site is in the Ni<sup>2+</sup> Ni-SIa state. Prior to the binding of H<sub>2</sub>, this state does not contain a ligand in the bridging position represented by 'X' in Figure 1.2 and so it is believed that it is this state which binds H<sub>2</sub> during H<sub>2</sub> oxidation. It is not yet clear however whether H<sub>2</sub> binds to the Ni or the Fe atom and studies using Density Functional Theory (DFT) have suggested that H<sub>2</sub> may be able to bind to both metal atoms.<sup>75</sup> Evidence that the hydrophobic gas channel connecting the active site to the molecular surface ends at the Ni atom<sup>37</sup> and that CO, which acts as a competitive inhibitor to H<sub>2</sub> oxidation, binds at the Ni,<sup>76</sup> indicates that the H<sub>2</sub> binding site may be at the Ni atom. However, the small distance between the Ni and Fe atoms means that the position of the gas channel may not accurately predict the binding position of H<sub>2</sub>. The vacant binding site and the unusual choice of ligands at the Fe, which maintains a low spin electron

configuration ideal for binding  $\text{H}_2$ ,<sup>74</sup> may point to the fact that it is the Fe atom at which  $\text{H}_2$  binds.



**Scheme 1.2:** A proposed mechanism for  $\text{H}_2$  cycling by [NiFe]-hydrogenases. Figure adapted from Reference 74. Previously characterised catalytic intermediates, as shown in Scheme 1.1, are indicated by a grey dashed rectangle.

Whether  $\text{H}_2$  binds to the Fe or the Ni atom the next step most likely involves heterolytic cleavage of the  $\text{H}_2$  resulting in a hydride which bridges the Ni and the Fe atoms (in position ‘X’ in Figure 1.2).<sup>74</sup> A nearby basic residue, such as one of the terminal or bridging cysteine residues,<sup>74</sup> or another nearby residue, may assist the cleavage of  $\text{H}_2$  resulting in the protonation of the residue. This results in the formation of the  $\text{Ni}^{2+}$  Ni-R state.

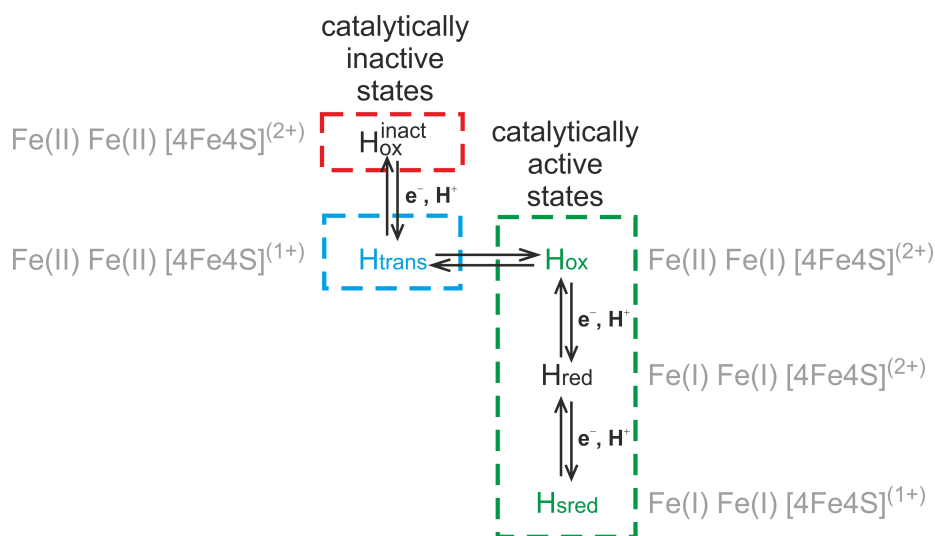
Oxidation of the Ni atom, forming  $\text{Ni}^{3+}$ , and removal of a proton *via* a proton transfer pathway, then generates the Ni-C state. A redox reaction then follows in

which the bridging hydride is lost resulting in the  $\text{Ni}^{3+}$  being reduced to  $\text{Ni}^{1+}$  and a proton being transferred to one of the cysteine ligands. The Ni-SIa state can now be regenerated *via* oxidation of  $\text{Ni}^{1+}$  to  $\text{Ni}^{2+}$  and loss of a proton.

Due to the recent detection of Ni-L species in the absence of light it has been proposed that this state may also be involved in catalysis.<sup>34,73</sup> Possible catalytic mechanisms that involve this state are discussed in detail in Chapter 6.

## 1.8 The Redox States of [FeFe]-Hydrogenases

Various redox states accessible to the [FeFe]-hydrogenases have been detected, with both the  $\text{Fe}_p$  and  $\text{Fe}_d$  atoms and the adjoining [4Fe4S]-cluster all found to be redox active. The different states, and the relationship between them, are outlined in Scheme 1.3.



**Scheme 1.3:** Catalytically active and inactive states of [FeFe]-hydrogenases and their interconversions. States depicted in colour refer to those visible by EPR spectroscopy (EPR active states). The formal oxidation state of the two Fe atoms of the binuclear Fe cluster and the adjoining [4Fe4S]-cluster is shown alongside each state in grey. The individual oxidation states of the  $\text{Fe}_p$  and  $\text{Fe}_d$  atoms are not specified.

The most oxidised state is the inactive  $\text{H}_{\text{ox}}^{\text{inact}}$  state, which is formed under highly oxidising conditions or as a result of aerobic purification of the enzyme.<sup>24,77</sup> This is an EPR inactive state, with both the  $\text{Fe}_p$  and  $\text{Fe}_d$  atoms in the  $\text{Fe}^{2+}$  oxidation state and the [4Fe4S]-cluster in the  $[\text{4Fe4S}]^{2+}$  state.<sup>24</sup> The labile CO ligand, found

either in a bridging position or bound terminally to the  $\text{Fe}_d$  atom depending upon the redox state of the enzyme, has been detected by X-ray crystallography to be bridging the  $\text{Fe}_p$  and  $\text{Fe}_d$  atoms in this state.<sup>78</sup> The vacant site at the  $\text{Fe}_d$  atom is occupied by an oxygenic species ( $\text{OH}^-/\text{H}_2\text{O}$ ).<sup>41</sup>

The  $\text{H}_{\text{ox}}^{\text{inact}}$  state can undergo a one-electron reduction to form the transient  $\text{H}_{\text{trans}}$  state.<sup>79</sup> This is an EPR active state in which the additional electron reduces the  $[\text{4Fe4S}]$ -cluster to  $[\text{4Fe4S}]^{1+}$ . In this state the bridging CO remains present and it is assumed that the oxygenic ligand bound to the otherwise vacant site on the  $\text{Fe}_d$  atom is retained.<sup>24</sup>

The  $\text{H}_{\text{ox}}$  state can then be formed by lowering the redox potential.<sup>79,80</sup> The  $\text{H}_{\text{ox}}$  state and the  $\text{H}_{\text{trans}}$  state are isoelectronic and the conversion between them reflects a conformational change in which the oxygenic ligand is removed resulting in a vacant coordination site on the  $\text{Fe}_d$  atom.<sup>24</sup> This promotes the transfer of an electron from the  $[\text{4Fe4S}]$ -cluster to the binuclear subcluster, resulting in a  $\text{Fe}^{2+}/\text{Fe}^{1+}$  mixed-valence state.<sup>81</sup>

The  $\text{H}_{\text{ox}}$  state is the most oxidised catalytically active state. It can be reduced with  $\text{H}_2$  to form the EPR silent  $\text{H}_{\text{red}}$  state. This involves the further one-electron reduction of the binuclear subcluster resulting in a  $\text{Fe}^{1+}/\text{Fe}^{1+}$  state with the previously bridging CO ligand now terminally bound to the  $\text{Fe}_d$  atom.<sup>78,79</sup>

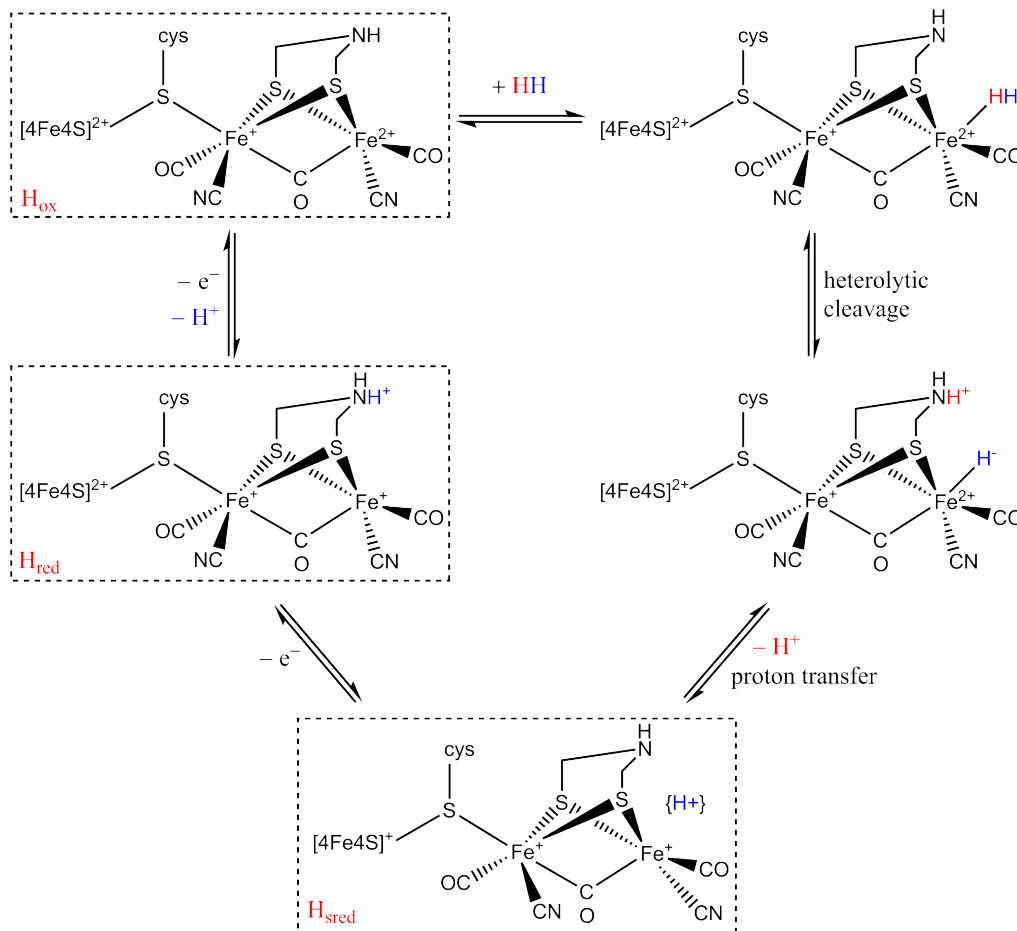
A further one-electron reduction can take place to form the recently characterised ‘super-reduced’  $\text{H}_{\text{sred}}$  state. This is the most reduced catalytically active state and is once again paramagnetic allowing EPR characterisation. It is believed that conversion from the  $\text{H}_{\text{red}}$  state to the  $\text{H}_{\text{sred}}$  state involves the reduction of the  $[\text{4Fe4S}]$ -cluster to  $[\text{4Fe4S}]^{1+}$ .<sup>47,82</sup> It is thought that the labile CO ligand remains in a terminal position.<sup>83</sup>

## 1.9 The Catalytic Cycle of [FeFe]-Hydrogenases

Although the existence of a catalytically active  $\text{H}_{\text{sred}}$  state had been proposed a number of times,<sup>79,84</sup> it has only been recently characterised.<sup>47,82</sup> There are therefore,



currently, very few catalytic schemes that have been proposed that involve all three active intermediate states ( $H_{ox}$ ,  $H_{red}$  and  $H_{sred}$ ). One such mechanism, proposed by Adamska *et al.*, is shown in Scheme 1.4.<sup>82</sup>



**Scheme 1.4:** A proposed mechanism for  $H_2$  cycling by  $[FeFe]$ -hydrogenases. Figure adapted from Reference 82. Previously characterised catalytic intermediates, as shown in Scheme 1.3, are indicated by a dashed rectangle.  $\{H^+\}$  in the  $H_{sred}$  state indicates that a proton is associated with the H-cluster and possibly bound to a nearby amino acid residue.

It is generally agreed that  $H_2$  binds to the most oxidised state,  $H_{ox}$ , and then undergoes heterolytic cleavage resulting in the binding of a hydride,  $H^-$ , to the binuclear subcluster. It is unclear as to whether the hydride is bound terminally to the  $Fe_d$  atom or bridges the  $Fe_p$  and  $Fe_d$  atoms and DFT studies have found that both are feasible.<sup>85,86</sup> In Scheme 1.4 the  $H^-$  is shown bound terminally for clarity. It has been suggested that role of the bridgehead N atom in the  $SCH_2NCH_2S$  dithiolate ligand is to act as a base for this heterolytic cleavage<sup>86,87</sup> resulting in a proton being

bound to the N atom. This proton can then be removed from the active site *via* a proton transfer pathway (see Section 1.5.5).

The next stage of catalysis involves loss of the hydride resulting in a bound proton (indicated by  $\{H^+\}$  in Scheme 1.4) and reduction of the  $[4Fe4S]$ -cluster and the bimetallic subcluster to form the  $[4Fe4S]^{1+}$ ,  $Fe_p^{1+}/Fe_d^{1+}$   $H_{red}$  state.<sup>47</sup> It is not yet known whether simultaneous proton-electron transfer occurs or whether there are discrete proton- and electron-transfer events.<sup>74</sup> Oxidation of the  $[4Fe4S]$  cluster and binding of the second proton to the bridgehead N atom results in the  $H_{red}$  state, before the second proton is removed from the active site *via* a proton transfer pathway and the  $[4Fe4S]$ -cluster can be reoxidised to  $[4Fe4S]^{2+}$  regenerating the  $H_{ox}$  state.<sup>82</sup>

## 1.10 Securing a Future Hydrogen Supply: The Technological Applications of Hydrogenases

Hydrogen ( $H_2$ ) has been proposed as an energy carrier playing a role in reducing the world's reliance on fossil fuels.<sup>8,88</sup> Ideally produced by water-splitting using a renewable source of electricity, such as wind, solar, tidal or hydro, it could then be used to generate electricity through the use of a hydrogen fuel cell.<sup>89</sup> However, there are many challenges to overcome before hydrogen can be seen as a viable energy carrier able to replace more conventional fuels such as oil and natural gas. One of these challenges, currently the center of significant levels of research, is the development of improved methods of hydrogen production. Hydrogen is currently produced largely from steam reformation of non-renewable fossil fuels, usually methane from natural gas, in which CO and  $CO_2$  are formed as by-products.<sup>88,90</sup> Research is underway investigating alternative catalysts for both  $H_2$  production and  $H_2$  oxidation (for use in fuel cells in order to convert the hydrogen back into electricity). Platinum is currently the catalyst of choice, however, if  $H_2$  is to be used as an energy carrier on a large scale, a cheaper, more abundant catalyst must be found. Able to carry out

the same reaction, with the much more abundant Fe and Ni atoms at their core, hydrogenases are currently under investigation as inspirational catalysts to further this research.

Currently hydrogen is used almost exclusively for synthesis and its continued supply is essential for use in the Haber-Bosch process in which it reacts with atmospheric nitrogen to produce ammonia, the majority of which is then converted into ammonium sulfate for use in fertilisers.<sup>90</sup> The production of hydrogen gas together with the high pressures and temperatures required means that the Haber-Bosch process makes up more than 1% of the total global energy consumption.<sup>91</sup> A more efficient process of producing  $H_2$  will help reduce the energy demand of this highly important industrial process.

Whether or not hydrogenases themselves will be used in technological devices in the future is not yet certain. Problems such as their sensitivity to  $O_2$ , their instability (due to their detachment from a surface on which they are adsorbed, or their eventual denaturing), the expensive and lengthy purification procedures required to produce them, and their large size relative to molecular catalysts, mean that their use may be limited. On the other hand, they are still, in terms of both efficiency and overpotential requirement, the best catalysts currently known for  $H_2$  cycling, a feat that they are able to achieve at room temperature and at neutral pH. This has sparked much interest in the development of synthetic catalysts which mimic the active sites of hydrogenases.<sup>92,93</sup>

Structural mimics based on the active sites of both  $[NiFe]$ -<sup>94-96</sup> (usually in an attempt to make a  $H_2$  oxidation catalyst) and  $[FeFe]$ -hydrogenases<sup>97-99</sup> (in an attempt to make a  $H_2$  production catalyst) have been made but these generally exhibit no catalytic activity or low catalytic activity that requires significant overpotentials. They also often exhibit low turnover numbers due to their susceptibility to rapid decomposition. A possible reason for the large difference in catalytic performance between current hydrogenase active site mimics and the enzymes themselves is that the synthetic mimics do not have the surrounding protein support that acts to sta-

bilise the active site, protecting it from solvent effects but also optimising the delivery and removal of substrate and products. Recent studies have shown that a synthetic version of the 2Fe binuclear part of the [FeFe]-hydrogenases active site is completely inactive towards  $H_2$  cycling, however, when it is incorporated into the apo-protein *in vitro* (where ‘apo-protein’ refers to the protein that includes the [4Fe4S] subcluster of the H-cluster only), making fully formed and intact holo-protein, the incorporated synthetic [FeFe]-hydrogenase active site successfully carries out  $H_2$  production with a turnover frequency as high as that of holo-protein which had been matured *in vivo*.<sup>45,46</sup> This study highlights the vital role that the protein plays within the enzyme. This study is discussed in more detail in Chapter 7.

Research has been undertaken that investigates the possibility of incorporating hydrogenases into a system that is able to carry out artificial photosynthesis, *i.e.* a system that is capable of making fuel from sunlight. There are a number of studies in which hydrogenase, semiconducting particles and organic dyes together successfully achieved photobiological  $H_2$  production, although current work uses sacrificial electron donors to supply the electrons required.<sup>100–102</sup> The need for direct adsorption of the different components of the system to each other is still being investigated and successful systems have involved hydrogenases being both directly adsorbed onto semiconducting particles and being free in solution as well as the co-adsorption of the organic dye.<sup>100,102</sup>

Another possible application involves coupling two different redox enzymes *via* a conducting particle in order to generate a catalyst capable of coupling a specific oxidation reaction to a reduction reaction. A system capable of catalysing the water gas shift reaction, which converts CO and  $H_2O$  into  $CO_2$  and  $H_2$ , has been made in this way by the co-adsorption of hydrogenase and carbon monoxide dehydrogenase onto graphite platelets.<sup>103</sup> Hydrogenases have also shown to be useful in the regeneration of NADPH (nicotinamide adenine dinucleotide phosphate), producing  $H^+$  as the only by-product of the reaction.<sup>104,105</sup>

There have been a number of ‘proof of principle’ demonstrations where  $O_2$ -

tolerant hydrogenases have successfully replaced Pt as the  $\text{H}_2$  oxidation catalyst in  $\text{H}_2/\text{O}_2$  fuel cells.<sup>106,107</sup> The specificity of enzymes means that the possibility of a membraneless fuel cell becomes viable leading to the minaturisation of the technology. If enzyme-based fuel cells can be shown to produce a stable current, of high enough power density, then hydrogenases may find a use in niche applications requiring a very small, portable fuel cell where the electrode could even be disposable.<sup>108,109</sup>

The above paragraphs highlight some of the main areas of research currently being undertaken in the quest to use hydrogenases, or synthetic mimics based on their structure, in a technologically useful role in the future. Possibilities include both their use as fuel producers, acting as  $\text{H}_2$  production catalysts, or in electricity generation through  $\text{H}_2$  oxidation. In order for this to be realised, a full and detailed understanding of the structure and mechanism of hydrogenases must be obtained. This understanding must include the role of the protein in substrate delivery and product removal, the importance of the [FeS]-clusters in being able to transfer electrons to and from the active site, as well as to accumulate the electrons required for the reaction close to the catalytic centre, and a detailed understanding of the substrate binding sites and bond breaking and forming processes that occur at the active site itself. This complex task can be simplified by compartmentalising the enzyme, separating it into sections in which each section is required to complete a certain task. This is an idea which is explored further in Chapters 3 and 4.

## 1.11 Topics Addressed in this Thesis

This thesis investigates the electrocatalytic activity of hydrogenases largely through the use of Protein Film Electrochemistry (PFE), a technique that is described in Chapter 2. Electron Paramagnetic Resonance spectroscopy (EPR) and Attenuated Total Reflectance Infrared spectrelectrochemistry (ATR-IR) is used to further this work, enabling various active and inactive states of hydrogenases to be distinguished.

In Chapter 3, PFE is used alongside mathematical modelling to quantify some

important differences between the catalytic performance of [FeFe]-hydrogenases and [NiFe]-hydrogenases. The model developed, which describes reversible surface-bound electrocatalysis and is non-specific to hydrogenases, compares the relative importance of interfacial electron transfer between the enzyme and the electrode and the inherent catalytic activity of the enzyme in determining the characteristic features of the catalyst such as overpotential and catalytic bias. This model is developed further in Chapter 4 to include the possibility that the catalyst in question is able to undergo potential-dependent changes that affect its inherent catalytic ability.

Chapter 5 presents PFE experiments that investigate the reaction between exogenous cyanide and the [NiFe]-hydrogenases *EcHyd1* and *EcHyd2* from *Escherichia coli*. Cyanide is found to reversibly inhibit the H<sub>2</sub> oxidation activity of both *EcHyd1* and *EcHyd2* and the use of EPR and ATR-IR enables the identity of the final inactive state to be determined.

Chapter 6 investigates the relationship between the Ni-C and Ni-L states of *EcHyd1*. In this chapter EPR and ATR-IR experiments detect the presence of Ni-L states in samples prepared in conditions free from visible light, challenging the widely held assumption that the Ni-L state is a light-induced artefact.

Finally, Chapter 7 details PFE experiments that investigate the spontaneous *in vitro* incorporation of a synthetic [2Fe] active site mimic complex into [FeFe]-hydrogenase protein that is originally missing the [2Fe] active site subcluster. Incorporation, which is observed *via* PFE as an increase in current, is studied in *Chlamydomonas reinhardtii* and *Clostridium pasteurianum*, and in both cases results in fully functional enzyme.

## Chapter 2

# Experimental Theory and Methods





## 2.1 Electrochemical Theory

### 2.1.1 Protein Film Electrochemistry

Protein Film Electrochemistry (PFE) is a suite of techniques in which a redox enzyme is directly adsorbed onto the surface of an electrode, creating a mono/submonolayer film<sup>110</sup> and allowing fast electron transfer between the enzyme and the electrode to take place.<sup>2</sup> The electrode acts as a source or sink for electrons involved in redox processes occurring at the enzyme and sets up a potential difference at the electrode/solution interface. The movement of electrons can be detected as a current, which is proportional to the catalytic rate and which varies with time and potential in a way characteristic to the enzyme. The precise potential control that PFE affords enables a detailed and quantitative description of enzyme activity to be obtained.<sup>2</sup>

Protein Film Electrochemistry provides a number of advantages over conventional solution electrochemical techniques: Through the direct adsorption of the protein onto the surface of the electrode, soluble redox mediators, used in solution techniques to shuttle electrons between the electrode and the redox-active protein, are no longer needed. This overcomes the limitations associated with the slow diffusion of the enzyme and mediator to the electrode. Furthermore, a major disadvantage of using solution electrochemistry techniques for proteins which are not commercially available and are difficult to purify is the large quantity of protein that is required. The use of PFE overcomes this with picomolar quantities of enzyme being sufficient to create a film. Lastly, PFE enables the oxidation state of redox centres in all protein molecules present in the sample to be altered instantaneously, allowing the kinetics of electron transfer reactions and activation/inactivation processes to be revealed.

Protein Film Electrochemistry incorporates a number of complementary techniques and in this thesis is used alongside cyclic voltammetry and chronoamperometry.

### 2.1.2 The Three-Electrode System

A voltage,  $E$ , applied between a working electrode and a reference electrode, can be described by Equation 2.1 (assuming that a finite current  $i$  is flowing between the two electrodes):<sup>111,112</sup>

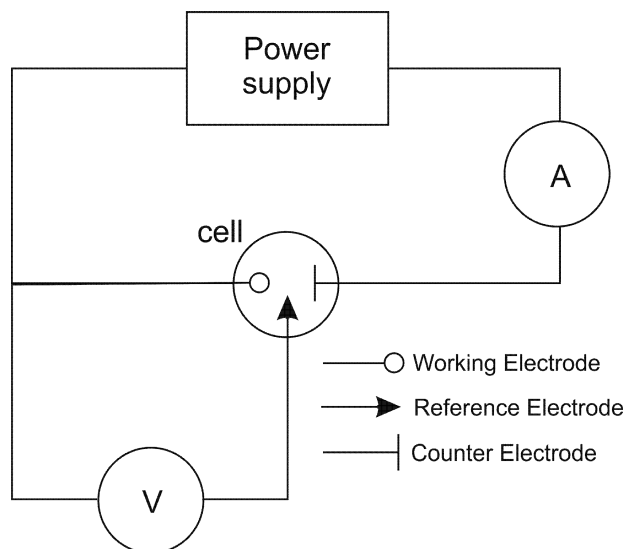
$$E = (\Phi_w - \Phi_s^w) + iR_s + (\Phi_s^{\text{REF}} - \Phi_{\text{REF}}) \quad (2.1)$$

where  $\Phi_w$  and  $\Phi_{\text{REF}}$  are the electric potentials of the working and reference electrodes, and  $\Phi_s^w$  and  $\Phi_s^{\text{REF}}$  represent the electric potential of the solution close to the working and reference electrode respectively. The term  $iR_s$  describes the voltage drop in solution due to the passage of current  $i$  between two electrodes. The intervening solution has an electrical resistance  $R_s$ .

Electrochemistry experiments involve the measurement of  $i$  as a function of changes in the potential difference at a working electrode and solution interface ( $\Phi_w - \Phi_s^w$ ), *i.e.* as a function of the driving force for electrolysis at the working electrode/solution interface. In an ideal case, often seen in microelectrode experiments,  $iR_s$  is negligible and the potential drop at the reference electrode/solution interface ( $\Phi_s^{\text{REF}} - \Phi_{\text{REF}}$ ) is fixed by the chemical composition of the reference electrode. In turn, changes in the applied potential are simply reflected as changes in ( $\Phi_w - \Phi_s^w$ ). However, with large electrodes,  $iR_s$  is no longer negligible and the passage of large currents through the reference electrode may change its chemical composition, altering ( $\Phi_s^{\text{REF}} - \Phi_{\text{REF}}$ ).<sup>111,112</sup>

Hence, in order to accurately measure ( $\Phi_w - \Phi_s^w$ ) a three-electrode system must be used in which a counter electrode is used alongside the working and reference electrodes.<sup>111,112</sup> In this system, represented in Figure 2.1, current flows between the working and counter electrodes whilst the potential of the working electrode relative to the reference electrode is measured using a device with high resistance, ensuring that only a very small amount of current is drawn through the reference electrode and so its potential remains constant. All PFE experiments in this thesis

were obtained using a three-electrode setup.



**Figure 2.1:** A schematic circuit diagram of the three-electrode system. Figure adapted from Reference 111.

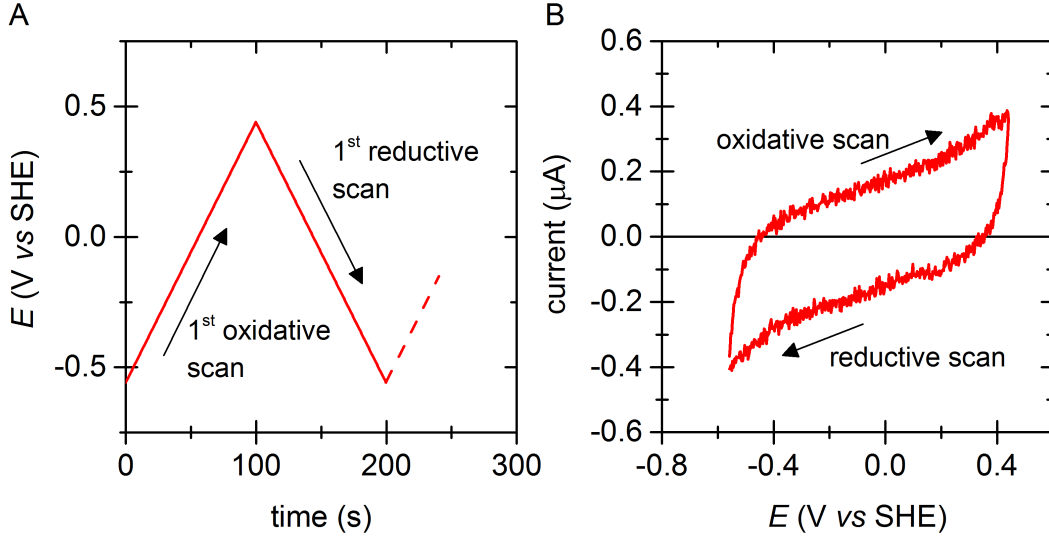
### 2.1.3 Cyclic Voltammetry

In a cyclic voltammetry experiment, the working electrode potential is swept back and forth between two limits at a chosen scan rate, whilst the current,  $i$ , is recorded as a function of the applied potential,  $E$ . This produces a cyclic voltammogram which is made up of an oxidative and a reductive scan, corresponding to a potential sweep from low to high potential and high to low potential respectively, as demonstrated in Figure 2.2.

The current recorded in a cyclic voltammogram consists of two components, a non-Faradaic and a Faradaic component.

#### 2.1.3.1 Non-Faradaic Current

Non-Faradaic current is a result of the formation of what is referred to as the ‘electrical double-layer’ and it gives rise to the cyclic voltammogram of a bare, unmodified electrode as shown in Figure 2.2B. The electrical double-layer arises due to a build up of charge at the solution-electrode interface.<sup>111</sup> For a given potential, there will be either a deficiency or excess of electrons at the electrode surface. The



**Figure 2.2:** (A) The triangular waveform obtained from the variation in applied potential with time for a  $10 \text{ mV s}^{-1}$  cyclic voltammetry scan. (B) The non-Faradaic current produced by a bare, unmodified pyrolytic graphite edge-plane rotating disk electrode (see Section 2.3.1.1) obtained from the potential *vs* time sweep shown in panel A.

interface as a whole must maintain electrical neutrality and so the charge on the electrode,  $q^m$ , must be matched by an equal but opposite charge at the solution interface,  $q^s$ , (*i.e.*  $q^m = -q^s$ ). This is achieved by the redistribution of ions at the electrode-solution interface resulting in an excess of either anions or cations (depending upon the charge of the electrode) near the electrode surface. As the applied potential is varied, the electrode-solution interface behaves like a capacitor as the charges at the interface rearrange, resulting in current flow between the working and counter electrode (in the case of a three-electrode system).

The charge on a capacitor,  $q$ , can be described by the product of the potential across the capacitor,  $E$ , and the capacitance,  $C$ :<sup>111</sup>

$$q = EC \quad (2.2)$$

The current,  $i$ , produced as the potential is modulated is equal to the change in charge with time:

$$i = \frac{dq}{dt} = C \frac{dE}{dt} \quad (2.3)$$

In cyclic voltammetry the change in potential with time ( $dE/dt$ ) is simply the scan rate ( $\nu$ ) so that:

$$i = C\nu \quad (2.4)$$

Therefore, it is clear that the magnitude of the non-Faradaic current produced will increase with scan rate.<sup>111</sup> The scan rate will also affect  $C$ , as will the electrode area, the potential range scanned and the solution composition, and so in turn all of these factors will also affect the non-Faradaic current produced.

### 2.1.3.2 Faradaic Current

The oxidation or reduction of an electroactive species at an electrode, which occurs alongside electron transfer between the electroactive species and the electrode surface, results in Faradaic current. During a cyclic voltammetry experiment, the Faradaic currents arising from these redox processes will be superimposed upon the non-Faradaic ‘background’ current discussed above.

Under equilibrium conditions there is no net flow of electrons and the ratio of the concentrations of oxidised and reduced species adsorbed on the electrode surface is given by the Nernst equation (derived in Appendix A);<sup>111</sup>

$$E_{\text{eqm}} = E^{\ominus} + \frac{RT}{nF} \ln \frac{[\text{Ox}]}{[\text{Red}]} \quad (2.5)$$

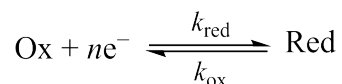
where  $E_{\text{eqm}}$  is the equilibrium potential,  $E^{\ominus}$  is the standard reduction potential for the redox couple,  $R$  is the gas constant,  $T$  is the temperature in Kelvin,  $n$  is the number of electrons involved in the process,  $F$  is Faraday’s constant and  $[\text{Ox}]$  and  $[\text{Red}]$  are the concentration of the electroactive species in the oxidised and reduced states respectively.

In dynamic electrochemistry experiments, a potential is applied which induces a current to flow. In a PFE experiment, this current provides a measure of the catalytic activity of the enzyme adsorbed onto the electrode surface. Importantly, in non-ideal situations a number of processes may contribute to the overall activity

of the enzyme-modified electrode and so the observed current may not be a direct measure of the inherent activity of the catalytic centre. Experiments should be designed to minimise these processes as far as possible, ensuring that they are not rate limiting and that the observed current is a true reflection of the rates and potentials of catalytic processes. The various non-catalytic processes that may affect the Faradaic current recorded during a PFE experiment are considered below.

### 2.1.3.3 Interfacial Electron Transfer

The rate of electron transfer between a redox-active entity and the electrode depends upon the applied potential as described by the Butler-Volmer equation. This equation can be derived by considering the general reaction;<sup>111</sup>



where Ox and Red represent two chemical species adsorbed onto an electrode surface that are interconverted by the transfer of  $n$  electrons ( $\text{e}^-$ ) and  $k_{\text{red}}$  and  $k_{\text{ox}}$  are the first-order potential-dependent rate constants which correspond to the reduction and oxidation processes respectively. At the standard potential of the system,  $E^\ominus$ ,  $k_{\text{red}} = k_{\text{ox}} = k^0$ , where  $k^0$  is the standard rate constant for the reaction. At other potentials, and under non-standard conditions,  $k_{\text{red}}$  and  $k_{\text{ox}}$  can be expressed in terms of  $k^0$  by the following equations;

$$k_{\text{red}} = k^0 \exp\left(\frac{-\alpha n F (E - E^\ominus)}{RT}\right) \quad (2.6)$$

$$k_{\text{ox}} = k^0 \exp\left(\frac{(1 - \alpha) n F (E - E^\ominus)}{RT}\right) \quad (2.7)$$

where  $\alpha$  is the transfer coefficient,  $E$  is the applied potential and  $n$ ,  $F$ ,  $R$  and  $T$  are as described previously. The transfer coefficient reflects the symmetry of the barrier to electron transfer and can range from zero to unity; a value close to zero implies that the transition state closely resembles the reactants, whereas a value close to one reflects a transition state that closely resembles the products. The value of  $\alpha$  is

usually assumed to be  $\approx 0.5$ , corresponding to a transition state with intermediate characteristics.

The total current,  $i$ , can be stated as in Equation 2.8;

$$i = i_c - i_a = nFA[k_{\text{red}}\Gamma_{\text{ox}} - k_{\text{ox}}\Gamma_{\text{red}}] \quad (2.8)$$

where  $i_c$  and  $i_a$  are the cathodic and anodic currents respectively,  $\Gamma_{\text{ox}}$  and  $\Gamma_{\text{red}}$  are the coverages of electroactive species in the oxidised and reduced states respectively, and  $A$  is the surface area of the electrode. Inserting Equations 2.6 and 2.7 into Equation 2.8 yields:

$$i = nFAk^0 \left[ \Gamma_{\text{ox}} \exp\left(\frac{-\alpha nF(E - E^\Theta)}{RT}\right) - \Gamma_{\text{red}} \exp\left(\frac{(1 - \alpha)nF(E - E^\Theta)}{RT}\right) \right] \quad (2.9)$$

At the equilibrium potential,  $E = E_{\text{eqm}}$ , the net current is zero, and:

$$nFAk^0\Gamma_{\text{ox}} \exp\left(\frac{-\alpha nF(E_{\text{eqm}} - E^\Theta)}{RT}\right) = nFAk^0\Gamma_{\text{red}} \exp\left(\frac{(1 - \alpha)nF(E_{\text{eqm}} - E^\Theta)}{RT}\right) \quad (2.10)$$

The Faradaic activity at the equilibrium potential can be expressed as the exchange current,  $i_0$ , which is equal in magnitude to  $i_a$  and  $i_c$  under these conditions. That is:

$$i_0 = nFAk^0\Gamma_{\text{ox}} \exp\left(\frac{-\alpha nF(E_{\text{eqm}} - E^\Theta)}{RT}\right) \quad (2.11)$$

and

$$i_0 = nFAk^0\Gamma_{\text{red}} \exp\left(\frac{(1 - \alpha)nF(E_{\text{eqm}} - E^\Theta)}{RT}\right) \quad (2.12)$$

At equilibrium, rearrangement of the Nernst equation (Equation 2.5) gives:

$$\exp\left(\frac{nF(E_{\text{eqm}} - E^\Theta)}{RT}\right) = \frac{\Gamma_{\text{ox}}}{\Gamma_{\text{red}}} \quad (2.13)$$

Both sides of this equation can be raised to the  $-\alpha$  power;

$$\exp\left(\frac{-\alpha nF(E_{\text{eqm}} - E^\Theta)}{RT}\right) = \left(\frac{\Gamma_{\text{ox}}}{\Gamma_{\text{red}}}\right)^{-\alpha} \quad (2.14)$$

which can then be substituted into Equation 2.11 to obtain:

$$i_0 = nFAk^0\Gamma_{\text{ox}}^{1-\alpha}\Gamma_{\text{red}}^\alpha \quad (2.15)$$

By using  $i_0$  instead of  $k^0$ , the current can now be described in terms of an overpotential,  $\eta$ , that is, a deviation from the equilibrium potential rather than a deviation from the standard potential of the system,  $E^\Theta$ . By dividing Equation 2.9 by Equation 2.15 we obtain;

$$\frac{i}{i_0} = \frac{\Gamma_{\text{ox}} \exp\left(-\alpha \frac{nF}{RT}(E - E^\Theta)\right)}{\Gamma_{\text{ox}}^{(1-\alpha)}\Gamma_{\text{red}}^\alpha} - \frac{\Gamma_{\text{red}} \exp\left(\frac{(1-\alpha)nF(E - E^\Theta)}{RT}\right)}{\Gamma_{\text{ox}}^{(1-\alpha)}\Gamma_{\text{red}}^\alpha} \quad (2.16)$$

which simplifies to:

$$\frac{i}{i_0} = \exp\left(\frac{-\alpha nF(E - E^\Theta)}{RT}\right) \left(\frac{\Gamma_{\text{ox}}}{\Gamma_{\text{red}}}\right)^\alpha - \exp\left(\frac{(1-\alpha)nF(E - E^\Theta)}{RT}\right) \left(\frac{\Gamma_{\text{ox}}}{\Gamma_{\text{red}}}\right)^{-(1-\alpha)} \quad (2.17)$$

Finally, substituting Equations 2.13 and 2.14 into Equation 2.17 gives:

$$i = i_0 \left[ \exp\left(\frac{-\alpha nF\eta}{RT}\right) - \exp\left(\frac{(1-\alpha)nF\eta}{RT}\right) \right] \quad (2.18)$$

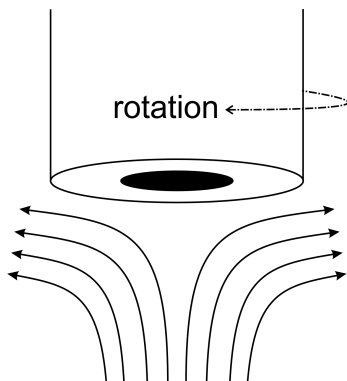
This expression is known as the Butler-Volmer equation and is central to the theory of electrochemical kinetics.

#### 2.1.3.4 Mass Transport

In order for the current observed during a PFE experiment to truly reflect the processes occurring at the enzyme, it is imperative that the catalytic reaction is not limited by the diffusion of the reactants (or products) to (or from) the electrode. In



this thesis, use of a rotating disk electrode (RDE) ensures that sufficient substrate is transported to the electrode. Rotation of a RDE results in a constant laminar flux of material from the bulk electrolyte solution to the electrode surface, as illustrated in Figure 2.3. The rotation pulls the electrolyte solution upwards, towards the electrode, before spinning it and releasing it outwards in a radial direction.



**Figure 2.3:** Schematic representation of the laminar flow at a rotating disk electrode.

The Levich equation (Equation 2.19) describes the variation in the mass transport limited current,  $i_{\text{MT}}$ , as a function of rotation rate;<sup>111</sup>

$$i_{\text{MT}} = 0.62nFA[S]_{\text{bulk}}D^{2/3}v^{-1/6}\omega^{1/2} \quad (2.19)$$

where  $n$  is the number of electrons involved in the process,  $F$  is Faraday's constant,  $A$  is the electrode area,  $[S]_{\text{bulk}}$  is the concentration of the substrate in the bulk solution,  $D$  is the diffusion coefficient of the substrate,  $v$  is the kinematic viscosity of the solvent ( $\text{cm}^2 \text{s}^{-1}$ ) and  $\omega$  is the angular velocity ( $\omega = 2\pi f$  where  $f$  is the number of revolutions per second of the electrode). As seen from Equation 2.19, the mass transport limited current will increase as a function of the square root of the rotation rate and therefore to ensure that the observed current,  $i_{\text{obs}}$ , is not limited by substrate transport the electrode rotation rate can simply be increased until it is no longer affects the observed current (*i.e.* until  $i_{\text{MT}} > i_{\text{obs}}$  and another factor becomes rate determining).

The electrode rotation rate required to ensure that mass transfer limitations

do not affect the observed current depends upon the ratio of the surface to bulk concentrations of substrate which is itself affected by the catalytic activity of the enzyme (*i.e.* how fast it uses up substrate) and the conditions of the experiment. The observed current can be related to the mass transport limited current by the equation;

$$\frac{[S]_{\text{sur}}}{[S]_{\text{bulk}}} = 1 - \frac{i_{\text{obs}}}{i_{\text{MT}}} \quad (2.20)$$

where  $[S]_{\text{sur}}$  and  $[S]_{\text{bulk}}$  are the concentrations of substrate at the electrode surface and in the bulk respectively. According to this expression, if  $i_{\text{obs}}$  is much smaller than  $i_{\text{MT}}$  then the substrate concentration at the electrode surface is approximately equal to that in the bulk and so the conditions will not be mass transfer limiting. However, as  $i_{\text{obs}}$  approaches  $i_{\text{MT}}$  the concentration of substrate at the electrode surface will tend to zero, leading to a large concentration gradient and increasingly mass transport limiting conditions.

The relationship between the observed current and the rotation rate is given by a modified form of Equation 2.19 known as the Koutecky-Levich equation;<sup>111</sup>

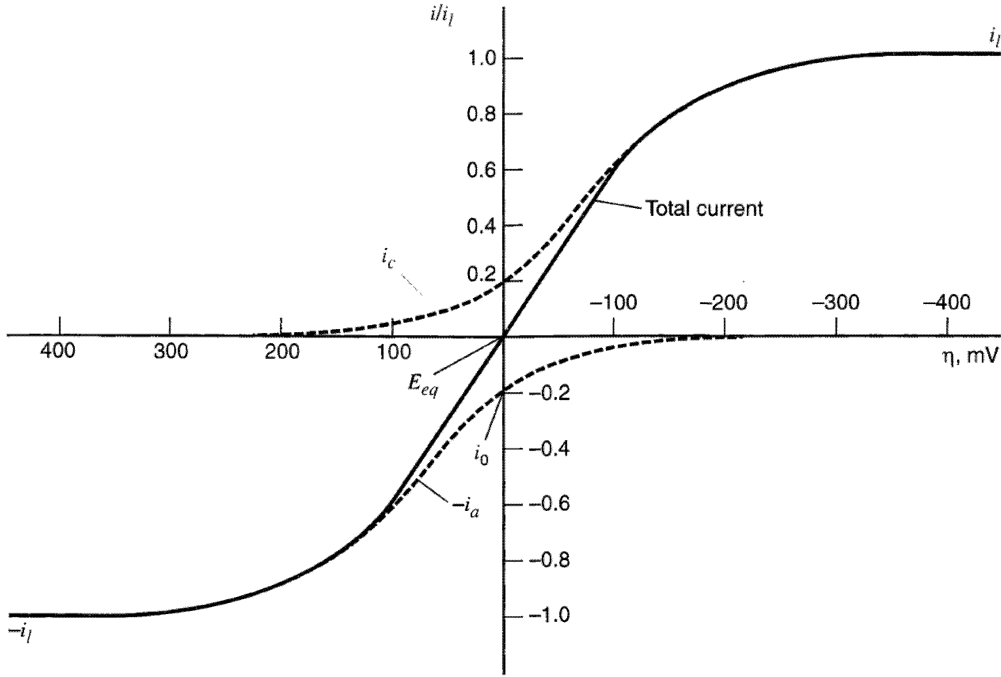
$$\frac{1}{i_{\text{obs,w}}} = \frac{1}{i_{\text{max}}} + \frac{1}{0.62nFA[S]_{\text{bulk}}D^{2/3}\nu^{-1/6}\omega^{1/2}} \quad (2.21)$$

where  $i_{\text{obs,w}}$  is the observed current at the angular velocity  $\omega$  and  $i_{\text{max}}$  corresponds to the maximum limiting current at an infinite rotation rate. The value of  $i_{\text{max}}$  can be determined from the  $y$ -intercept of a so-called Koutecky-Levich plot, that is a plot of  $1/i_{\text{obs,w}}$  against  $1/\omega^{1/2}$ . Alternatively, the value of  $i_{\text{max}}$  can be estimated from the observed current at a rotation rate above which no further increase in current is observed.

Unless otherwise specified, all PFE work presented in this thesis employed a rotation rate sufficient to ensure that the observed current was not affected by mass transport limitations (typically  $\omega \geq 1500$  rpm, where  $\omega$  represents the electrode rotation rate in revolutions per minute).

### 2.1.3.5 Catalytic Voltammetry

In the presence of a substrate, interfacial electron transfer between the enzyme and the electrode is coupled to a catalytic process. If we consider a reversible catalyst then the catalytic current produced by a redox enzyme will show a sigmoidal potential dependence, as shown in Figure 2.4.<sup>111</sup>



**Figure 2.4:** Current-overpotential curves for the system  $\text{Ox} + e^- \leftrightarrow \text{Red}$  with  $\alpha = 0.5$ ,  $T = 298 \text{ K}$ ,  $i_{l,c} = -i_{l,a} = i_l$  and  $i_0/i_l = 0.2$ . The dashed lines show the component currents  $i_c$  and  $i_a$  and the solid curve shows the net current. Figure taken with permission from Reference 111.

This behaviour is predicted by the Butler-Volmer model, and going either direction from  $E_{\text{eq}}$ , the rate of catalysis is determined by the rate of interfacial electron transfer. The solid line reflects the net current, the sum of the anodic ( $i_a$ ) and cathodic ( $i_c$ ) currents shown by the dashed lines. At large negative overpotentials, the anodic current is negligible and the total current tends to  $i_c$  in this region. At large positive overpotentials, the cathodic current is negligible and the total current will be essentially  $i_a$ . At extreme overpotentials the current reaches a plateau. In this potential region, the observed rate of catalysis is no longer limited by the rate of electron transfer but is instead limited by potential-independent steps. In the

absence of mass transport limiting conditions,  $i_l$  will reflect the maximum turnover frequency of the enzyme,  $k_{\text{cat}}^l$ , in the form of Equation 2.22

$$i_l = nFA\Gamma_m k_{\text{cat}}^l \quad (2.22)$$

where  $\Gamma_m$  is the electroactive coverage of the enzyme on the electrode surface.

In the absence of catalysis (for example, in the absence of a substrate or whilst the enzyme active site is inhibited) non-turnover signals may be detected. These are due to the reversible electron transfer between the electrode surface and redox centres present in the immobilised enzyme molecules. Oxidative and reductive peaks will occur in the cyclic voltammogram for each redox centre present, each pair of peaks centred around the reduction potential of the redox-couple ( $E^0$ ). The electroactive coverage of enzyme molecules on the electrode surface can be calculated from the the area under these peaks using Equation 2.23;<sup>111</sup>

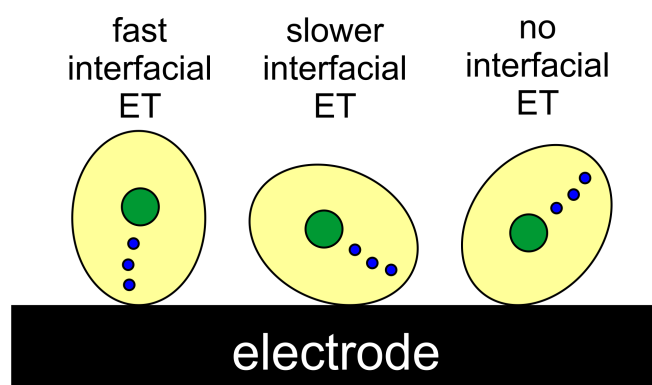
$$i = \pm \frac{n^2 F^2 \nu A \Gamma_m}{RT} \left( \frac{\exp\left(\frac{nF}{RT}(E - E^0)\right)}{\left(1 + \exp\left(\frac{nF}{RT}(E - E^0)\right)\right)^2} \right) \quad (2.23)$$

where  $\nu$  represents the scan rate,  $E$  is the applied potential,  $A$  is the electrode area and  $n$ ,  $F$ ,  $R$  and  $T$  have their usual meanings.

Although non-turnover signals are relatively easy to observe for small redox proteins which enable a high density of electroactive sites on an electrode surface, these signals can be difficult to detect for larger enzymes which contain multiple redox sites and give a low electroactive coverage. There are very few examples where non-turnover signals have been detected for hydrogenases, namely the [NiFe]-hydrogenase *Allochromatium vinosum*<sup>113</sup> and the [NiFeSe]-hydrogenase from *Desulfomicrobium baculatum*.<sup>114</sup>

The ideal sigmoidal waveform shown in Figure 2.4 is often not observed experimentally: A potential-dependent process may affect the activity of an enzyme within a certain potential region, a well documented example of this being the potential-

dependent conversion of catalytically active [NiFe]-hydrogenase into the inactive Ni-B state at oxidising potentials. This process is visualised experimentally by a decrease in current in the high potential region of a cyclic voltammogram instead of the plateau seen in Figure 2.4. A distribution of enzyme orientations on the electrode surface may also result in deviations from the ideal sigmoidal waveform.<sup>2</sup> As demonstrated in Figure 2.5, very efficient electron transfer between the electrode and active site may be possible for some enzyme molecules present in the protein film, whereas for others it may be far less efficient. This will cause a dispersion in the rates of interfacial electron transfer, resulting in a residual slope being observed in the waveform even at a high driving force.<sup>115</sup>



**Figure 2.5:** A demonstration of the various orientations that enzyme molecules may adopt on an electrode surface and how the orientation will affect the rate of interfacial electron transfer (ET) between the electrode and the electron relay chain. Enzyme molecules are depicted in light yellow, the electron relay chain is shown in blue, the active site is represented in green and the electrode is shown in black.

The various factors which affect the catalytic waveforms observed *via* cyclic voltammetry are discussed in greater detail in Chapters 3 and 4.

## 2.1.4 Chronoamperometry

Chronoamperometry is a method in which the potential of the working electrode is held at a constant value allowing the current to be measured as a function of time.<sup>111</sup> This technique is particularly useful when determining the rates of processes such as enzyme inactivation and reactivation because, unlike in cyclic voltammetry, the potential and time domains are deconvoluted. A variation of this method is

potential step chronoamperometry, where the potential of the working electrode is instantaneously stepped between different potentials whilst the resulting changes in current are monitored.

Similar to cyclic voltammetry experiments, chronoamperometry measurements consist of both Faradaic and non-Faradaic components: Upon a change in potential, the non-Faradaic response will be observed as a charging current superimposed upon the enzymatic Faradaic current. The non-Faradaic charging current decays exponentially with time and is significant for approximately the first 10 s following a potential step.<sup>116</sup> This means that accurate rates cannot be obtained for rapid potential step-driven reactions, in which a considerable proportion of the process is completed within this ‘charging time’. As for cyclic voltammetry, in order to ensure that the Faradaic component of the observed current is a true reflection of the processes occurring at the enzyme, care must be taken to minimise mass transport and interfacial electron transfer limiting conditions.

## 2.2 Electron Paramagnetic Resonance Theory

### 2.2.1 The Resonance Condition and the $g$ -Factor

An electron with spin  $S = 1/2$  can have two orientations in a magnetic field,  $\mathbf{B}$ : It can be orientated parallel to  $\mathbf{B}$ , where  $m_s = +1/2$ ; or anti-parallel to  $\mathbf{B}$ , where  $m_s = -1/2$ . An electron orientated parallel to  $\mathbf{B}$  will be energetically higher than an electron in the anti-parallel configuration, the electronic Zeeman energies being given by:<sup>117</sup>

$$E = g\mu_B \mathbf{B} m_s \quad (2.24)$$

In this equation  $g$  takes a value inherent to the property of the matter and  $\mu_B$  is the Bohr magneton.

By absorbing electromagnetic radiation of energy  $\Delta E$  an electron can change orientation, or ‘resonate’. Noting the Bohr frequency condition,  $\Delta E = h\nu$ , this

defines the resonance condition for an electron with  $\mathbf{S} = 1/2$  as:

$$h\nu = g\mu_B\mathbf{B} \quad (2.25)$$

where  $h$  is Planck's constant,  $\nu$  is the (microwave) frequency and  $g$ ,  $\mu_B$  and  $\mathbf{B}$  are as defined before. In a continuous wave Electron Paramagnetic Resonance (CW EPR) experiment,  $\mathbf{B}$  is varied at constant  $\nu$  until the resonance condition is met. In theory, resonance could also be detected by varying  $\nu$  at a constant  $\mathbf{B}$ , however, the use of resonant cavities to increase the sensitivity of the experiment, designed so that their dimensions correspond to a specific wavelength, dictate that in practice it is  $\mathbf{B}$  which is varied.

For practical purposes Equation 2.25 can be rewritten as:

$$g = \frac{0.714484\nu \text{ (in MHz)}}{\mathbf{B} \text{ (in Gauss)}} \quad (2.26)$$

For a free electron *in vacuo* the value of  $g$  is  $g_e = 2.00232$ . However, when an electron orbits around one or more nuclei it will experience a second magnetic field,  $\delta\mathbf{B}$ , as a result of spin-orbit coupling. The resonance condition for this 'perturbed' electron then becomes;

$$h\nu = g_e\mu_B(\mathbf{B}_e + \delta\mathbf{B}) \quad (2.27)$$

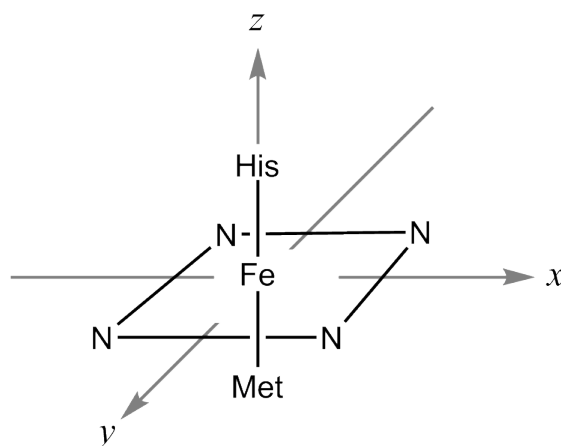
which, as we do not know the value of  $\delta\mathbf{B}$ , can be more conveniently written as:

$$h\nu = (g_e + \delta g)\mu_B\mathbf{B} \quad (2.28)$$

The concept of  $\delta g$  is vital to EPR. It is this quantity that contains the chemical information on the nature of the bond coupling the electron and the nucleus and in general the electronic structure of the molecule. The  $g$ -value associated with a species is simply the value of  $g_e + \delta g$  for that species.

### 2.2.2 Anisotropy

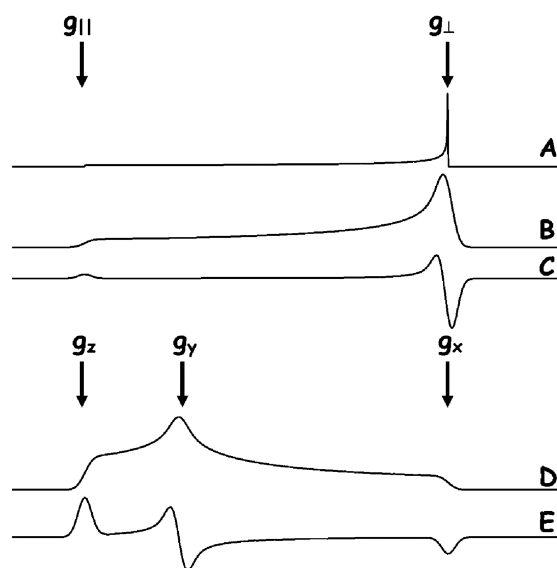
Molecular properties, such as  $\delta g$  in Equation 2.28, are angular dependent and will reflect the 3D electronic structure of the molecule. Consider an oxidised *c*-type cytochrome molecule with a low-spin ferric heme, shown in a simplified representation in Figure 2.6, with the tetrapyrrole structure simply considered as four nitrogens in a plane at equal distance from the Fe.<sup>117</sup> The axial ligands are the amino acid residues histidine and methionine, labelled as His and Met respectively in Figure 2.6.



**Figure 2.6:** A simplified representation of the porphyrin prosthetic group in cytochrome *c*, where His and Met represent histidine and methionine respectively. This molecule exhibits axial paramagnetic anisotropy. The *z*-axis is defined as parallel to the histidine-Fe and methionine-Fe bonds with the *x*- and *y*-axis in the porphyrin plane. Figure adapted from Reference 117.

When the molecule represented in Figure 2.6 is placed in an external dipolar magnetic field  $\mathbf{B}$  with the field vector  $\mathbf{B}$  along the *x*-axis, then  $\delta g$  in Equation 2.28 will be largely determined by the heteroaromatic electrons of the porphyrin plane. If the molecule is rotated so that the *z*-axis is now parallel to  $\mathbf{B}$ , then  $\delta g$  will now be determined by the electronic structure of the histidine and methionine ligands. If the molecule was instead rotated so that the field vector changed from being parallel to the *x*-axis to being parallel to the *y*-axis then, because the simplified porphyrin plane structure is symmetric, no change in  $\delta g$  would be seen. This is an example of axial symmetry where  $g_x = g_y \neq g_z$  (where  $g_j = g_e + \delta g_j$ ) giving a spectrum similar to that shown in Figures 2.7A to 2.7C.





**Figure 2.7:** The affect of anisotropy in absorption and derivative EPR spectra: (A) axial intensity pattern where  $g_x = g_y = g_{\perp}$  and  $g_z = g_{\parallel}$ ; (B) axial EPR absorption; (C) axial EPR derivative; (D) rhombic EPR absorption where  $g_x \neq g_y \neq g_z$ ; (E) rhombic EPR derivative. Figure reproduced with permission from Reference 117.

In a sample consisting of randomly orientated cytochrome molecules there will be many more molecules with the  $\mathbf{B}$  vector anywhere in the  $xy$ -plane than molecules where  $\mathbf{B}$  is parallel to the unique  $z$ -axis. This is reflected in the intensity distribution seen in Figure 2.7A. A real EPR spectrum is obtained when the stick spectrum shown in Figure 2.7A is convoluted with a line shape of finite line width, as shown in Figure 2.7B. This reflects the various orientations that a molecule can adopt where the  $\mathbf{B}$  vector lies somewhere between the two extremes of the  $xy$ -plane and the  $z$ -axis.

Standard EPR spectrometers employ field modulation plus phase-sensitive detection in order to increase the sensitivity of the measurement by filtering out non-resonant noise.<sup>118</sup> This results in the EPR absorption pattern being differentiated with respect to the magnetic field (see Section 2.2.4), and it is this which is most often recorded (Figure 2.7C).

Figures 2.7D and 2.7E show the EPR absorption pattern and the equivalent derivative spectrum obtained when there is no axial symmetry. This would be the case if one of the nitrogen atoms of the porphyrin plane in Figure 2.6 were

made inequivalent to the others, so that the molecular  $x$ -axis and  $y$ -axis became inequivalent. In this case, a rhombic spectrum is obtained and  $g_x \neq g_y \neq g_z$ .

### 2.2.3 Multiple Magnetic Interactions

Electronic Zeeman interactions between the external magnetic field  $\mathbf{B}$  and the electron spin  $\mathbf{S}$  (a  $\mathbf{S}^*\mathbf{B}$  interaction) are not necessarily the only interactions which gives rise to features visible in an EPR spectrum. Although not relevant for the work discussed in this thesis, electron spins can also interact with each other, as well as with nuclear spins, and nuclear spins can interact with the applied magnetic field.<sup>117</sup>

Hyperfine interactions are the result of an interaction between an electron spin  $\mathbf{S}$  and a nuclear spin  $\mathbf{I}$  (a  $\mathbf{S}^*\mathbf{I}$  interaction), and they are usually at least one order of magnitude weaker than the electronic Zeeman interaction. These interactions result in ‘perturbations’ of the features which arise from  $\mathbf{S}^*\mathbf{B}$  interactions, with each of these features being split into  $2\mathbf{I}+1$  lines. The magnitude of the splitting,  $\mathbf{A}$ , reflects the strength of the interaction.  $\mathbf{A}$  is an anisotropic quantity and so each spectral feature may be split by a different  $\mathbf{A}$ , giving rise to, for example for a rhombic system,  $\mathbf{A}_x$ ,  $\mathbf{A}_y$  and  $\mathbf{A}_z$ , which correspond to the magnitude of the splitting of the  $g_x$ ,  $g_y$  and  $g_z$  features respectively.

Hyperfine interactions may result from an interaction between the electronic spin and the nuclear spin of the metal ion with which the electron is associated (referred to as central or metal hyperfine splitting) or as a result of the interaction between a coordination sphere ligand nuclei (referred to as ligand hyperfine or superhyperfine splittings). The splittings from the latter are typically an order of magnitude less than those from the central metal ion and those from higher sphere nuclei are usually not resolved.

In systems with multiple unpaired electrons, which give rise to  $\mathbf{S} > 1/2$ , a  $\mathbf{S}^*\mathbf{S}$  interaction may occur, giving rise to a zero-field interaction. Unlike electronic Zeeman interactions, this mutual interaction between unpaired electrons is always

present and is independent of any external magnetic field.

Nuclear Zeeman interactions ( $\mathbf{I}^* \mathbf{B}$  interactions) and spin-spin interactions between nuclei with  $\mathbf{I} > 1/2$  ( $\mathbf{I}^* \mathbf{I}$  interactions also known as quadrupolar interactions) are usually too weak to be detected in standard continuous wave EPR (CW EPR); however, they can be resolved in some pulsed EPR techniques, notably with ENDOR (electron nuclear double resonance) and ESEEM (electron spin echo envelope modulation).

### 2.2.4 Continuous Wave EPR

In a continuous wave EPR (CW EPR) experiment the microwave frequency,  $\nu$ , is held constant whilst the applied magnetic field,  $\mathbf{B}$ , is swept between two values. When the value of  $\mathbf{B}$  satisfies the resonance condition (Equation 2.25) for an electron spin at the particular microwave frequency of the experiment, energy will be absorbed by the sample and a signal will be observed.

The CW EPR signal is recorded as a derivative spectrum as a result of the modulation-amplitude detection method used to greatly increase the sensitivity of the experiment (see Figures 2.7C and 2.7E).<sup>118</sup> This method prevents the microwave-diode, which detects the reflected microwaves from the resonator (the EPR signal), from also detecting noise and interference. To achieve this, modulation coils operating with alternating current (typically at 100 KHz) are attached near the resonator and produce an additional oscillating magnetic field. The microwaves reflected from the cavity are then modulated at a particular frequency which is then selectively filtered out by a phase detector. Although the intensity of the detected EPR signals increases with increasing modulation amplitude, the EPR lineshape can become distorted when the modulation amplitude is too large. To prevent this, the modulation amplitude should be less than one third of the peak-to-peak intrinsic linewidth.

To further increase the sensitivity of the CW EPR experiment a microwave cavity that stores microwave energy very efficiently is used. This efficiency is indicated by a quality factor,  $Q$ . The sensitivity of the CW EPR measurement is directly propor-

tional to  $Q$  and measurements are therefore carried out with  $Q$  as high as possible. When measuring an EPR spectrum, microwave radiation that is not resonant with the electron spins should not be reflected and should not reach the microwave-diode, hence no signal should be seen. To achieve this, the microwave frequency should be adjusted to the resonator frequency,  $\nu_R$ , prior to the measurement. This is called ‘critical coupling’. The resonator bandwidth,  $\Delta\nu_R$ , is inversely proportional to  $Q$ :

$$\Delta\nu_R = \frac{\nu_R}{Q} \quad (2.29)$$

The applied microwave power is another experimental factor which can be used to enhance the signal intensity of an EPR spectrum. Regulated by an attenuator, the signal intensity grows with the square root of the power, up until the point where the microwave power is so high that the sample becomes ‘saturated’. This describes the situation when there is no longer a finite difference in the population levels between the lowest and highest energy state and so no net microwave radiation can be adsorbed.

For an  $S = 1/2$  system, the number of molecules in the two different energy states can be described by the Boltzmann distribution;

$$n_1 = n_0 \exp(-\Delta E/k_B T) \quad (2.30)$$

where  $n_1$  and  $n_0$  are the number of molecules in the highest (spin parallel to  $\mathbf{B}$ ) and lowest (spin anti-parallel to  $\mathbf{B}$ ) energy levels, respectively;  $\Delta E$  is the difference in energy between the two states,  $k_B$  is the Boltzmann constant and  $T$  is the temperature of the sample. When  $T = 0$ , all molecules will be in the ground state and  $n_1 = 0$ . At infinitely high  $T$ ,  $n_1 = n_0$ . As mentioned above, in order to detect an EPR signal there must be a finite difference in the population of the energy levels ( $n_1 < n_0$ ). Upon absorption of microwave radiation, a molecule in the ground state will move to the excited state. Spin-lattice relaxation then enables the spin system to get rid of the excess energy obtained and return to the original state of thermal

equilibrium. The lifetime of the excited state,  $T_1$ , is inherent to the sample and is temperature dependent and will affect the microwave power at which a system becomes saturated. A lower temperature is required to study a system where  $T_1$  is short (the sample relaxes fast) compared one with a longer  $T_1$ .

As dictated by the Heisenberg uncertainty principle, the energy and the time associated with an atomic-scale system cannot be determined jointly with precision:

$$\Delta E \Delta t = \frac{h}{2\pi} \quad (2.31)$$

In terms of EPR this means that if spin-lattice relaxation is infinitely fast, then the excited state lifetime would be zero and the uncertainty in the excited state energy would be maximal. This would lead to an EPR spectrum broadened beyond detection. If, on the other hand, the spin-lattice relaxation rate is slow, then the excited state lifetime is increased and the excited state energy can be determined with more certainty. This will lead to a decrease in the homogenous line width and an increase in signal amplitude. Here the term ‘homogenous linewidth’ indicates that the distribution applies equally well to all molecules in the sample that are chemically identical.<sup>118</sup>

In reality samples of proteins come with a distribution of conformations so even if each molecule in the sample has the same chemical formula, the relative coordinates of a given atom within a protein, with respect to a reference atom within that protein, are not exactly the same for each protein molecule present in the sample. This results in inhomogenous line broadening, a temperature independent process due to the distribution in conformations of molecules in frozen solutions giving rise to slightly different  $g$ -values.<sup>118</sup>

## 2.3 Experimental Methods

### 2.3.1 Protein Film Electrochemistry

#### 2.3.1.1 Working Electrodes

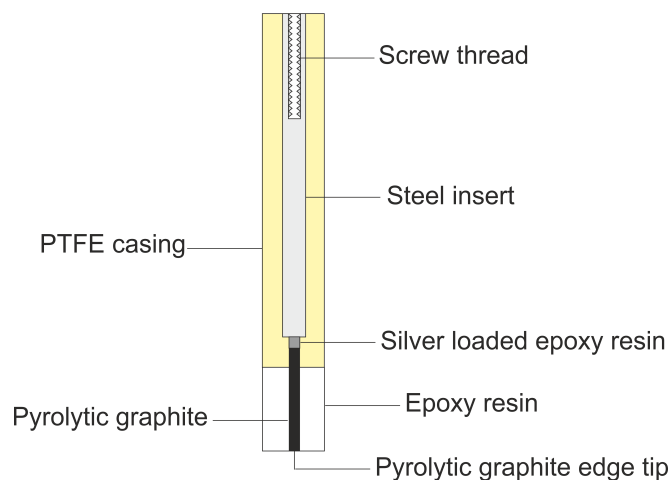
All PFE experiments detailed in this thesis made use of working electrodes made ‘in-house’ through the incorporation of a pyrolytic graphite edge-plane (PGE) tip (geometric surface area of *ca.*  $0.03\text{ cm}^2$ ) into a rotating disc working electrode. ‘Edge-plane’ describes graphite in which the plane perpendicular to the basal (aromatic) layers is exposed. Pyrolytic graphite is chemically inactive over a wide potential range, from *ca.*  $-1$  to  $+0.7\text{ V}$  *vs.* standard hydrogen electrode (SHE), and over a large pH range, making it a suitable electrode material for a wide variety of proteins.

Abrasion of the edge-plane surface creates a rough surface with polar  $-\text{COOH}$  and  $-\text{COH}$  functional groups, as well as hydrophobic regions. Enzyme solution is directly applied to the abraded edge-plane surface, with the rough surface and range of functional groups increasing the likelihood of effective enzyme adsorption.<sup>110</sup>

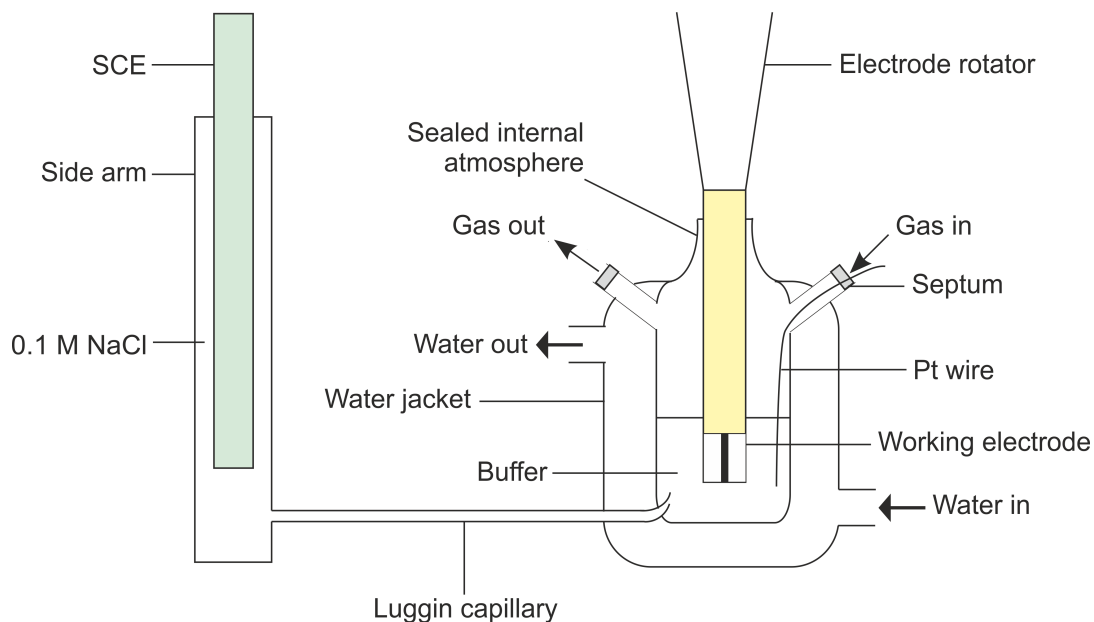
The rotating disc working electrode (RDE) was constructed as shown in Figure 2.8. The screw threaded steel insert, housed within a Teflon (PTFE) casing, allowed the electrode to be screwed onto an electrode rotator (EcoChemie). To ensure a good conductive connection between the steel insert and the pyrolytic graphite tip, silver loaded epoxy resin (RS Electronics) was used to attach the pyrolytic graphite firmly in place. The graphite tip was then surrounded with an insulating epoxy resin (Robnor Resins). Once constructed, the tip of the electrode was sanded with decreasing grade sandpapers to expose a clean PGE surface.

#### 2.3.1.2 Electrochemical Apparatus

All electrochemical experiments described in this thesis were carried out in a glass electrochemical cell constructed in-house by Mrs Terri Adams (ICL Glass Workshop), shown schematically in Figure 2.9.



**Figure 2.8:** A schematic diagram showing the construction of the pyrolytic graphite edge-plane RDEs used in this thesis.



**Figure 2.9:** A schematic diagram of the glass electrochemical cell used in all PFE experiments detailed in this thesis.

The working electrode compartment contained a Pt wire counter electrode, chosen for its resistance to oxidation and corrosion, and was thermostated by means of a water jacket connected to a water circulator (VWR International), allowing the experimental temperature to be precisely controlled. A side arm contained a saturated calomel reference electrode (SCE) in 0.1 M NaCl (Fisher) electrolyte solution. The side arm was connected to the main compartment of the cell *via* a Luggin capillary which prevented mass transport of the electrolyte solution from the side arm into the cell solution. It also enabled the side arm to be separated from the main cell compartment allowing the reference electrode to be maintained at ambient temperature throughout.

All potentials quoted in this thesis have been corrected to give the potential value with respect to SHE using the relation:<sup>111</sup>

$$E_{\text{SHE}}(\text{V}) = E_{\text{SCE}}(\text{V}) + [0.2412 - 6.61 \times 10^{-4}(T - 25) - 1.75 \times 10^{-6}(T - 25)^2 - 9.0 \times 10^{-10}(T - 25)^3] \quad (2.32)$$

where  $T$  is the temperature of the reference electrode environment in degrees Celsius.

A rotating disk working electrode was used in conjunction with an electrode rotator (see Section 2.3.1.1) that fitted snugly into a ground glass joint above the working compartment of the electrochemical cell. Two inlets, both sealed using septa, allowed gases to be flushed through the headspace of the cell *via* inlet and outlet needles. These inlets also enabled liquids to be introduced into the cell solution *via* a syringe.

Due to the  $\text{O}_2$  sensitivity of the enzymes studied in this thesis, experiments were performed in a glove box (M. Braun or Belle Technologies) under an anaerobic atmosphere ( $< 2$  ppm  $\text{O}_2$ ). Cyclic voltammetry and chronoamperometry experiments were carried out using Nova software (EcoChemie) to control an Autolab PGSTAT 10, Autolab PGSTAT128N or Autolab PGSTAT101 electrochemical analyser (all EcoChemie).



### 2.3.1.3 Preparation of Hydrogenase Films

To adsorb protein molecules onto a PGE electrode surface, creating an enzyme ‘film’, the surface was abraded with sandpaper (grade P400, Norton) for about 30 s to provide a clean, rough surface with a range of functionalities (see Section 2.3.1.1). The electrode was then placed in a sonicator for *ca.* 5 s to remove any traces of loose graphite or sandpaper residue before being rinsed with purified water (Millipore: 18 M $\Omega$  cm). The enzyme was applied to the electrode tip by pipetting 1  $\mu$ L of dilute enzyme solution (*ca.* 20  $\mu$ M) onto the PGE surface and leaving it to adsorb for approximately 20 s before using a pipette to remove any excess enzyme not attached to the electrode surface. This last stage is important to ensure that no free enzyme is present in the cell solution complicating the electrochemistry due to slow enzyme diffusion to the electrode.

### 2.3.1.4 Hydrogenase Activation

An aerobic procedure was used to obtain the [NiFe]-hydrogenases Hydrogenase-1 and Hydrogenase-2 from *Escherichia coli* (referred to as *EcHyd1* and *EcHyd2* respectively, see Section 2.3.3 for details on the preparation procedure) meaning that the isolated enzyme was in a mixture of the catalytically inactive Ni-B and Unready states (see Section 1.6). Therefore, it was necessary to ‘activate’ these enzymes prior to an experiment.

For PFE experiments, this was achieved by preparing an enzyme film and then poisoning the electrode at low potential (−560 mV). Every 5 minutes a cyclic voltammogram (scanned between −560 mV and +240 mV at 10 mV s<sup>−1</sup> under 100% H<sub>2</sub>) was completed allowing the activation process to be monitored. This process was continued until two consecutive cyclic voltammograms overlaid, indicating that the enzyme was fully active. For *EcHyd1* full activation took around 90 minutes, whilst for *EcHyd2* this process took approximately 40 minutes.

For Electron Paramagnetic Resonance (EPR) and Attenuated Total Reflectance Infrared spectroscopy (ATR-IR) studies the enzyme was activated whilst in solution

by blowing H<sub>2</sub> gas over the solution surface for a least four hours in the case of *EcHyd1* and for one hour in the case of *EcHyd2*.

### 2.3.1.5 Film Loss

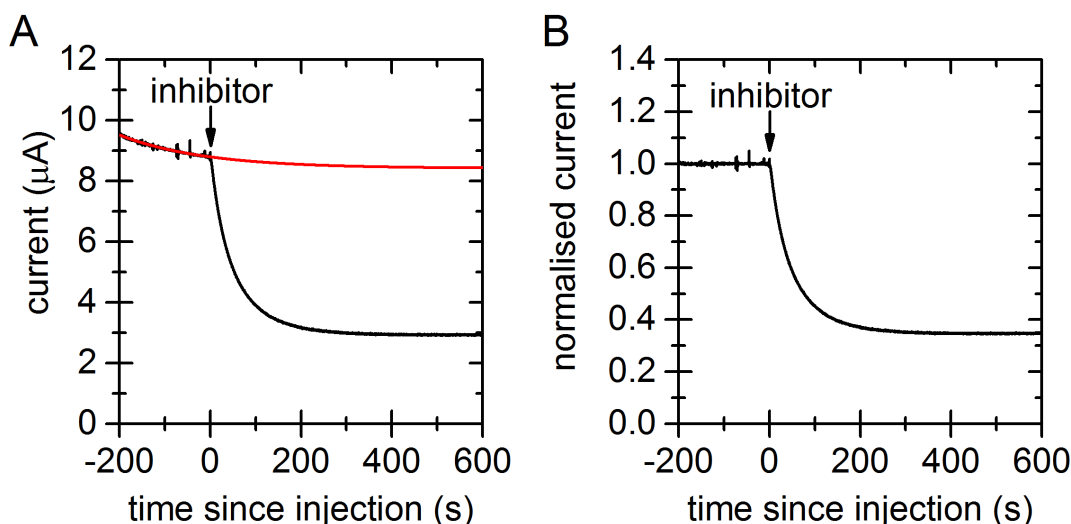
The catalytic current produced by an enzyme modified electrode decreases over time, even when the electrode is held under constant conditions. This decrease in current is known as ‘film loss’ and is considered to be due to either: enzyme molecules dissociating from the electrode surface, enzyme molecules reorientating on the electrode surface causing them to lose electronic contact with the electrode, enzyme molecules denaturing on the electrode surface, or a combination of these events. Often the rate of film loss is enhanced at higher overpotentials and at higher temperatures, as well as through rapidly stepping the potential from one value to another.

The effect of film loss is apparent in chronoamperometry experiments as a slow decrease in the observed baseline current. This decay in current can be fitted to an exponential model like that outlined in Equation 2.33;

$$i_t = (i_{t=0} - i_{t=\infty}) \exp\left(\frac{t_0 - t}{\tau}\right) + i_{t=\infty} \quad (2.33)$$

where  $i_t$  denotes the current at time  $t$ ,  $i_{t=0}$  is the current at time  $t_0$ ,  $i_{t=\infty}$  is the limiting current and  $\tau$  is the decay constant. Data can be fitted to Equation 2.33 using non-linear least-squares regression analysis (for example by using the Solver tool incorporated in Microsoft Excel). Normalisation, by dividing the experimental data by the exponential fit to the baseline current, extrapolating over the time course of the experiment, can then be used to account for film loss (Figure 2.10).

The convolution of potential and time in cyclic voltammetry experiments, together with the influence that potential has on the rate of film loss, means that this phenomenon is harder to account for in cyclic voltammetry data where the affect of film loss is seen as a decrease in the maximum current reached in successive scans. It is possible to account for film loss to some extent by plotting cyclic voltammetry



**Figure 2.10:** The procedure used to normalise chronoamperometry data to account for the effect of film loss in this thesis. (A) The raw data (black line) and the exponential fit (Equation 2.33) to the baseline current, recorded prior to the injection of an inhibitor (red line). (B) The normalised current, obtained by dividing the raw data in Panel A by the exponential fit.

data as a current *vs.* time trace and then fitting an exponential curve as described by Equation 2.33 to the data points obtained at a chosen potential (usually the potential which gives the maximum current is used). The experimental data can then be normalised to account for film loss by dividing the experimental current by the exponential curve similar to the film loss correction of chronoamperometry data.

## 2.3.2 Electron Paramagnetic Resonance

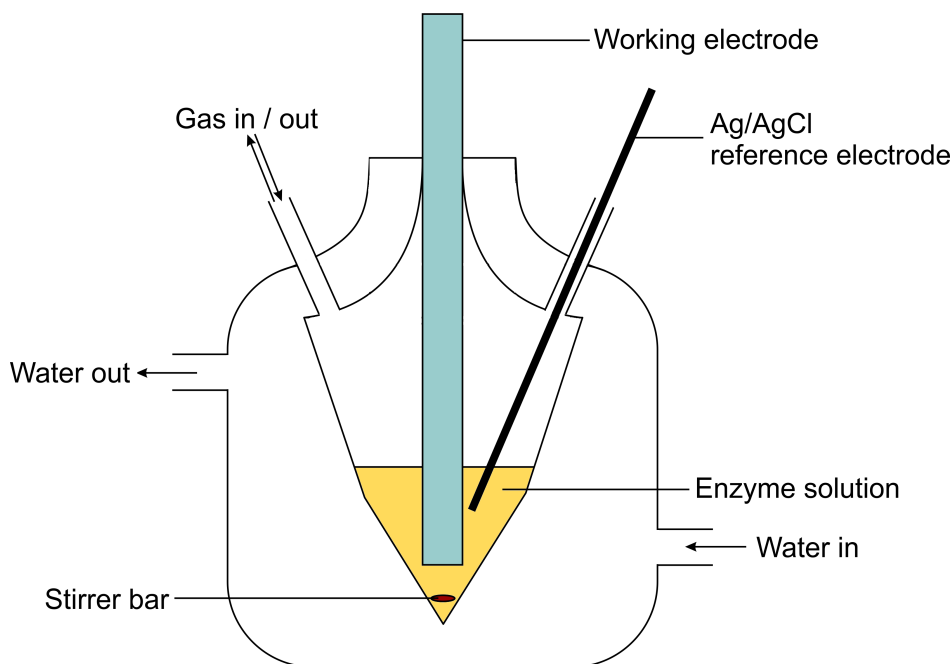
### 2.3.2.1 Preparation of EPR Samples at Defined Potentials

The majority of Electron Paramagnetic Resonance (EPR) samples measured in this thesis were prepared at a defined potential in an attempt to ‘trap’ a particular state of the active site. Unlike in PFE, where the potential is set directly by an electrode, when preparing EPR samples the potential of the bulk enzyme solution is set chemically by the addition of oxidising and reducing agents. Electrodes are still incorporated into the setup, but in this case they are used to measure the solution potential at zero current (a chronopotentiometry technique). Redox-active mediators, chosen to cover the potential range required, can be added to the en-

zyme solution to help communicate the potential of the enzyme/mediator solution to the working electrode. When preparing EPR samples any mediators used must be chosen carefully so that they do not decompose, do not modify the enzyme's redox component, react reversibly with the enzyme as well as the working electrode, and do not interfere with the EPR measurement itself. Ideally, the redox potential of an enzyme solution should be measured at equilibrium, however, for hydrogenase samples this is sometimes not the case. This is due to the difficulty in depriving the enzyme of its substrate ( $\text{H}^+$ ) and so persistent catalytic activity prevents equilibration at low potential. Measurements made in this thesis were taken at higher potentials, sufficient to ensure that the hydrogenase is thermodynamically favoured to carry out the  $\text{H}_2$  oxidation reaction, or even to form the catalytically inactive Ni-B state (see Section 1.6). By preparing hydrogenase samples in the absence of  $\text{H}_2$ , the substrate for the catalytic oxidation reaction, (near) equilibrium conditions can be reached within the potential region in which net  $\text{H}_2$  oxidation would otherwise occur.

Throughout this thesis, EPR samples at defined potentials were prepared using a glass titration cell constructed in-house by Mrs Terri Adams (ICL Glass Workshop), shown schematically in Figure 2.11. The set-up used was based on a method first developed by Dutton,<sup>119</sup> for spectroelectrochemical titrations monitored *via* visible spectroscopy and then later adapted by Cammack<sup>120</sup> for the preparation of EPR samples.

The enzyme solution and redox mediators were placed inside the gas tight cell along with a very small stirrer bar that ensured constant mixing of the enzyme solution. A water jacket allowed precise temperature control. A two-electrode system was used to determine the equilibrium solution potential: The working electrode used was either a Pt wire or the Pt ring of a combination electrode (Mettler Toledo, InLab Redox Micro) and the reference electrode used was a micro Ag/AgCl electrode (WPI, DRIREF-2). The combination electrode was not used as such because to do so a large minimum volume of enzyme solution was needed. Custom made



**Figure 2.11:** A schematic diagram of the titration cell used in this thesis to prepare EPR samples at well-defined potentials. The set-up is based on that first described by Dutton.<sup>119</sup>

inlets allowed both electrodes to be securely fastened into the cell, creating a gas tight seal. The potential was monitored using Nova software (EcoChemie) that controlled an Autolab PGSTAT 10, Autolab PGSTAT128N or Autolab PGSTAT101 electrochemical analyser (all EcoChemie).

Gas flowing into and out of the cell passed through small bubblers (not shown) enabling the gas flow rate through the cell to be monitored. This also increased the humidity of the gas before it entered the cell, limiting the rate of evaporation of the enzyme solution. Stainless steel tubing was used to direct the gases into and out of the cell.

The cell was kept at a positive pressure, allowing EPR samples to be removed from the cell and directly transferred into an EPR tube by removing the gas outlet tube from the outlet gas bubbler and placing it within an EPR tube. The other end of the tube was then briefly dipped into the enzyme solution, the positive pressure of the cell forcing the enzyme solution through the stainless steel tube into the EPR tube. The enzyme solution was then flash frozen in isopentane which had been cooled to cryogenic conditions ( $-160\text{ }^{\circ}\text{C}$ ) using liquid  $\text{N}_2$ .

All potentials quoted for EPR samples in this thesis have been corrected to give the potential value with respect to SHE using the relation;<sup>111</sup>

$$E_{\text{SHE}}(\text{V}) = E_{\text{Ag}/\text{AgCl}}(\text{V}) + 0.195 \quad (2.34)$$

All samples were prepared in a glove box (M. Braun or Belle Technologies) under an anaerobic atmosphere ( $< 2$  ppm  $\text{O}_2$ ).

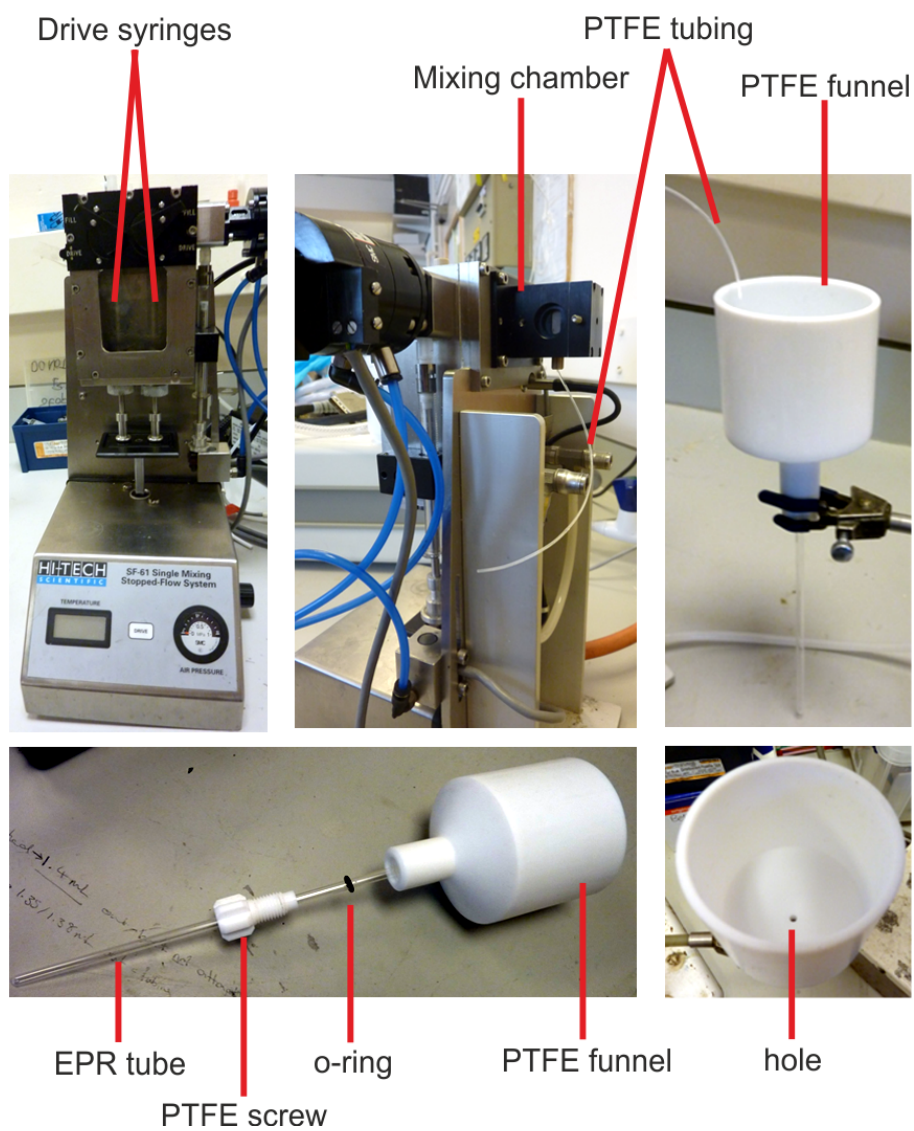
### 2.3.2.2 The Freeze-Quench Setup

In order to detect intermediate species that are formed during a reaction, a freeze-quench technique can be employed. This technique allows reagents to be mixed together and frozen on a sub-second timescale.

A freeze-quench apparatus (Figure 2.12) was made in-house by adapting the sample handling unit from a stopped-flow instrument (Hi-Tech) allowing reagents to be mixed rapidly at room temperature and then instantaneously frozen, quenching the reaction. The entire setup was used inside a glove box (Belle Technologies) enabling samples to be prepared under an anaerobic atmosphere ( $< 2$  ppm  $\text{O}_2$ ).

This setup allows equal amounts of reagents, loaded into two drive syringes, to be mixed very rapidly upon ejection out of the syringes and into a mixing chamber. The mixed solution is immediately directed out of the unit through PTFE tubing and sprayed into a PTFE funnel containing isopentane cooled to cryogenic temperatures ( $-160$  °C). The funnel is specially designed to be attachable to an EPR tube, with a PTFE screw and an o-ring clamping the EPR tube in place when tightened. A needle was used to push the frozen sample through a hole in the bottom of the funnel into the EPR tube. It was imperative that after making a sample the mixing chamber and other ‘dead space’ was cleaned to avoid contamination from one sample to the next.

With this setup, it is not possible to measure the potential of the mixed solution prior to freezing. In this thesis this method was used to make samples at a high potential only, with enough redox mediator ( $\text{K}_3[\text{Fe}(\text{CN})_6]$ ) present to ensure a high



**Figure 2.12:** The apparatus used to make freeze-quench EPR samples.

driving force for Ni-B formation was maintained, and so the precise potential of the sample was of less importance.

### 2.3.2.3 EPR Spectrometer and Resonator

All EPR experiments in this thesis were performed at the CAESR ESR facility in Oxford. All measurements were carried out using an EMX X-band (9-10 GHz) CW EPR spectrometer with an X-band Super High Sensitivity resonator (both Bruker GmbH, Germany) equipped with a helium flow cryostat (Oxford Instruments CF935A) to allow temperature control. Data analysis was performed in MATLAB (Mathworks) using the program EasySpin.<sup>121</sup>

### 2.3.2.4 EPR of Hydrogenases

In order for signals to be recorded EPR measurements of hydrogenases must be made under cryogenic conditions ( $< 120$  K). In the case of [NiFe]-hydrogenases, electron delocalisation of the FeS clusters which make up the electron relay chain within these enzymes means that they relax faster than the Ni species at the active site. At high temperatures ( $> 40$  K) FeS cluster signals are extensively broadened and only signals originating from the Ni ion can be detected. At low temperatures ( $< 60$  K) signals from the Ni species are typically saturated (see Section 2.2.4) and are complicated by other nearby paramagnets, such as the FeS cluster proximal to the active site (see Section 1.4.3). Electron paramagnetic resonance measurements in this thesis deal only with signals originating from the Ni ion at the active site of [NiFe]-hydrogenases and so all measurements have been made at *ca.* 80 K.

## 2.3.3 Enzyme Preparation

The [NiFe]-hydrogenase samples of *EcHyd1* and *EcHyd2* used in this thesis were purified using a previously published protocol<sup>122</sup> with the kind help of Elena Nomerotskaia working within the Armstrong Group. Quoted concentrations of *EcHyd1* and *EcHyd2* were estimated spectroscopically by Elena Nomerotskaia using the Bradford



method.<sup>123</sup>

The [FeFe]-hydrogenase samples of *CaHydA* and *CrHydA1* were prepared by Danuta Krawietz and Julian Esselborn under the supervision of Prof. Thomas Happe at the Ruhr-Universität in Bochum, Germany according to published procedures.<sup>124</sup> Samples of apo-*CrHydA1* and apo-*CpI* used in Chapter 7 were prepared by Julian Esselborn using previously published protocols.<sup>46</sup>

### 2.3.4 Buffer Solutions

All solutions were prepared using deionised water (Millipore: 18 M $\Omega$  cm). Unless otherwise specified, a mixed hydrogenase buffer system was used in all PFE experiments involving *EcHyd1* and *EcHyd2*, which allowed investigations to be made over a wide pH range. This consisted of 15 mM each of MES (2-[*N*-morpholino]-ethane-sulfonic acid, Melford); HEPES (4-[2-hydroxyethyl]-1-piperazine-*N*-2-ethane-sulfonic acid, Fisher); TAPS (*N*-tris(hydroxymethyl) methyl-3-amino-propane-sulfonic acid, Melford); CHES (2-[*N*-cyclohexylamino]ethane-sulfonic acid, Melford); and anhydrous sodium acetate (Analytical grade, Fisher); with 0.1 M NaCl (Analytical grade, Fisher) as the supporting electrolyte. The pH was adjusted by titrating with NaOH and HCl (both AnalaR grade, BDH) as required and measured using a pH meter calibrated at the required temperature.

Experiments with *CaHydA*, *CrHydA1*, apo-*CrHydA1* and apo-*CpI* were conducted using a phosphate buffer. This consisted of 50 mM di-sodium hydrogen orthophosphate (Na<sub>2</sub>HPO<sub>4</sub>, Analytical grade, Fisher) and 50 mM sodium dihydrogen orthophosphate (NaH<sub>2</sub>PO<sub>4</sub>, Analytical grade, Fisher) titrated against each other to the required pH. Again, 0.1 M NaCl (Analytical grade, Fisher) was used as the supporting electrolyte.

All buffers were degassed prior to being taken into the glove box by purging the solution with house N<sub>2</sub> for at least 3 hours. Once in the glove box the buffer solutions were stirred overnight to remove any residual O<sub>2</sub> from solution prior to use.

### 2.3.5 Gas Control

Gas flow into the electrochemical cell was achieved *via* a closed-loop system with all outlets of the electrochemical glass cell being sealed from the atmosphere of the glove box. Water bubblers were used to monitor the gas flow into and out of the cell. High purity gases were used in all experiments detailed in this thesis: H<sub>2</sub> (Premier Plus Grade) and O<sub>2</sub> were purchased from Air Products, N<sub>2</sub> (Oxygen-free) and Ar (Pureshield Argon) were procured from BOC. Precise mixtures of gases were created through the use of mass flow controllers (Sierra Instruments).

## Chapter 3

# Modelling Reversible Surface Electrocatalysis and Distinctions Between the Major Classes of Hydrogenases



## Abstract

The study of enzymes can facilitate our understanding of electrocatalysis by allowing complex processes to be dissected systematically. Composed of specialised parts designed to perform a specific task, enzymes consist of a multi-component assembly with a buried active site that is relatively insensitive to the presence of an electrode surface. This chapter combines variable-temperature protein film electrochemistry with mathematical modelling to highlight important differences between the catalytic performance of [FeFe]-hydrogenases and [NiFe]-hydrogenases, the H-cluster of the [FeFe]-hydrogenases emerging as the superior catalyst.

Experimental catalytic voltammograms provide an electrochemical ‘fingerprint’ of the four hydrogenases studied: the [NiFe]-hydrogenases *EcHyd1* and *EcHyd2*, both from *Escherichia coli* and the [FeFe]-hydrogenases *CaHydA* from *Clostridium acetobutylicum* and *CrHydA1* from *Chlamydomonas reinhardtii*. These results are used to justify a simple model for reversible surface-bound electrocatalysis. The model compares the relative importances of interfacial electron transfer between the enzyme and the electrode *vs.* the inherent catalytic activity of the enzyme (including intramolecular electron transfer, proton transfers, substrate binding and product release) in determining the overpotential requirement, catalytic current, and the catalytic bias (the ability of a redox enzyme to operate more effectively in the oxidative direction over the reductive direction or *vice versa*) of the enzyme in question. The model can be applied equally well to voltammetry obtained from the four-electron reduction of O<sub>2</sub> by the ‘blue’ Cu oxidase, bilirubin oxidase. The results provided a basis for quantifying and explaining the differences between H<sub>2</sub> evolving and H<sub>2</sub> oxidising enzymes and highlight some important considerations for efficient, reversible electrocatalysis.

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The work described in this chapter has been published: Hexter, S. V.; Grey, F.; Happe, T.; Climent, V.; Armstrong, F. A. *Proc. Natl. Acad. Sci.*, **2012**, *109*, (29), 11516–11521.

## 3.1 Introduction

Protein Film Electrochemistry (PFE) has enabled the extensive study of hydrogenases and has yielded detailed information on the reactions which occur at the active sites, particularly those which involve inhibitors. The change in the relative rate of catalysis over a potential region can be observed electrochemically and it is clear that many hydrogenases are able to act reversibly, operating effectively with a minimal overpotential (driving force).<sup>2</sup> Principles accounting for why enzymes attached to electrodes often behave as reversible electrocatalysts include low reorganisation energies for electron transfer, a close match between the reduction potentials of redox-active sites and the substrate, and catalytic sites that stabilise intermediates and allow synchronous proton-coupled electron transfer.<sup>2</sup>

Generally, [FeFe]-hydrogenases are regarded as the more active catalysts.<sup>125</sup> They are usually considered as better H<sub>2</sub> evolvers, whereas [NiFe]-hydrogenases are better H<sub>2</sub> oxidisers;<sup>126</sup> however, until now, no quantitative studies comparing these characteristics, and how they change with potential, have been undertaken. Protein Film Electrochemistry allows a detailed thermodynamic study of the H<sub>2</sub> cycling mechanism of hydrogenases to be achieved. In comparison to spectroscopic techniques it allows unrivaled potential control enabling relatively modest potential dependent changes to the rate of turnover (seen as a change in catalytic current) to be detected.

This chapter describes a study of the temperature and potential dependences of electrocatalytic H<sub>2</sub> cycling by a number of hydrogenases, all from mesophilic microorganisms. One O<sub>2</sub>-tolerant and one O<sub>2</sub>-sensitive [NiFe]-hydrogenase have been studied; the O<sub>2</sub>-tolerant *EcHyd1* and the O<sub>2</sub>-sensitive *EcHyd2* both from *Escherichia coli*. These are membrane bound hydrogenases *in vivo* with a membrane-extrinsic domain, consisting of the large and the small subunits, which projects into the periplasm.<sup>122</sup> The [FeFe]-hydrogenases are *CaHydA* from *Clostridium acetobutylicum* and *CrHydA1* from *Chlamydomonas reinhardtii*, a fermentative anaerobe and a photosynthetic green alga respectively.<sup>127</sup> In all of these hydrogenases, apart from *CrHydA1*, electron transport between the protein surface and active site is

mediated by a [FeS]-cluster relay system (see Sections 1.4.3 and 1.5.3).

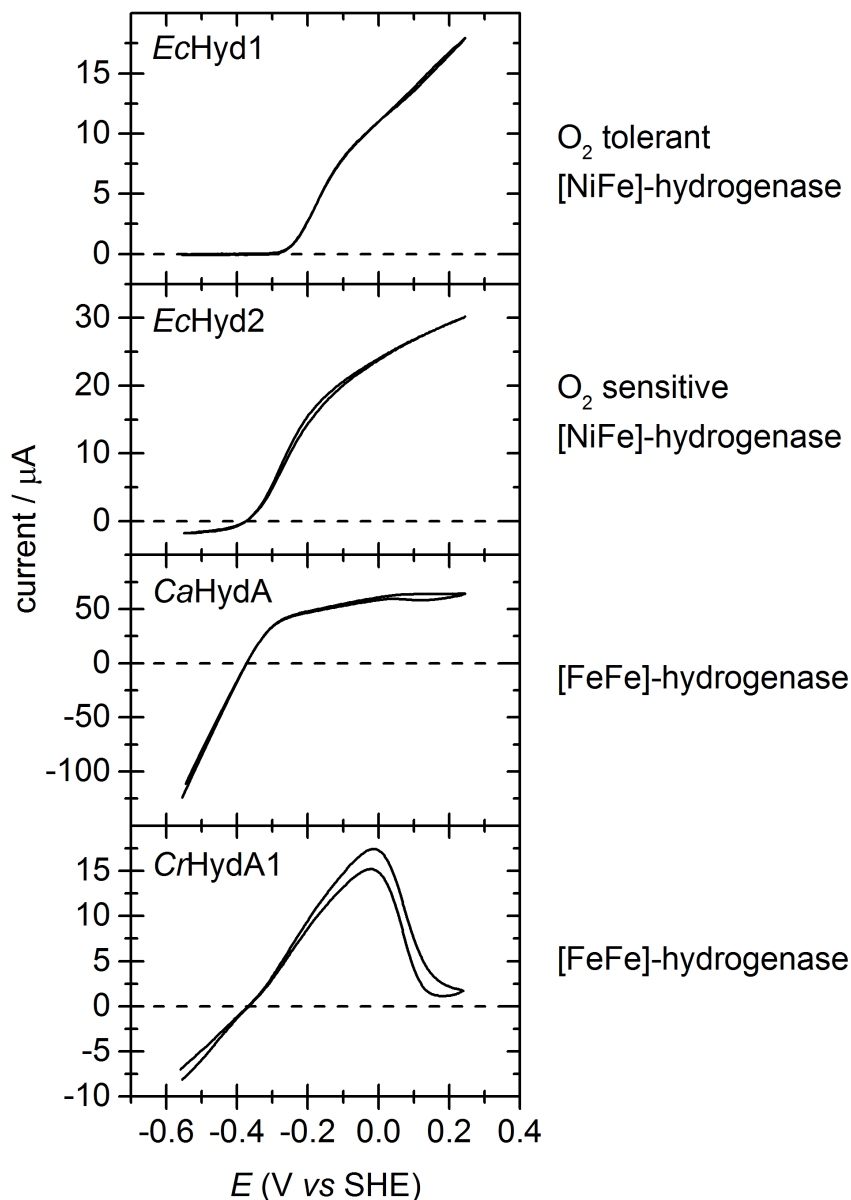
The different voltammetric waveforms obtained when these hydrogenases are adsorbed onto a pyrolytic graphite ‘edge’ (PGE) electrode (Section 2.3.1.1) can be compared and analysed using a mathematical model proposed in this chapter.

## 3.2 The Voltammetric Waveforms Obtained from Surface Immobilised Hydrogenases

Figure 3.1 shows cyclic voltammograms obtained from the four hydrogenases studied in this chapter. All are measured under 100 % H<sub>2</sub> in order to allow comparison.

Apart from *EcHyd1*, all voltammograms show reversible electrocatalysis with the voltammogram intercepting the current axis at the value of  $E_{2\text{H}^+/\text{H}_2}$  for the experimental conditions ( $E_{2\text{H}^+/\text{H}_2} = -0.35$  V at pH 6, 22 °C). The voltammogram of *EcHyd2* shows only a very small amount of H<sub>2</sub> production activity under these conditions reflecting the significant effect of H<sub>2</sub> as a product inhibitor of [NiFe]-hydrogenases.<sup>122</sup> In this case, the reduction current tends towards a limiting value upon scanning negative of  $E_{2\text{H}^+/\text{H}_2}$ , consistent with H<sup>+</sup> reduction becoming controlled by a chemical rather than electrochemical step. Consistent with the observed activity of other O<sub>2</sub>-tolerant hydrogenases, *EcHyd1* shows negligible H<sup>+</sup> reduction activity under these conditions<sup>122,129,130</sup> and an overpotential of approximately 70 mV is required to drive the H<sub>2</sub> oxidation reaction.

The [FeFe]-hydrogenases are good catalysts of both H<sub>2</sub> oxidation and H<sup>+</sup> reduction, and a small overpotential either side of  $E_{2\text{H}^+/\text{H}_2}$  is sufficient to result in a significant increase in the catalytic current. In the case of *CaHydA*, the oxidation current reaches an almost flat limiting value at high, positive overpotentials. This is consistent with H<sub>2</sub> oxidation becoming controlled by a chemical rather than electrochemical step. Importantly, for all hydrogenases shown in Figure 3.1, under 100 % H<sub>2</sub> the oxidation current is independent of a rotation rate above *ca.* 2000 rpm indicating that the currents shown are not limited by mass transport of H<sub>2</sub> to the



**Figure 3.1:** Cyclic voltammograms obtained from different hydrogenases (as indicated in each panel) attached to a PGE electrode. Current indicates direct electrocatalysis of  $\text{H}^+$  reduction/ $\text{H}_2$  oxidation. Conditions: pH 6 calibrated at 30 °C,  $\omega = 2500$  rpm,  $\nu = 5$  mV s $^{-1}$ , 100%  $\text{H}_2$ . Temperature: 22 °C, except for *CrHydA1* where the voltammogram was measured at 18 °C. Adapted from Reference 128. Throughout this chapter the buffer composition used is as specified in Section 2.3.4.



electrode. In the voltammogram obtained from *CrHydA1* a small inflection is observed at potentials slightly above and below  $E_{2\text{H}^+/\text{H}_2}$ , as reported previously.<sup>131</sup> Both [FeFe]-hydrogenases show an increase in  $\text{H}^+$  reduction activity with driving force, consistent with this reaction being controlled by an electrochemical rather than chemical step, even at large overpotentials.

A residual slope is seen, to different extents, in all voltammograms, most prominently in those obtained from the [NiFe]-hydrogenases. This is indicative of a distribution of interfacial electron-transfer rate constants which arise due to the different orientations that enzyme molecules may adopt upon the electrode surface.<sup>115</sup>

The voltammogram of *CrHydA1* shows evidence of an oxidative inactivation process at high potentials. This inactivation is the transformation of enzyme from the active  $\text{H}_{\text{ox}}$  state into the inactive  $\text{H}_{\text{ox}}^{\text{inact}}$  state (see Section 1.8 for a description of the various redox states available to the H-cluster). In the voltammogram of *CaHydA* a very small amount of this inactivation process is observed. A similar oxidative inactivation process also occurs within the [NiFe]-hydrogenases, forming the catalytically inactive Ni-B state (see Section 1.6), although this inactivation is not visible in the voltammograms in Figure 3.1. This transformation is slow especially in the presence of  $\text{H}_2$ , which acts to protect the active site from the formation of the Ni-B state, and therefore is more visible when a voltammogram is carried out at a very slow scan rate, or in the presence of a low level of  $\text{H}_2$ .<sup>122,132</sup> Oxidative inactivation to form the  $\text{H}_{\text{ox}}^{\text{inact}}$  or Ni-B state is usually reversible, however voltammograms often show hysteresis when the interconversions are slow relative to the scan rate.

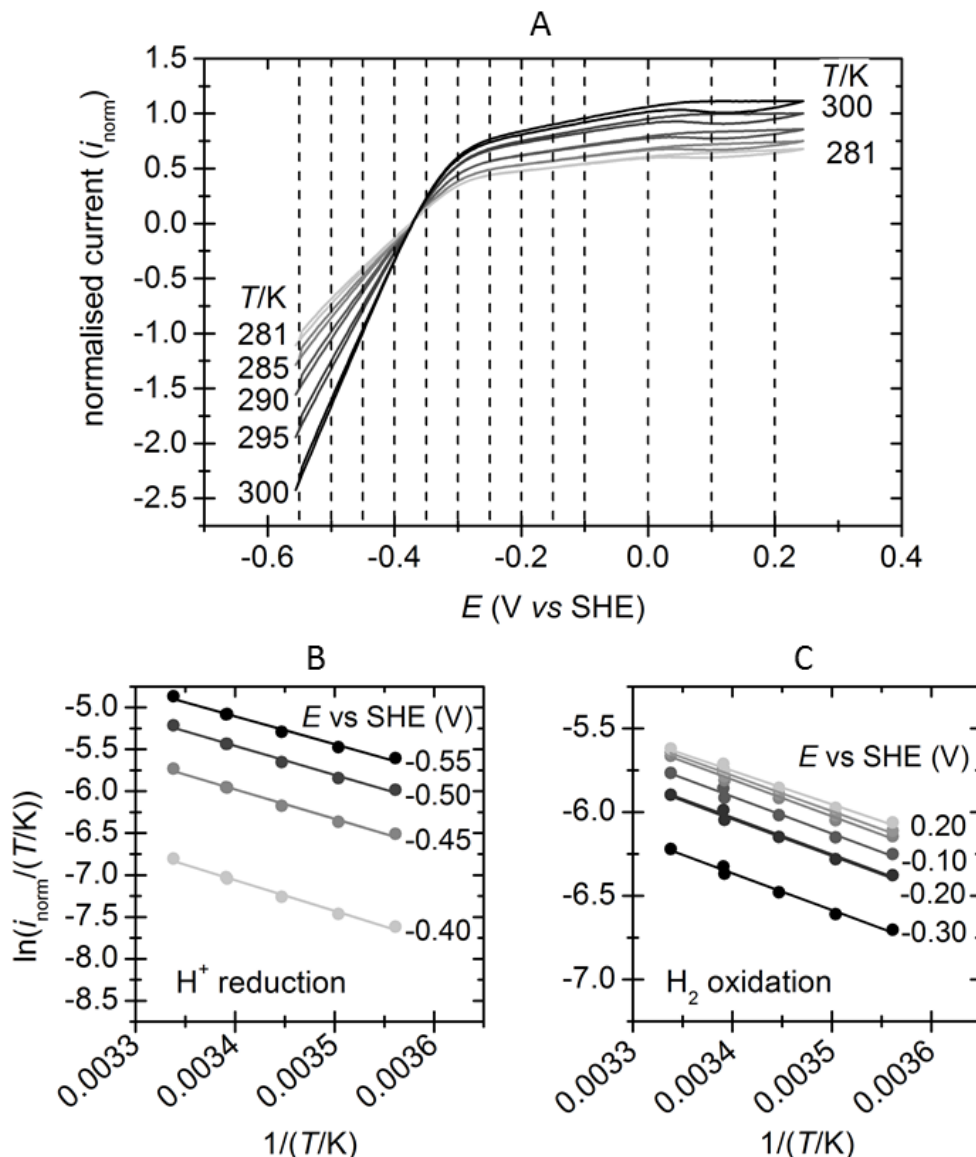
### 3.3 Activation Enthalpies for $\text{H}_2$ Cycling

The voltammetry of a single enzyme film was recorded at different temperatures for each of the hydrogenases shown in Figure 3.1. By measuring the change in current with temperature at a number of potentials an Eyring analysis (see Appendix B) could be used to obtain the potential dependence of the activation enthalpy  $\Delta\text{H}^\ddagger$  for

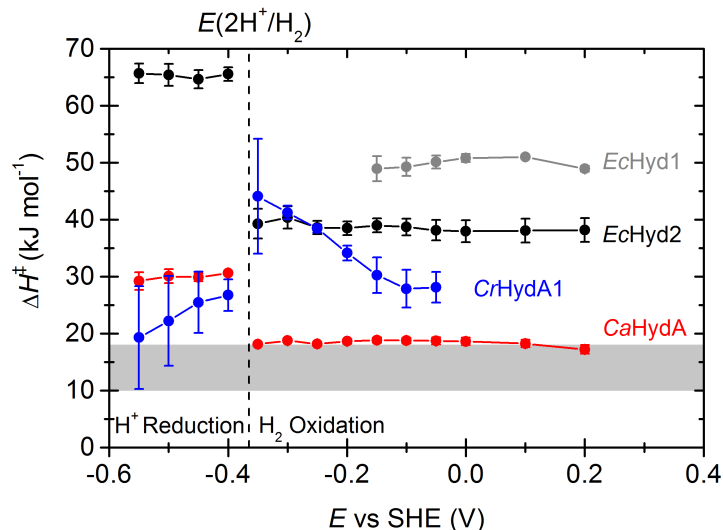
the catalytic reaction. In this way  $\Delta H^\ddagger$  for the  $\text{H}_2$  oxidation reaction and the  $\text{H}^+$  reduction reaction could be compared for each of the hydrogenases studied. This method of analysis is exemplified for *CaHydA* in Figure 3.2.

Figure 3.2A shows a number of cyclic voltammograms obtained at different temperatures from a single film of *CaHydA*. From these data the Eyring plots shown in Figures 3.2B and 3.2C, for the  $\text{H}^+$  reduction and  $\text{H}_2$  oxidation regions respectively, were derived. These give two sets of parallel lines, with gradients that differ between  $\text{H}^+$  reduction and  $\text{H}_2$  oxidation, yielding  $\Delta H^\ddagger$  values of approximately 29 and 19  $\text{kJ mol}^{-1}$  respectively. A summary of the data obtained for all four hydrogenases studied is shown in Figure 3.3. For each enzyme, only potentials at which oxidative inactivation does not occur are included in the analysis.

When considering the data in Figure 3.3, it is important to note that the  $\Delta H^\ddagger$  values almost span the entire range (15–70  $\text{kJ mol}^{-1}$ ) of typical enzyme activation energies,<sup>134</sup> indicating that the differences between the enzymes are substantial. Other than for *CrHydA1*, the  $\Delta H^\ddagger$  values derived from within the  $\text{H}^+$  reduction or  $\text{H}_2$  oxidation potential region for each hydrogenase are almost invariant with potential. This is in contrast with the expected potential dependent behaviour of  $\Delta H^\ddagger$  values for simple electrochemical reactions, where a linear slope of  $\pm\alpha F/RT$  is expected.<sup>111</sup> Although  $\Delta H^\ddagger$  values differ significantly between  $\text{H}^+$  reduction and  $\text{H}_2$  oxidation, the lack of potential dependence of  $\Delta H^\ddagger$  within each region reflects the importance of the chemical steps as opposed to the interfacial electron transfer. However, in the case of *CrHydA1* the  $\Delta H^\ddagger$  values are strongly potential-dependent and decrease as the overpotential is raised in either direction. This is indicative of catalysis being controlled by a potential dependent electron transfer between the enzyme’s active site and the electrode throughout the entire potential region and is fully consistent with the fact that *CrHydA1* is the only hydrogenase in this study to lack an electron relay system to mediate electron transfer to and from the buried active site (see Section 1.5.3). For all enzymes studied other than *CrHydA1*, values of  $\Delta H^\ddagger$  for  $\text{H}^+$  reduction are higher than the equivalent value for  $\text{H}_2$  oxidation, with the  $[\text{FeFe}]$ -



**Figure 3.2:** (A) Cyclic voltammograms of *CaHydA* obtained at different temperatures. All scans were obtained from the same enzyme ‘film’ and the data have been normalised to account for film loss. Vertical dotted lines show the potentials used for the Eyring analysis. The temperature corresponding to each voltammogram is indicated in the panel in kelvin. (B) and (C) show Eyring plots for the  $\text{H}^+$  reduction and  $\text{H}_2$  oxidation potential regions, respectively, using the data from Panel A. Conditions: pH 6 calibrated at 30 °C,  $\omega = 2500$  rpm,  $\nu = 5$  mV s $^{-1}$ , 100%  $\text{H}_2$ . These figures are adapted from Reference 128.

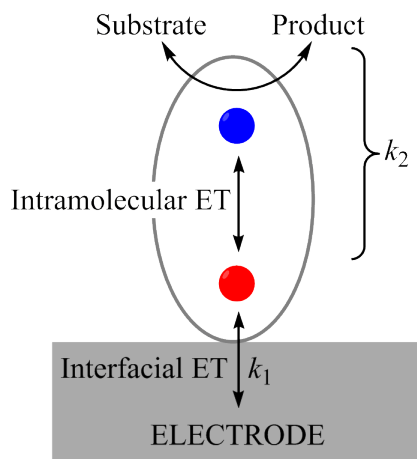


**Figure 3.3:** The apparent activation enthalpies across a range of approximately 0.8 V for four different hydrogenases, as indicated in the figure. The data were obtained from Eyring plots of the voltammetric currents recorded over a range of temperatures. The error bars reflect the variations in the slopes of Eyring plots obtained from different enzyme films. Data from potentials at which oxidative inactivation occurs have not been included. The shaded area depicts the range of  $\Delta H^\ddagger$  values reported for the exchange current at Pt.<sup>133</sup> Adapted from Reference 128.

hydrogenases exhibiting lower values of  $\Delta H^\ddagger$  in both potential regions compared to the [NiFe]-hydrogenases. Under these conditions, *EcHyd1* carries out very little  $H^+$  reduction<sup>130</sup> and so a value for  $\Delta H^\ddagger$  could not be obtained for this enzyme within this potential region. The  $H^+$  reduction capabilities of *EcHyd2* are strongly product inhibited and this may be the reason for the high, potential independent value of  $\Delta H^\ddagger$  found in the  $H^+$  reduction region for this enzyme under 100%  $H_2$ . A lower  $\Delta H^\ddagger$  may be observed under the more physiological condition of low  $H_2$ . The grey area in Figure 3.3 represents the  $\Delta H^\ddagger$  values for the exchange current at Pt, which lie in the region 10–18  $\text{kJ mol}^{-1}$  depending upon the surface structure of the metal.<sup>133</sup> Values obtained from *CaHydA* fall within 1  $\text{kJ mol}^{-1}$  of this range for  $H_2$  oxidation. However, it should be noted that values for Pt were obtained at pH values close to 1 while those reported in this chapter were measured close to pH 6.

### 3.4 Developing a Model for Reversible Surface-Bound Electrocatalysis

A model was developed in order to compare the relative importances of interfacial electron-transfer between the enzyme and the electrode *versus* the inherent catalytic activity of the enzyme in determining catalytic current, overpotential requirement and a bias towards  $\text{H}_2$  oxidation or  $\text{H}^+$  reduction. The model describes reversible electrocatalysis as carried out by an electron transport enzyme immobilised onto an electrode. A pictorial representation of the model is shown in Scheme 3.1.



**Scheme 3.1:** A pictorial representation of interfacial and intramolecular electron transfer (ET) through an enzyme attached to an electrode. Interfacial electron transfer (described by  $k_1$ ) is dependent upon the electrode potential. All catalytic events which occur within the enzyme, such as substrate binding, product release, proton transfers and intramolecular electron transfers, are represented by  $k_2$ . The blue sphere represents the enzyme's catalytic site and the red sphere depicts the electrochemical control centre responsible for the potential determining step. In this chapter it is assumed that the electrochemical control centre is the distal [FeS]-cluster, that is, the [FeS]-cluster furthest from the active site.

The electrocatalytic process is separated into two stages: (*i*) interfacial electron transfer between the electrode and the electron relay centre that acts as the electron entry/exit site and here regarded as the electrochemical control centre responsible for the potential determining step (in the case of hydrogenases this would be the distal [FeS]-cluster, apart from in *CrHydA1* in which electrons would be directly transferred to the [4Fe4S]-cluster at the active site, see Section 1.5.3 for a description

of the monomeric structure of *CrHydA1*) and; (ii) catalytic chemical events within the enzyme. In this way energy input (thermodynamics and electrochemical rates) is separated from the reversible chemical steps of catalysis. The model ignores any potential dependent processes that control the inherent catalytic activity of the enzyme, such as the oxidative inactivation process that leads to the formation of the  $H_{ox}^{inact}$  or Ni-B states (see Chapter 4).

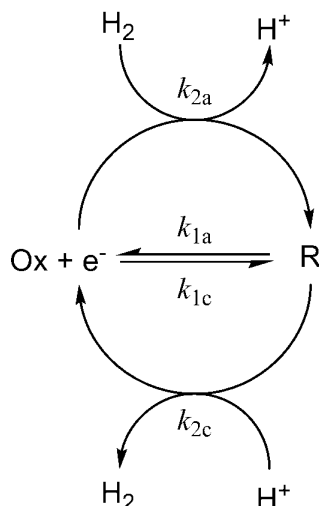
A corresponding mathematical model, describing reversible electrocatalysis, is shown in Scheme 3.2. This model further assumes that (i) the enzyme is adsorbed onto a rotating disc electrode that can be rotated at a fast enough rate to minimise substrate mass transport limitations; (ii) the enzyme is always substrate-saturated and the catalytic rate measured is proportional to the catalytic constant  $k_{cat}$  (the maximum rate of product formation, in other words the turnover frequency) rather than  $k_{cat}/K_M$ , known as the specificity constant<sup>135</sup> ( $K_M$  is known as the Michaelis constant and reflects how well the enzyme and substrate interact); (iii) the catalytic centre adopts a single state that behaves uniformly across the potential range studied; (iv) electrons enter or exit the catalytic cycle by repetitive one-electron transfers, as is realistic for electron-transport enzymes that carry out long-range one-electron tunnelling.

The terms  $k_{1a}$  and  $k_{1c}$  are potential-dependent rate constants that describe the interfacial electron transfer between the electrode and the electrochemical control centre (electron entry/exit site) responsible for the potential-determining step.<sup>136</sup> Assuming Butler-Volmer kinetics,  $k_{1a}$  and  $k_{1c}$  can be described by;

$$k_{1a} = k^0 \exp \left[ \alpha_a \frac{nF}{RT} (E - E_{Ox/R}^0) \right] = k^0 e_1^{1-\alpha_c} \quad (3.1)$$

$$k_{1c} = k^0 \exp \left[ -\alpha_c \frac{nF}{RT} (E - E_{Ox/R}^0) \right] = k^0 e_1^{-\alpha_c} \quad (3.2)$$

where  $k^0$  is the standard rate constant for the electron transfer process,  $\alpha_a$  and  $\alpha_c$  are the respective transfer coefficients (both assumed to be equal to 0.5 throughout this thesis),  $E_{Ox/R}^0$  is the reduction potential of the electrochemical control centre,



**Scheme 3.2:** A representation of a mathematical model for reversible electrocatalysis, adapted from Reference 128. ‘Ox’ and ‘R’ refer to the oxidised and reduced forms of the electrochemical control centre, the site at which electrons enter or exit the enzyme at a rate described by  $k_{1a}$  and  $k_{1c}$ . All other catalytic events which occur within the enzyme are described by  $k_{2a}$  and  $k_{2c}$ .

$n$  represents the number of electrons required for the stoichiometric catalytic reaction (the relay system mediates  $n$  one-electron transfers per catalytic cycle, each at  $E_{\text{Ox/R}}^0$ ),  $R$  and  $F$  are the ideal gas constant and the Faraday constant respectively,  $T$  is the temperature and  $E$  is the potential of the electrode, and:

$$e_1 = \exp \left[ \frac{nF}{RT} (E - E_{\text{Ox/R}}^0) \right] = \frac{k_{1a}}{k_{1c}} \quad (3.3)$$

Thus  $e_1$  represents the driving force provided by the applied potential for interfacial electron transfer.

The rate constants  $k_{2a}$  and  $k_{2c}$  describe the potential-independent substrate turnover frequencies for the oxidation (anodic) and reduction (cathodic) reactions respectively. These rate constants take account of all chemical reactions that follow the potential determining step; intramolecular electron transfer to accumulate within the enzyme the electrons required for the catalytic reaction, proton transfers, substrate binding and product release. The ratio  $k_{2c}/k_{2a}$  describes the catalytic bias of the enzyme, that is, the degree to which a redox enzyme operates more effectively in a particular direction (oxidation or reduction). This ratio, represented in the

model by the parameter  $e_2$ , can be expressed in terms of the difference between the reduction potential of the electrochemical control centre,  $E_{\text{Ox/R}}^0$ , and the equilibrium potential of the substrate,  $E_{\text{eqm}}$ , according to Equation 3.4:

$$e_2 = \frac{k_{2c}}{k_{2a}} = \exp \left[ \frac{-nF}{RT} (E_{\text{Ox/R}}^0 - E_{\text{eqm}}) \right] \quad (3.4)$$

In the case of hydrogenases,  $n = 2$  and the equilibrium potential of the substrate can be described as  $E_{2\text{H}^+/\text{H}_2}$ , which varies with pH and  $\text{H}_2$  partial pressure, so that:

$$e_2 = \frac{k_{2c}}{k_{2a}} = \exp \left[ \frac{-2F}{RT} (E_{\text{Ox/R}}^0 - E_{2\text{H}^+/\text{H}_2}) \right] \quad (3.5)$$

Importantly, the presence of a catalyst cannot change the position of equilibrium for a given reaction. In this model any catalytic bias is determined by the potential of the electrochemical control centre,  $E_{\text{Ox/R}}^0$ , described as the electron entry/exit site, and not the redox properties of the catalytic site itself.

In order for a catalyst to be intrinsically competent it must have a high turnover frequency, hence  $k_{2a}$  and  $k_{2c}$  must be large values. Therefore, the electrocatalytic waveform will be controlled by the electron entry and exit into and out of the catalyst, and the rates and potentials at which this occurs. In other words, the electrocatalytic waveform will be controlled by the rate constants  $k_{1a}$  and  $k_{1c}$ . With this in mind, the net current can be given by;

$$j = nF\Gamma_m[k_{1a}[\text{Red}] - k_{1c}[\text{Ox}]] \quad (3.6)$$

where  $\Gamma_m$  is the total electroactive coverage of the enzyme on the electrode surface and  $[\text{Ox}]$  and  $[\text{Red}]$  are the proportion of enzyme in which the electrochemical control centre is, respectively, oxidised and reduced.

Under steady state conditions the expression for  $j$  reduces to (detailed derivation shown in Appendix C);

$$j = j_{\text{lim}} \frac{e_1 - e_2}{1 + e_1 + pe_1^{\alpha_c}} \quad (3.7)$$



where  $j_{\text{lim}}$  is the limiting current as defined for an oxidative process;

$$j_{\text{lim}} = nF\Gamma_{\text{m}}k_{2\text{a}} \quad (3.8)$$

and  $p$  is a ratio comparing the proficiency of an electrocatalyst for its catalytic function to its proficiency in performing interfacial electron transfer:

$$p = \frac{k_{2\text{a}} + k_{2\text{c}}}{k^0} = \frac{k_{2\text{a}}(1 + e_2)}{k^0} \quad (3.9)$$

If  $j_{\text{lim}}$  is defined as the limiting current for an reductive process, then;

$$j = j_{\text{lim}} \frac{\frac{e_1}{e_2} - 1}{1 + e_1 + pe_1^{\alpha_{\text{c}}}} \quad (3.10)$$

where

$$j_{\text{lim}} = nF\Gamma_{\text{m}}k_{2\text{c}} \quad (3.11)$$

and  $p$  is defined as before.

Finally, the various orientations that a molecule may adopt upon an electrode surface, leading to a dispersion in interfacial electron-transfer rate constants, was taken into consideration by the introduction of an effective tunnelling factor  $\beta d_0$ , where  $\beta$  is a medium-dependent decay constant and  $d_0$  is the distance above closest approach of the donor and acceptor. This allows for a distribution in interfacial electron-transfer rate constants between the limiting values  $k_{\text{min}} = k^0 \exp(-\beta d_0)$  and  $k_{\text{max}} = k^0$ , where  $k^0$  is the standard rate constant for interfacial electron transfer.<sup>115,137</sup> A large value of  $\beta d_0$  represents a greater distribution of interfacial electron-transfer rate constants. Seen *via* voltammetry, this has the effect of reducing the apparent dependence of catalytic current on increasing overpotential, resulting in a residual slope even at high overpotentials. Integrating Equation 3.7 over all possible rate constants (see Appendix C for details of the integration) leads to the final

equation;

$$j = \frac{j_{\text{lim}}}{\beta d_0} \frac{e_1 - e_2}{1 + e_1} \ln \frac{pe_1^{\alpha_c} + (1 + e_1)}{pe_1^{\alpha_c} + (1 + e_1) \exp(-\beta d_0)} \quad (3.12)$$

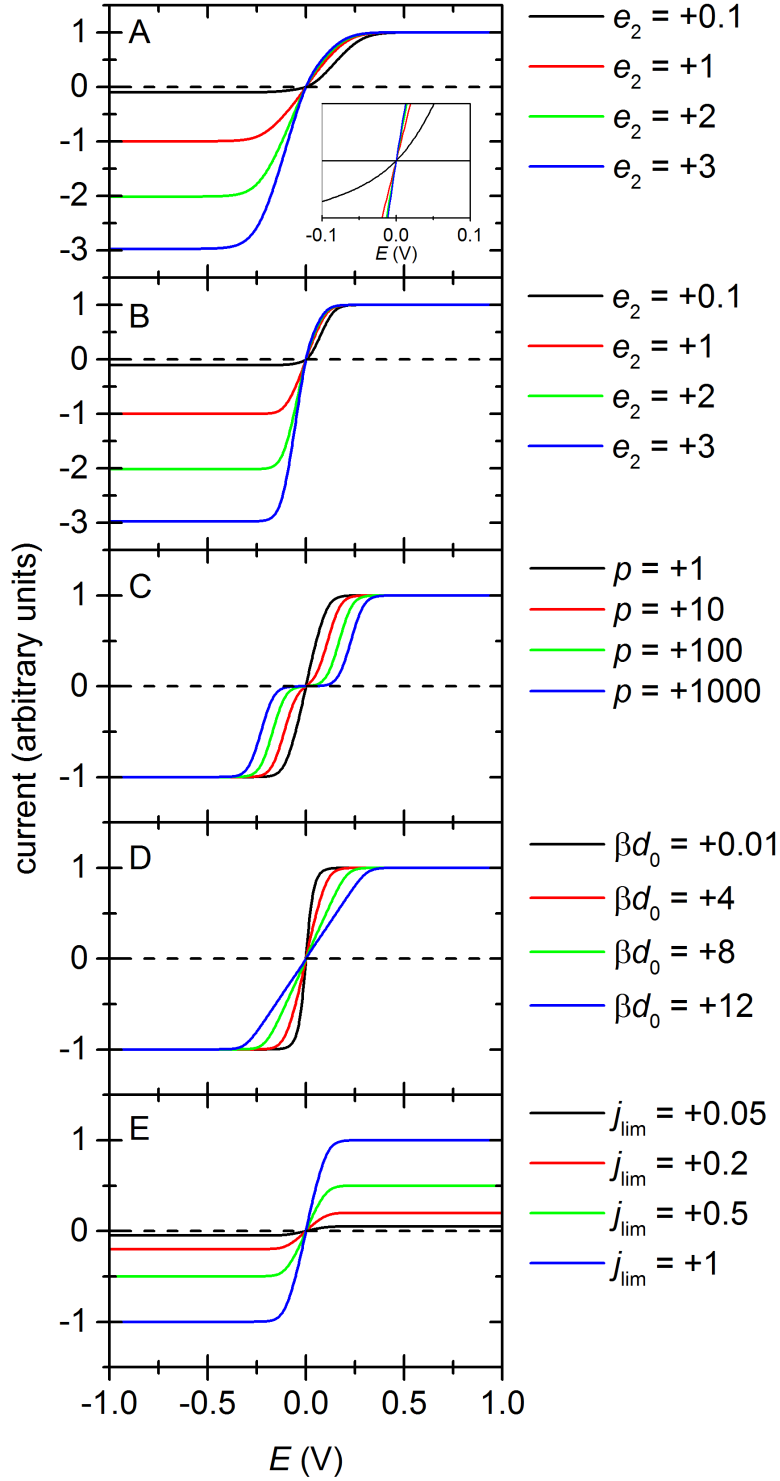
for a system that reaches a limiting current in the oxidative direction (*i.e.* where  $j_{\text{lim}} = nF\Gamma_{\text{m}}k_{2a}$ ). If a limiting current is defined in the reductive region ( $j_{\text{lim}} = nF\Gamma_{\text{m}}k_{2c}$ ) then integration of Equation 3.10 results in:

$$j = \frac{j_{\text{lim}}}{\beta d_0} \frac{\frac{e_1}{e_2} - 1}{1 + e_1} \ln \frac{pe_1^{\alpha_c} + (1 + e_1)}{pe_1^{\alpha_c} + (1 + e_1) \exp(-\beta d_0)} \quad (3.13)$$

In the case of  $\beta = 0$  or  $d_0 = 0$  then Equation 3.12 and Equation 3.13 will not be defined and Equation 3.7 and Equation 3.10 must be used respectively.

The effect of varying the parameters  $E_{\text{Ox/R}}^0$  and  $n$  (both resulting in a change to  $e_1$  and  $e_2$ ),  $p$ ,  $\beta d_0$  and  $j_{\text{lim}}$  on the electrocatalytic waveform, as predicted by Equation 3.12, is simulated in Figure 3.4.

The simulations resemble the characteristic behaviour for an electron transfer reaction occurring at an electrode which is limited by mass transport at high overpotentials. However in this case, where the electrode is rotated at a sufficient enough speed to avoid such conditions, the limiting factor at high overpotentials is in fact the rate of catalytic turnover. Under all conditions, the voltammograms cross the potential axis at  $E_{\text{eqm}}$  as required by thermodynamics. Figure 3.4A shows the effect of varying  $e_2$ . When  $e_2 = 1$ , *i.e.*  $E_{\text{Ox/R}}^0 = E_{\text{eqm}}$ , then the absolute magnitude of the anodic and cathodic currents approach the same limiting values. A value of  $e_2 < 1$  ( $E_{\text{Ox/R}}^0 > E_{\text{eqm}}$ ) indicates a catalytic entity which is more proficient at catalysing the anodic (oxidation) reaction relative to the cathodic (reductive) reaction whilst the opposite is true when  $e_2 > 1$  ( $E_{\text{Ox/R}}^0 < E_{\text{eqm}}$ ). This is demonstrative of the role that  $E_{\text{Ox/R}}^0$  (as defined by the electrochemical control centre) has in defining any catalytic bias opposed to the catalytic centre itself. The parameter  $e_2$  is important for defining the magnitude of the catalytic current but not the overpotential required to transfer electrons into or out of the enzyme. Figure 3.4B in comparison to Figure 3.4A shows the effect on the voltammetry of changing  $n$ , the number of



**Figure 3.4:** Simulated curves from Equation 3.12 with values of: (A)  $e_2$  as indicated, where values of  $e_1$  and  $e_2$  are based on  $n = 1$ . Corresponding values of  $E_{\text{Ox/R}}^0$  are:  $+0.058$  V ( $e_2 = +0.1$ ),  $0$  V ( $e_2 = 1$ ),  $-0.018$  V ( $e_2 = +2$ ) and  $-0.028$  V ( $e_2 = +3$ ). The inset shows an enlarged version of (A) centred on the potential region close to  $E_{\text{eqm}}$ : (B) As for (A) but now values of  $e_1$  and  $e_2$  are based on  $n = 2$ . Corresponding values of  $E_{\text{Ox/R}}^0$  are:  $+0.029$  V ( $e_2 = +0.1$ ),  $0$  V ( $e_2 = 1$ ),  $-0.009$  V ( $e_2 = +2$ ) and  $-0.014$  V ( $e_2 = +3$ ): (C) Different values of  $p$ ; (D)  $\beta d_0$ , and; (E)  $j_{\text{lim}}$  as indicated. Unless stated, all other parameters are:  $E_{\text{eqm}} = 0$  V,  $E_{\text{Ox/R}}^0 = 0$  V,  $n = 2$ ,  $p = +1$ ,  $\beta d_0 = +4$  and  $j_{\text{lim}} = +1$ ,  $T = 298$  K. Adapted from Reference 128.

electrons required for the stoichiometric reaction. A larger value of  $n$  results in a steeper gradient of the observed sigmoidal curve.

Figure 3.4C demonstrates the effect of changing  $p$ . For values of  $p \gg 1$ , the standard rate constant for interfacial electron transfer,  $k_0$ , is small compared to rate constants for the catalytic reaction and significant catalytic current is not observed until the driving force for electron transfer has increased to a sufficient value. When  $p \approx 1$ , the rate of electron transfer is fast and the voltammogram passes cleanly through the current axis, the proportion of electrochemical control centres present in the oxidised or reduced state being governed by the Nernst potential. When  $p \ll 1$  the rate constants for the catalytic reaction are small compared to  $k_0$ . This implies a poor catalyst which, although may be proficient at interfacial electron transfer with the electrode, has a very low turnover frequency. In this case Equation 3.6 is no longer valid and so this scenario will not be considered further in here.

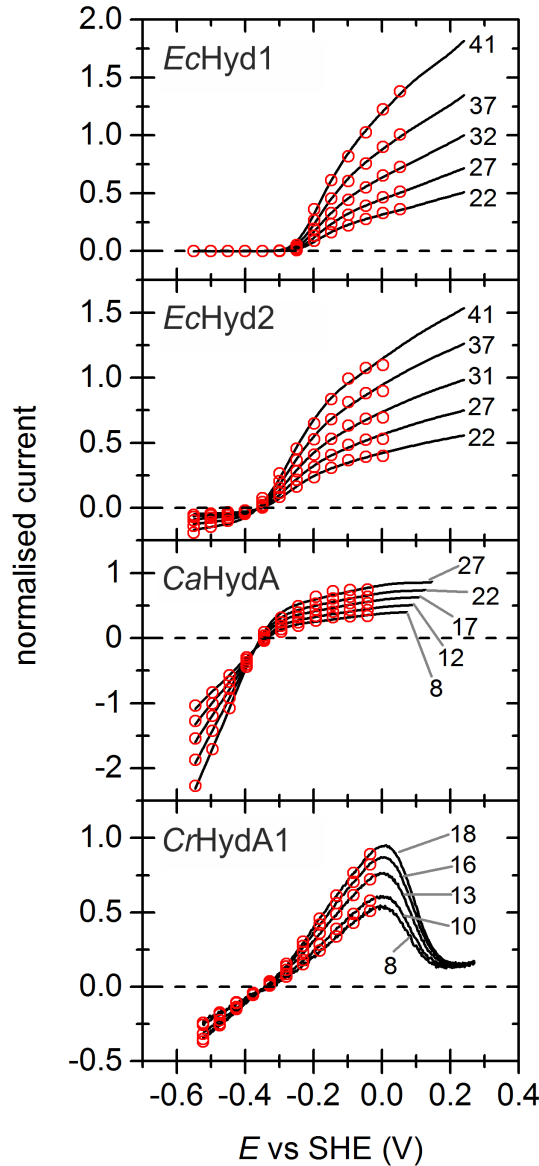
The effect of dispersion is considered in Figure 3.4D, a larger value of  $\beta d_0$  resulting in a more shallow increase in current with increasing overpotential.

Finally, Figure 3.4E displays the effect of varying the parameter  $j_{\text{lim}}$ , the value of which determines the maximum current reached in either the anodic or cathodic direction. In this case,  $j_{\text{lim}} = nF\Gamma_{\text{m}}k_{2\text{a}}$ , and so determines the limiting current in the anodic direction.

## 3.5 Application of the Model to the Voltammetric Waveforms Obtained from Hydrogenases

The above model, expressed by Equation 3.12, can be used to fit voltammograms obtained from a number of different hydrogenases, as shown in Figure 3.5. This figure shows voltammetric currents measured at different temperatures for the four enzymes shown in Figure 3.1. Data obtained from the high potential region of each voltammogram, where anaerobic inactivation occurs, was not included in the fittings as this potential-dependent inactivation process is not accounted for in this simple

model (see Chapter 4 for an extended model which discusses these processes).

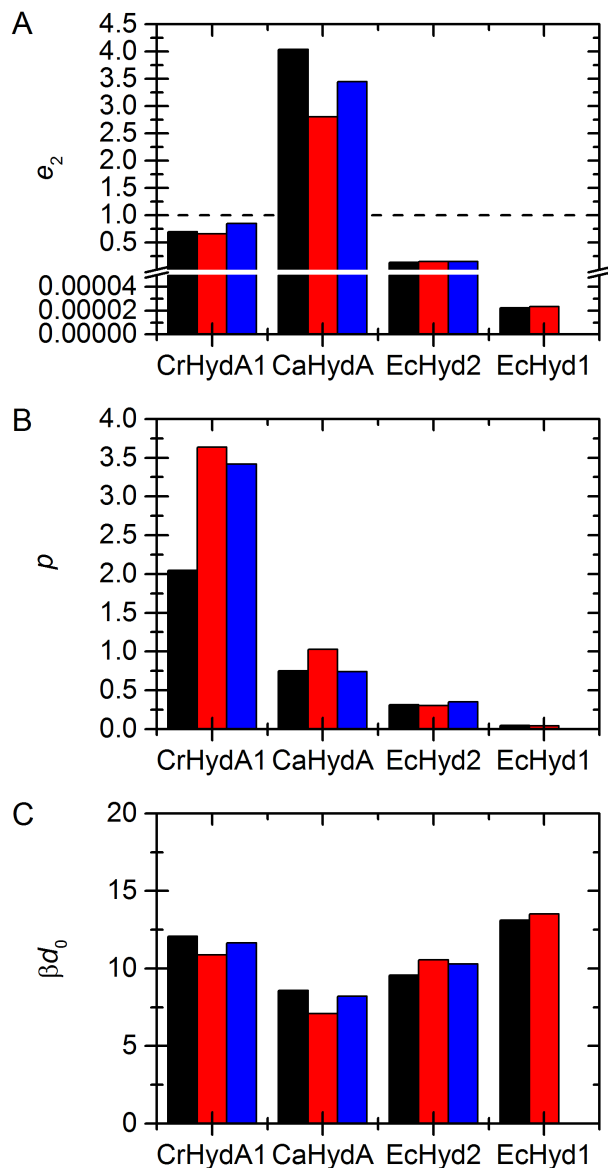


**Figure 3.5:** Voltammograms obtained from the average of the forward and backward sweeps of cyclic voltammograms recorded at different temperatures for four hydrogenases, as indicated in the figure. Red circles show the fit of each voltammogram to Equation 3.12. The numbers alongside each voltammogram refer to the experimental temperature in degrees Celsius. Conditions: pH 6 calibrated at 30 °C,  $\omega = 2500$  rpm,  $\nu = 5$  mV s<sup>-1</sup>, 100% H<sub>2</sub>.

For these fittings, values of  $j_{\text{lim}}$ ,  $p$  and  $E_{\text{Ox/R}}^0$  were allowed to vary with temperature. For each hydrogenase a common value of  $\beta d_0$  was considered for all temperatures.

Within the potential region at which oxidative inactivation does not occur, the intricate shapes of the catalytic voltammograms can be reproduced very well by the

model. The model is also able to describe how these shapes alter with temperature. The good fit of the model to the experimental data enables relative rates to be extracted from the voltammograms as well as quantitative comparisons between the different hydrogenases to be made. Figure 3.6 and Table 3.1 summarise these results.



**Figure 3.6:** Values of the parameters (A)  $e_2$ , (B)  $p$  and (C)  $\beta d_0$  corresponding to the best fits of Equation 3.12 for experiments at 22 °C, except for *CrHydA1* where data was obtained at 18 °C. The dashed line in Panel A represents  $e_2 = 1$ , the case when there is no catalytic bias. Black, red and blue bars represent different experimental repeats.

Figure 3.6A shows the value of the parameter  $e_2$  as calculated, using Equation 3.4, from the value of  $E_{\text{Ox/R}}^0$  found from each fit at 22 °C except for the values

|                | $E_{2\text{H}^+/\text{H}_2}$ | $E_{\text{Ox/R}}^0$ | $e_2$                  |
|----------------|------------------------------|---------------------|------------------------|
| <i>CrHydA1</i> | −0.346 V                     | −0.342 V            | 0.730                  |
| <i>CaHydA</i>  | −0.351 V                     | −0.366 V            | 3.303                  |
| <i>EcHyd2</i>  | −0.351 V                     | −0.327 V            | 0.147                  |
| <i>EcHyd1</i>  | −0.351 V                     | −0.215 V            | $2.206 \times 10^{-5}$ |

**Table 3.1:** The average value of  $E_{\text{Ox/R}}^0$  found from the fits of Equation 3.12 to voltammograms obtained from each of the hydrogenases studied and the corresponding values of  $e_2$  found using Equation 3.4. Data was obtained from the fits at 22 °C apart from for *CrHydA1* where data was obtained at 18 °C. The values of  $E_{2\text{H}^+/\text{H}_2}$  quoted are as found from the Nernst equation at pH 6, 22 °C for *CaHydA*, *EcHyd2* and *EcHyd1* and at pH 6, 18 °C for *CrHydA1*.

corresponding to *CrHydA1* for which data at 18 °C was used. Black, red and blue bars in Figure 3.6 represent different experimental repeats, three repeats were carried out for all enzymes, except for *EcHyd1* for which only two repeats were completed. Table 3.1 shows the average value of  $E_{\text{Ox/R}}^0$  found for each enzyme, the value of  $E_{2\text{H}^+/\text{H}_2}$  as found from the Nernst equation at the experimental conditions, and the corresponding value of  $e_2$  found from Equation 3.4. As outlined previously, a value of  $e_2 < 1$  represents a bias towards  $\text{H}_2$  oxidation, whereas a value of  $e_2 > 1$  represents a bias towards  $\text{H}^+$  reduction. The low value of  $e_2$  for *EcHyd2* reflects the poor  $\text{H}^+$  reduction ability of this enzyme. *EcHyd1* is an extreme case in which an average value for  $e_2$  in the region of  $2 \times 10^{-5}$  represents a catalyst barely able to produce  $\text{H}_2$  under these conditions.<sup>130</sup> On the other hand, the relatively high average value of  $e_2$  found for *CaHydA* represents the catalytic bias that this enzyme has for performing the  $\text{H}^+$  reduction reaction.

The ratio of the net rate of the chemical steps to the standard interfacial electron-transfer rate ( $[k_{2a}+k_{2c}]/k^0$ ) is given by the parameter  $p$  (Equation 3.9). A comparison of the values found for the parameter  $p$ , as shown in Figure 3.6B, makes it clear that a significantly higher value of  $p$  is found for *CrHydA1* than for the other enzymes studied. The value of the parameter  $p$  found from the best fits to data

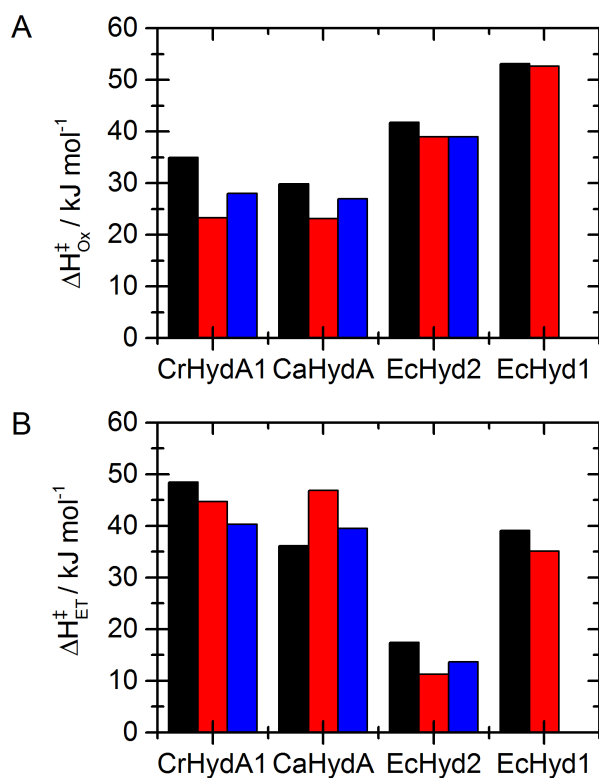
of *EcHyd1* is particularly small, with values of  $p = 0.0447$  and  $p = 0.0388$  being obtained.

Values of  $\beta d_0$  vary between *ca.* 7 for *CaHydA* to *ca.* 13.5 for *EcHyd1*. Considering a typical value of  $\beta$  around  $10\text{-}12\text{ nm}^{-1}$ , this implies that distances between the electrode surface and the electron entry/exit point of the protein ( $d_0$ ) range between 0.6 and 1.2 nm depending upon the enzyme.

A lack of knowledge regarding the electroactive coverage of enzyme on the electrode surface ( $\Gamma_m$ ) means that explicit values for  $k^0$  and  $k_{2a}$  cannot be obtained. In principle, the electroactive enzyme coverage could be obtained by integrating the current peaks recorded through cyclic voltammetry during non-turnover conditions (see Section 2.1.3.5); however, the low enzyme coverage and the relatively large double layer capacity of the PGE electrode surface mean that this is very hard to achieve. Instead, a maximum value of  $1\text{ pmol cm}^{-2}$  for the density of enzyme upon the surface of the electrode was adopted based on the assumption that a higher coverage would render the enzyme visible under non-turnover conditions.<sup>110</sup> Assuming an electrode area of  $0.03\text{ cm}^2$ <sup>110</sup> this gives a value of  $\Gamma_m$  of  $0.03\text{ pmol}$ . Using this assumption, the variation of  $k_{2a}$  with temperature could be found for each enzyme from the temperature dependence of  $j_{\text{lim}}$  (Equation 3.8). Using the Eyring equation, the values of  $k_{2a}$  were then used to find the activation enthalpy for the  $\text{H}_2$  oxidation reaction at  $E_{2\text{H}^+/\text{H}_2}$ ,  $\Delta H_{\text{Ox}}^\ddagger$ , as predicted from the fits of Equation 3.12 to the experimental data. Activation enthalpies for the electrochemical step at  $E_{2\text{H}^+/\text{H}_2}$ ,  $\Delta H_{\text{ET}}^\ddagger$ , were found in a similar way from the values of  $k_0$  obtained at different temperatures for each enzyme, using the values of the parameters  $p$ ,  $k_{2a}$  and  $e_2$  (found from the parameter  $E_{\text{Ox/R}}^0$ ) along with Equation 3.9. A summary of these results is shown in Figure 3.7.

The values obtained for  $\Delta H_{\text{Ox}}^\ddagger$  are highest for *EcHyd1* and lowest for the [FeFe]-hydrogenases, *CrHydA1* and *CaHydA*. The activation enthalpies for the interfacial electron-transfer step,  $\Delta H_{\text{ET}}^\ddagger$ , show no clear trend with *CrHydA1*, *CaHydA* and *EcHyd1* all giving values of approximately  $40\text{ kJ mol}^{-1}$ . *EcHyd2* has a much lower





**Figure 3.7:** Values of (A)  $\Delta H_{\text{Ox}}^\ddagger$  and (B)  $\Delta H_{\text{ET}}^\ddagger$  found from the temperature dependence of the values of  $j_{\text{lim}}$ ,  $k_{2a}$ ,  $E_{\text{Ox/R}}^0$ ,  $e_2$  and  $p$  corresponding to the best fits of Equation 3.12 to the experimental data. See Appendix D for the specific values of  $j_{\text{lim}}$ ,  $k_{2a}$ ,  $E_{2\text{H}^+/\text{H}_2}$ ,  $E_{\text{Ox/R}}^0$ ,  $e_2$ ,  $p$  and  $k_0$  used in the calculation. Black, red and blue bars represent different experimental repeats.

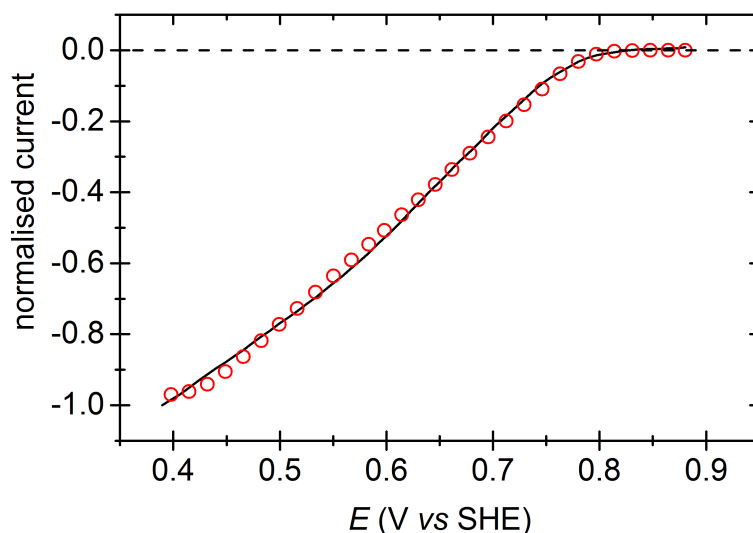
value of *ca.* 15 kJ mol<sup>-1</sup>.

### 3.6 Application of the Model to Other Enzymes

In order to test the model more fully, it was also applied to voltammetry data obtained from the reduction of O<sub>2</sub> by the ‘blue’ Cu oxidase, bilirubin oxidase. Voltammetry obtained from this enzyme shows an onset potential for the catalytic wave that corresponds to the potential of the ‘blue Cu’ centre - this being the natural electron exit site for this enzyme. When the environment of this centre is modified by genetic engineering, the onset potential shifts dramatically.<sup>138</sup> Within the realms of the model, the blue Cu centre is the electrochemical control centre and will dictate the value of  $E_{\text{Ox/R}}^0$ .

Figure 3.8 shows a voltammogram for electrocatalytic four-electron reduction of O<sub>2</sub> by bilirubin oxidase immobilised onto a PGE electrode. In the case of this irreversible electrocatalyst, the limiting current is reached at high overpotentials in the cathodic direction, and so  $j_{\text{lim}}$  should be defined in terms of  $k_{2c}$  (Equation 3.11). It follows that Equation 3.13 can now be used to model the data, the result of which is shown as red circles in Figure 3.8.

The model can be used to obtain a good fit to voltammetry data obtained from the four-electron reduction of O<sub>2</sub> by bilirubin oxidase using the theoretical value of  $E_{\text{eqm}}$  (+0.960 V) to predict a value of +0.828 V for the potential of the electrochemical control centre, in this case the potential of the ‘blue Cu’ centre. This is in agreement with the known high potential values of the blue Cu centres in bilirubin oxidase and the related enzymes fungal laccases, which fall within the range of +0.6 to +0.8 V. The value of  $E_{\text{Ox/R}}^0 = +0.828$  V obtained from the fit gives a value of  $e_2 = 3.404 \times 10^7$ , fully consistent with O<sub>2</sub> reduction by bilirubin oxidase being irreversible under the conditions of the experiment.



**Figure 3.8:** Catalytic voltammogram showing  $O_2$  reduction by bilirubin oxidase immobilised onto a PGE electrode. The voltammogram has been digitally reproduced from Reference 139. The data shown represent the average of the forward and reverse potential sweeps. The average current was then normalised so that the maximum reductive current is equal to  $-1$  (line). The fit to Equation 3.13 is shown as red circles. For the fit the parameters  $E_{eqm}$  and  $n$  were fixed at their theoretical values of  $+0.960$  V and  $4$  respectively. Other parameters obtained from the fit are as follows:  $E_{Ox/R}^0 = +0.828$  V (giving  $e_2 = 3.4039186 \times 10^7$ ),  $p = 35.57033$ ,  $\beta d_0 = 30.69564$  and  $j_{lim} = 0.97273$ . Other conditions:  $0.1$  M sodium phosphate buffer solution at pH  $5$ ,  $0^\circ\text{C}$ ,  $\omega = 2500$  rpm,  $\nu = 5$  mV s $^{-1}$ ,  $1$  bar  $O_2$ .

### 3.7 Conclusions and Perspectives

The results presented in this chapter highlight important differences between the catalytic performance of [NiFe]-hydrogenases and [FeFe]-hydrogenases, using both experimental data and a simple model.

In all hydrogenases studied, apart from *CrHydA1*, catalysis seems to be limited by a chemical, rather than electronic, step as indicated by the lack of a potential effect on the values obtained for  $\Delta H^\ddagger$  (Figure 3.3). The decrease in  $\Delta H^\ddagger$  with driving force observed for *CrHydA1* for both the  $H_2$  oxidation and  $H^+$  reduction reaction follows the behaviour expected for a simple electrochemical reaction as predicted by the Tafel equation. Although this is in agreement with *CrHydA1* being the only hydrogenase in this study to lack an electron relay system, in its physiological role in *Chlamydomonas reinhardtii*, the H-cluster of *CrHydA1* exchanges electrons directly with a [2Fe-2S]-ferredoxin that docks specifically with the enzyme. We cannot be certain that the differences that are seen between the hydrogenases *in vitro* are the same *in vivo*.

The catalytic voltammograms for each of the four hydrogenases (Figure 3.1) which can be seen as the electrochemical ‘fingerprint’ of each enzyme, can be reproduced very well by the simple model proposed in this chapter (Figure 3.5). The parameters obtained from the fits are rich in information but it is important to establish which aspects provide the most realistic insight.

Figure 3.6A compares the catalytic bias of each hydrogenase which, according to the model, is determined by the reduction potential of the electrochemical control centre (opposed to the catalytic centre itself) and represented by the parameter  $e_2$ . The values of  $e_2$  produced from the fits to the data obtained from *CrHydA1* are close to 1, indicating only a very small amount of bias, in this case towards the  $H_2$  oxidation reaction. This may be due to the lack of a separate electron relay system, distinct from the H-cluster, which is able to bias the catalyst towards one direction. In the case of *CrHydA1*, the H-cluster can be seen as a combination of a [4Fe-4S] centre used for electron entry, exit and storage (as two electrons are required per

turnover) which in this case acts as the electrochemical control centre, and a 2Fe centre which acts as the catalytic site. The average value of  $e_2$  was found to be 0.730 for *CrHydA1* suggesting that the H-cluster may have a small inherent bias towards catalysis of the  $H_2$  oxidation reaction. The bias portrayed in Figure 3.6A otherwise show that [FeFe]-hydrogenases are less biased towards  $H_2$  oxidation than [NiFe]-hydrogenases, and *CaHydA* is clearly strongly biased towards  $H^+$  reduction as shown by a value of  $e_2 > 1$ . It is important to note that these results, obtained under an atmosphere of 100%  $H_2$ , underestimate the bias that would be observed under the more physiological conditions of low  $H_2$ : This is because compared to [NiFe]-hydrogenases, [FeFe]-hydrogenases have larger values of  $K_M$  for  $H_2$  oxidation, whilst [NiFe]-hydrogenases have much lower values of  $K_I$  for the product inhibition of  $H^+$  reduction.

The model predicts the following values for the potentials of the electrochemical control centres ( $E_{Ox/R}^0$ ) of the hydrogenases studied, which should correspond to the reduction potentials of the distal FeS clusters in these enzymes: *CaHydA*,  $E_{Ox/R}^0 = -0.366$  V, 14 mV more negative than  $E_{2H^+/H_2}$  at pH 6 ( $-0.351$  V); *EcHyd2*,  $E_{Ox/R}^0 = -0.327$  V, 24 mV less negative than  $E_{2H^+/H_2}$  at pH 6; and *EcHyd1*,  $E_{Ox/R}^0 = -0.215$  V, 136 mV less negative than  $E_{2H^+/H_2}$  at pH 6. This large, positive displacement of  $E_{Ox/R}^0$  relative to  $E_{2H^+/H_2}$  for *EcHyd1* is in full agreement with experimental evidence suggesting that at pH 6 this enzyme is not able to evolve  $H_2$  and requires an overpotential in order to oxidise  $H_2$  because the potential of the distal FeS cluster is far higher than the potential of  $E_{2H^+/H_2}$  under these conditions.<sup>130</sup> In a paper by Murphy *et al.*<sup>130</sup> it was shown that under different pH conditions the potential of  $E_{Ox/R}^0$  in *EcHyd1* does not change in line with  $E_{2H^+/H_2}$ . This results in a hydrogenase which, although at pH 6 is heavily biased towards  $H_2$  oxidation, at pH 3 it is able to reversibly reduce  $H^+$  with only a minimal overpotential. In the case of *CrHydA1*,  $E_{Ox/R}^0 = -0.342$  V, a value only 4 mV less negative than the value of  $E_{2H^+/H_2}$ . In this case the value of  $E_{Ox/R}^0$  is reflective of the reduction potential of the [4Fe-4S] subunit at which electrons enter and leave the H-cluster.

Figure 3.6B compares the relative magnitudes of the chemical rate constants ( $k_{2a}$  and  $k_{2c}$ ) to the standard interfacial electron transfer rate constant ( $k^0$ ), a relationship which is expressed in the model as the parameter  $p$ . The values found for this ratio for the [FeFe]-hydrogenases are higher than those for the [NiFe]-hydrogenases. Taking account of the diversity of the protein structures for these two [FeFe]-hydrogenases an interpretation of this result is that the H-cluster is an inherently superior catalyst compared to the [NiFe]-hydrogenase active site. However, this interpretation excludes the observation that the potential dependence of  $\Delta H^\ddagger$  seen for *CrHydA1* indicates that this enzyme has a relatively small value of  $k^0$ . It may be this factor which accounts for the much larger value of  $p$  for this enzyme compared to all others studied.

The values of  $\Delta H_{Ox}^\ddagger$  found from the model (Figure 3.7A) closely resemble those found experimentally (Figure 3.3), apart from for *CrHydA1* where the variation in experimental  $\Delta H^\ddagger$  means useful comparisons are difficult to make.

Finally, Figure 3.8 exemplifies the generality of this model. The same principles which apply to a model designed to reproduce the electrochemistry of hydrogenases immobilised onto an electrode surface can be used to reproduce the four-electron reduction of  $O_2$  by bilirubin oxidase.

In summary, this simple model reproduces key features of the catalytic voltammograms of different enzymes. The model helps to clarify why many enzymes are efficient, reversible electrocatalysts, but also why some, which contain the same active site, are not. The catalytic centre must convert, with high efficiency, the electrochemical free energy that is provided by the relay centre that is undergoing interfacial electron transfer. In order for a catalyst to carry out efficient, reversible catalysis then the potential of the relay centre undergoing interfacial electron transfer and the catalytic site undergoing substrate conversion must be well matched.

## Chapter 4

# A Unified Model for Surface Electrocatalysis Based on Observations with Enzymes





## Abstract

This chapter extends the model developed in Chapter 3 for electrocatalysis by surface confined enzymes. A central assertion in the study of catalysis is that, for a given driving force, a true catalyst has no influence on the direction of the reaction. Consequently, a catalytic bias (the preference of a catalyst to work in a certain direction) must arise from secondary effects. In the model outlined in Chapter 3 it was the potential of an electrochemical control centre, the site at which electrons enter or leave the enzyme, that determined a catalytic bias. In this chapter the model is extended to consider a catalyst which undergoes potential-dependent changes in its inherent catalytic ability. These changes, resulting in different states of the catalyst, may be due to potential-dependent changes in its composition or arrangement of atoms. This extension of the model allows the oxidative inactivation that is observed at high potential in voltammograms obtained from many hydrogenases, and which was disregarded in Chapter 3, to now be considered.

The model, that is not only applicable to hydrogenases, can be used to describe many potential-dependent events including inhibitor binding (in this chapter exemplified for cyanate inhibition of carbon monoxide dehydrogenase) or product inhibition of a specific redox state which dominates within a certain potential window. Finally, the model is adapted to consider metal surface catalysts allowing a comparison to be made between the oxidative inactivation of hydrogenases and the passivation of metal surfaces (in this case a single crystal Ru(0001) surface) that occurs at oxidising potentials.

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## 4.1 Introduction

In Chapter 3 the complication of oxidative inactivation, observed in the high potential region of voltammograms obtained from many hydrogenases, was not regarded during the fitting of experimental data to the proposed model. The model assumed that the absolute activity of the catalytic centre was not dependent upon the potential applied. However, there are a number of known instances where this is not the case. The ability of a catalytic site to bind a substrate or an inhibitor may depend upon it being in a particular redox state that prevails only under certain electrode potentials. In the case of [NiFe]-hydrogenases, the formation of the Ni-B state at oxidising potentials is an example of potential dependent inhibition and can be directly linked to a known structural change at the active site involving the bridging of  $\text{OH}^-$  across the bi-metallic centre when the Ni ion is oxidised to  $\text{Ni}^{3+}$  (see Section 1.6).<sup>24,26,68</sup> There are a number of examples of potential dependent inhibition of both [FeFe]-hydrogenases and [NiFe]-hydrogenases; the complementary potential dependence of the inhibition of [FeFe]-hydrogenases by CO and HCHO has previously been attributed to the change in the prevalency of the  $\text{H}_{\text{ox}}$ ,  $\text{H}_{\text{red}}$  and  $\text{H}_{\text{sred}}$  states with potential,<sup>84</sup> whilst the inhibitory effect of both  $\text{O}_2$ <sup>140</sup> and KCN (see Chapter 5) is known to increase with increasing potentials. A similar effect is also observed in other enzymes: In the presence of cyanate,  $\text{CO}_2$  reduction activity by Ni-containing carbon monoxide dehydrogenase is inhibited to a much greater extent than CO oxidation activity due to cyanide preferentially binding to the most reduced state of the enzyme,  $\text{C}_{\text{red2}}$ .<sup>141</sup> The affinity of a catalyst for its substrate, represented by the Michaelis constant  $K_{\text{M}}$ , may also vary as the electrode potential causes a particular state to prevail.<sup>142</sup> Similarly, product inhibition may be characteristic of one particular state of the enzyme but not another. A related but subtly different effect which may lead to potential dependent changes in absolute catalytic activity are conformational changes to the region surrounding the active site or electron relay centres which are dependent upon the prevailing redox state. Another possibility, that changes in potential alter the coupling of the enzyme to the electrode, may

also arise and can be assessed by varying the electrode material. The occurrence of these catalytic transformations over a potential window is observed by protein film electrochemistry and, if they are much slower than catalytic turnover, will give rise to hysteresis when viewed by cyclic voltammetry.

## 4.2 Extending the Basic Model

The basic model introduced in Chapter 3 and represented in Scheme 3.2 accounts for a catalyst immobilised on an electrode surface. Electrons enter or exit the catalyst in multiple one electron transfers *via* an electrochemical control centre, the potential of which determines the catalytic bias (the preference of a catalyst to work in a certain direction). The potential dependent rate of electron transfer between the electrode and electrochemical control centre is described by  $k_{1a}$  and  $k_{1c}$ . All other catalytic events which occur are described by the potential independent rate constants  $k_{2a}$  and  $k_{2c}$ . In this chapter this model is extended to encompass more catalytic states, differing in atomic arrangements and linked by potential-dependent transformations.

For simplification we can consider that the catalyst can adopt two possible states, referred to as State I and State II, that prevail under different electrochemical potentials. If conversion between State I and State II involves electron transfer described by the rate constants  $k_{3I}$  and  $k_{3II}$  as shown in Scheme 4.1, then, assuming Butler-Volmer kinetics, we obtain Equations 4.1 and 4.2;

$$k_{3I} = k_3^0 \exp \left[ -\alpha_I \frac{n'F}{RT} (E - E_{\text{switch}}) \right] = k_3^0 e_3^{-(1-\alpha_{II})} \quad (4.1)$$

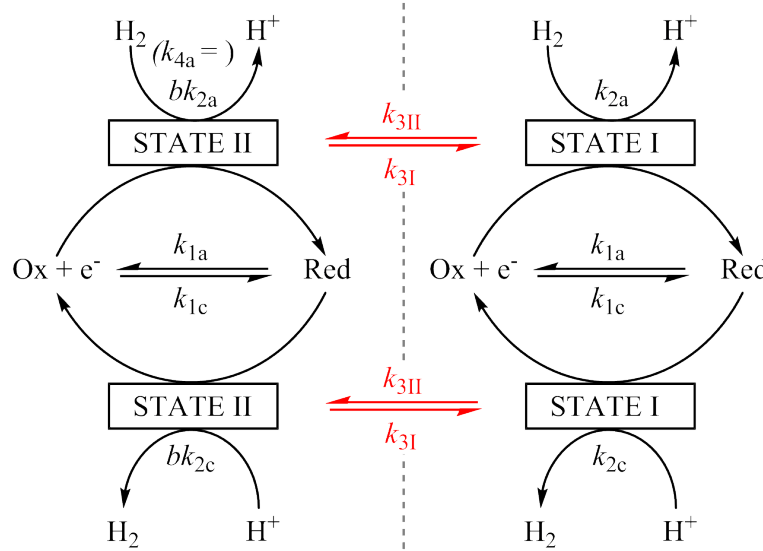
$$k_{3II} = k_3^0 \exp \left[ \alpha_{II} \frac{n'F}{RT} (E - E_{\text{switch}}) \right] = k_3^0 e_3^{\alpha_{II}} \quad (4.2)$$

where  $k_3^0$  is the standard rate constant for the interconversion of the two states,  $n'$  is the number of electrons transferred during the transformation, and  $\alpha_I$  and  $\alpha_{II}$  are

the respective transfer coefficients, and;

$$e_3 = \exp \left[ \frac{n'F}{RT} (E - E_{\text{switch}}) \right] = \frac{k_{3\text{II}}}{k_{3\text{I}}} \quad (4.3)$$

where  $E_{\text{switch}}$  is the characteristic potential at which State I and State II interconvert.



**Scheme 4.1:** A representation of a mathematical model for reversible electrocatalysis, reproduced from Reference 143 with permission from the PCCP Owner Societies. ‘Ox’ and ‘Red’ refer to the oxidised and reduced forms of the electrochemical control centre, the site at which electrons enter or exit the enzyme at a rate described by  $k_{1a}$  and  $k_{1c}$ . All other catalytic events which occur within the enzyme are described by  $k_{2a}$  and  $k_{2c}$  where  $b$  is a multiplication factor. The scheme depicts the potential dependent conversion of a catalytic site in ‘State I’ into a catalytic site in ‘State II’ described by  $k_{3I}$  and  $k_{3II}$ . Throughout this Chapter, State II is assumed to be the more oxidised state unless specified otherwise.

We now consider the proportion of enzyme in State I and State II relative to the total enzyme coverage so that;

$$\Gamma_m = \Gamma_I + \Gamma_{II} \quad (4.4)$$

where  $\Gamma_m$  is the total electroactive coverage of enzyme on the electrode and  $\Gamma_I$  and  $\Gamma_{II}$  are the coverages of enzyme in State I and State II respectively. The proportion

of enzyme in State I changes with time according to:

$$\frac{d(\Gamma_I/\Gamma_m)}{dt} = \left(1 - \frac{\Gamma_I}{\Gamma_m}\right) k_{3I} - \left(\frac{\Gamma_I}{\Gamma_m}\right) k_{3II} \quad (4.5)$$

Likewise, the proportion of enzyme in State II changes with time according to:

$$\frac{d(\Gamma_{II}/\Gamma_m)}{dt} = \left(1 - \frac{\Gamma_{II}}{\Gamma_m}\right) k_{3II} - \left(\frac{\Gamma_{II}}{\Gamma_m}\right) k_{3I} \quad (4.6)$$

If interconversion between State I and State II is sufficiently fast then the relative amounts of each state is given by the Nernst equation (Appendix A). Under steady-state conditions, and assuming that State II is the more oxidised state, then  $\Gamma_I$  and  $\Gamma_{II}$  are given by:

$$\Gamma_I = \frac{\Gamma_m}{1 + \exp\left[\frac{n'F}{RT}(E - E_{\text{switch}})\right]} = \frac{\Gamma_m}{1 + e_3} \quad (4.7)$$

$$\Gamma_{II} = \frac{\Gamma_m \exp\left[\frac{n'F}{RT}(E - E_{\text{switch}})\right]}{1 + \exp\left[\frac{n'F}{RT}(E - E_{\text{switch}})\right]} = \frac{\Gamma_m e_3}{1 + e_3} \quad (4.8)$$

In Chapter 3 the limiting current represented by  $j_{\text{lim}}$  was proportional to the total electroactive coverage of enzyme on the electrode  $\Gamma_m$ . If we now consider the proportion of enzyme in State I and State II separately, new expressions for the limiting current, now expressed as  $j_{\text{lim}}^{\Gamma_I+\Gamma_{II}}$ , arise;

$$j_{\text{lim}}^{\Gamma_I+\Gamma_{II}} = nFk_{2a}(\Gamma_I + \Gamma_{II}) \quad (4.9)$$

as defined for an oxidative process, and

$$j_{\text{lim}}^{\Gamma_I+\Gamma_{II}} = nFk_{2c}(\Gamma_I + \Gamma_{II}) \quad (4.10)$$

for a reductive process.

We now assume that the enzyme is able to undergo catalysis in both State I and State II, as described by the model shown in Scheme 4.1, but that the maximal activity of the enzyme differs depending upon its state. If the maximum rate of catalysis in the anodic direction is given by  $k_{2a}$  for enzyme in State I and  $k_{4a}$  for enzyme in State II, we can express  $k_{4a}$  such that;

$$k_{4a} = bk_{2a} \quad (4.11)$$

where  $b$  is a multiplication factor representing the ratio between the inherent catalytic activities of the two states. The expression for  $j_{\text{lim}}^{\Gamma_{\text{I}}+\Gamma_{\text{II}}}$  in the anodic direction then becomes:

$$j_{\text{lim}}^{\Gamma_{\text{I}}+\Gamma_{\text{II}}} = nFk_{2a}(\Gamma_{\text{I}} + b\Gamma_{\text{II}}) \quad (4.12)$$

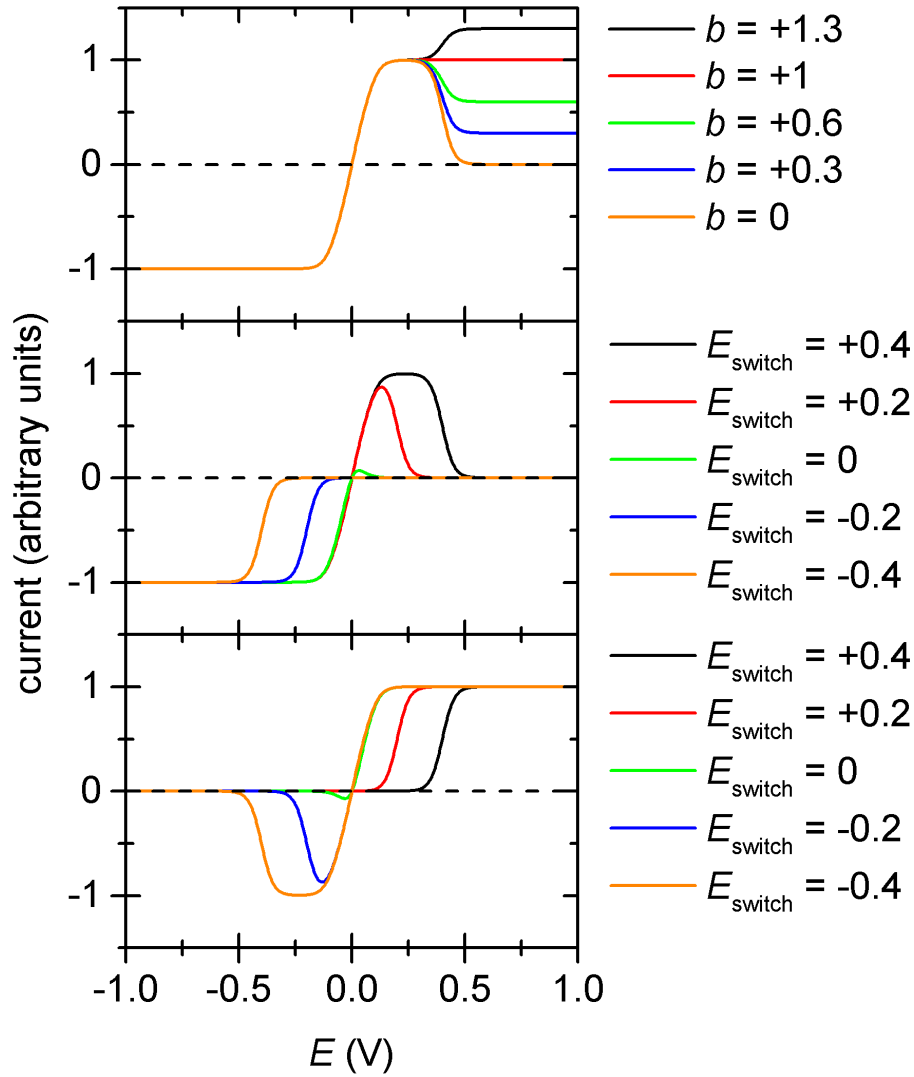
Substituting Equations 4.7 and 4.8 into Equation 4.12,  $j_{\text{lim}}^{\Gamma_{\text{I}}+\Gamma_{\text{II}}}$  in the anodic direction can be finally expressed as:

$$j_{\text{lim}}^{\Gamma_{\text{I}}+\Gamma_{\text{II}}} = nFk_{2a}\Gamma_{\text{m}} \left( \frac{1}{1+e_3} + \frac{be_3}{1+e_3} \right) = j_{\text{lim}} \left( \frac{1}{1+e_3} + \frac{be_3}{1+e_3} \right) \quad (4.13)$$

where  $j_{\text{lim}}$  is as previously defined in Equation 3.8. Taking into account enzyme in State I and State II, Equation 3.12 derived in Chapter 3 now becomes:

$$j = \frac{j_{\text{lim}}}{\beta d_0} \left( \frac{1}{1+e_3} + \frac{be_3}{1+e_3} \right) \frac{e_1 - e_2}{1+e_1} \ln \frac{pe_1^{\alpha_c} + (1+e_1)}{pe_1^{\alpha_c} + (1+e_1)\exp(-\beta d_0)} \quad (4.14)$$

The effect of changing the values of the parameters  $b$  and  $E_{\text{switch}}$  is demonstrated in Figure 4.1. The effect of changing all other parameters is as shown in Figure 3.4. In these simulations it is assumed that interconversion between State I and State II is fast. This is typically not the case and hysteresis is usually observed during cyclic voltammetry experiments. As shown in Figure 4.1A, when  $b = 1$ , States I and II have the same catalytic activity and conversion from one state to the other cannot be detected through voltammetry. When  $b \neq 1$ , the activity of State II is  $b$  times that of State I. This is observed during a voltammetry experiment, and in



**Figure 4.1:** Simulated curves from Equation 4.14 with differing values of: (A)  $b$ ; (B)  $E_{\text{switch}}$  with State II the more oxidised state, and; (C)  $E_{\text{switch}}$  with State II the more reduced state. Values of  $b$  and  $E_{\text{switch}}$  are as indicated in the figure. Unless stated otherwise, parameters are:  $E_{\text{Ox/R}}^0 = E_{\text{eqm}} = 0$  V (and so  $e_2 = 1$ ),  $n = 2$ ,  $p = +1$ ,  $\beta d_0 = +4$ ,  $j_{\text{lim}} = +1$ ,  $b = 0$ ,  $n' = 1$  and  $E_{\text{switch}} = 0.4$ . Adapted from Reference 143.

Figure 4.1A, by a decrease (when  $b < 1$ ) or an increase (when  $b > 1$ ) in current upon scanning to more oxidising potentials (this is for the case where State II is more oxidised than State I, see below for when this is not the case). Ideally, the current reaches a plateau at a value determined by  $bk_{2a}$ .

Figures 4.1B and 4.1C show the effect of varying  $E_{\text{switch}}$ , the parameter that represents the potential at which the interconversion between State I and State II takes place. If we first consider Figure 4.1B in which State II is completely inactive ( $b = 0$ ) and more oxidised than State I then the value of  $E_{\text{switch}}$  affects the peak position and intensity for catalytic oxidation activity. Oxidation activity occurs between  $E_{\text{eqm}}$  and  $E_{\text{switch}}$  and so  $E_{\text{switch}}$  also determines the ‘window’ of catalytic oxidation activity. When  $E_{\text{switch}} < E_{\text{eqm}}$  the catalyst converts into a catalytically inactive state before net oxidation activity can be observed. This results in the catalyst appearing to have a bias towards the reductive reaction.

In Figure 4.1C the catalytically inactive State II is more reduced than State I. In this case,  $\Gamma_{\text{I}}$  is now described by Equation 4.8 and  $\Gamma_{\text{II}}$  is described by Equation 4.7. It follows that;

$$j_{\text{lim}}^{\Gamma_{\text{I}}+\Gamma_{\text{II}}} = j_{\text{lim}} \left( \frac{b}{1+e_3} + \frac{e_3}{1+e_3} \right) \quad (4.15)$$

and;

$$j = \frac{j_{\text{lim}}}{\beta d_0} \left( \frac{b}{1+e_3} + \frac{e_3}{1+e_3} \right) \frac{e_1 - e_2}{1+e_1} \ln \frac{pe_1^{\alpha_c} + (1+e_1)}{pe_1^{\alpha_c} + (1+e_1) \exp(-\beta d_0)} \quad (4.16)$$

$E_{\text{switch}}$  now determines the window of catalytic reduction activity and a catalytic bias towards oxidation activity is observed when  $E_{\text{switch}} > E_{\text{eqm}}$ .

### 4.3 Applying the Extended Model

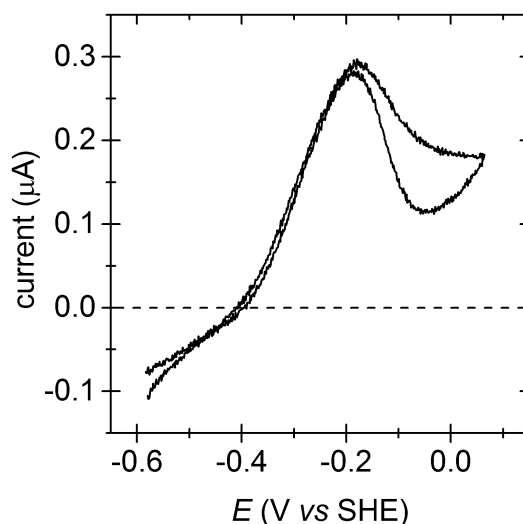
As for the basic model introduced in Chapter 3, the extended model described above can be applied to voltammograms showing reversible electrocatalysis by enzymes. In this section, comparisons with voltammetry that derives from surface electro-



chemistry on metal catalysts will also be made.

### 4.3.1 Modelling the Oxidative Inactivation of Hydrogenase

The inactive state, referred to as the Ni-B state, which forms when a [NiFe]-hydrogenase undergoes a one-electron oxidation at high potential, has been well characterised and is now known to be a  $\text{Ni}^{3+}$  species in which a hydroxide ligand bridges the Ni and Fe atoms of the active site (see Section 1.6). Evidence of Ni-B formation can be seen as a decrease in current with increasing overpotential as exemplified in Figure 4.2 for *EcHyd2* from *Escherichia coli*. The transformation to this inactive state is usually relatively slow compared to catalysis and because of this a slow scan rate over the required potential region is necessary to observe this inactivation (in Figure 3.1 a scan rate of  $5 \text{ mV s}^{-1}$  is too fast to see substantial evidence of conversion to the Ni-B state for *EcHyd2*). High hydrogen concentrations and a low pH value also protect the active site to some extent from Ni-B formation. The voltammogram shown in Figure 4.2 was carried out at pH 8 under 1%  $\text{H}_2$  in Ar further increasing the rate of Ni-B formation relative to the scan shown in Figure 3.1.



**Figure 4.2:** An example of a cyclic voltammogram of *EcHyd2* attached to a PGE electrode. Conditions: pH 8, 25 °C,  $\omega = 3000 \text{ rpm}$ ,  $\nu = 0.05 \text{ mV s}^{-1}$ , 1%  $\text{H}_2$  in Ar.

A similar one-electron oxidation to form an inactive state also occurs when [FeFe]-hydrogenases are subjected to oxidising conditions, forming the inactive  $\text{H}_{\text{ox}}^{\text{inact}}$  state.

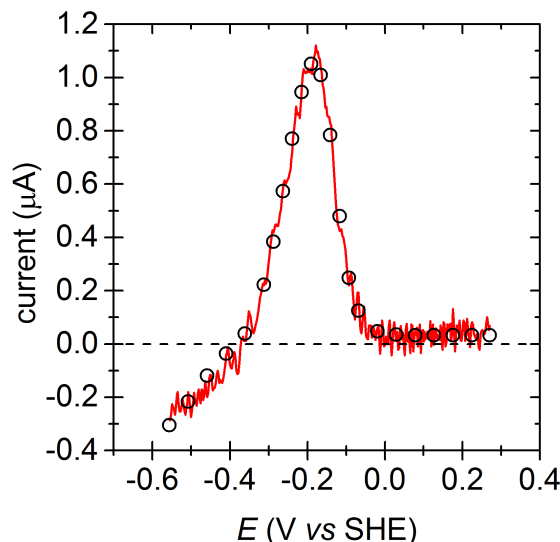
For the [FeFe]-hydrogenase *CrHydA1* from *Chlamydomonas reinhardtii* this inactivation is particularly quick and a scan rate of  $5 \text{ mV s}^{-1}$  is sufficiently slow to observe a large reduction in current, indicative of the formation of an inactive state, as shown in Figure 3.1.

Unlike the more basic model highlighted in Chapter 3, the model outlined above can be used to describe the changing activity of hydrogenases over a potential region which incorporates inactive states. Figure 4.3 shows a voltammogram of *EcHyd2* at which transformation to the oxidised inactive Ni-B state takes place and the corresponding fit to the extended model. In the case of *EcHyd2*, the very slow conversion to the Ni-B state means that the potential at which this process takes place, as described by  $E_{\text{switch}}$ , can be more accurately assessed by studying the faster reactivation reaction. To do this, the enzyme was first fully inactivated by poisoning the electrode at  $+0.270 \text{ V}$  for *ca.* 2.5 hours under an atmosphere of 10%  $\text{H}_2$  in Ar before a voltammogram was performed from  $+0.270 \text{ V}$  to  $-0.560 \text{ V}$ . The higher rate of the reactivation reaction meant that a slightly faster scan rate of  $1 \text{ mV s}^{-1}$  was sufficiently slow to accurately monitor this reaction. The electrode was rotated at a sufficient speed to ensure that the current was not limited by mass transport.

Under the conditions of the experiment,  $E_{2\text{H}^+/\text{H}_2} = -0.384 \text{ V}$ , a value much lower than the value of  $E_{\text{switch}}$  determined by the fit ( $E_{\text{switch}} = -0.140 \text{ V}$ ). In this case,  $E_{\text{switch}}$  determines the potential window at which *EcHyd2* is able to oxidise  $\text{H}_2$ . The parameter  $b$  obtained by the fit is very close to zero ( $b = +0.02016$ ) reflecting the complete inactivation of the enzyme at potentials more positive than  $E_{\text{switch}}$ .

### 4.3.2 Modelling Cyanate Inhibition of Carbon Monoxide Dehydrogenase

Carbon monoxide dehydrogenases (CODH) are enzymes capable of catalysing  $\text{CO}_2$  and CO interconversion at potentials close to the thermodynamic value.<sup>144</sup> Spectroscopic and protein film electrochemistry experiments have shown that cyanate ( $\text{NCO}^-$ ), an analogue of  $\text{CO}_2$ , binds to the most reduced state of the enzyme,  $\text{C}_{\text{red}2}$ ,

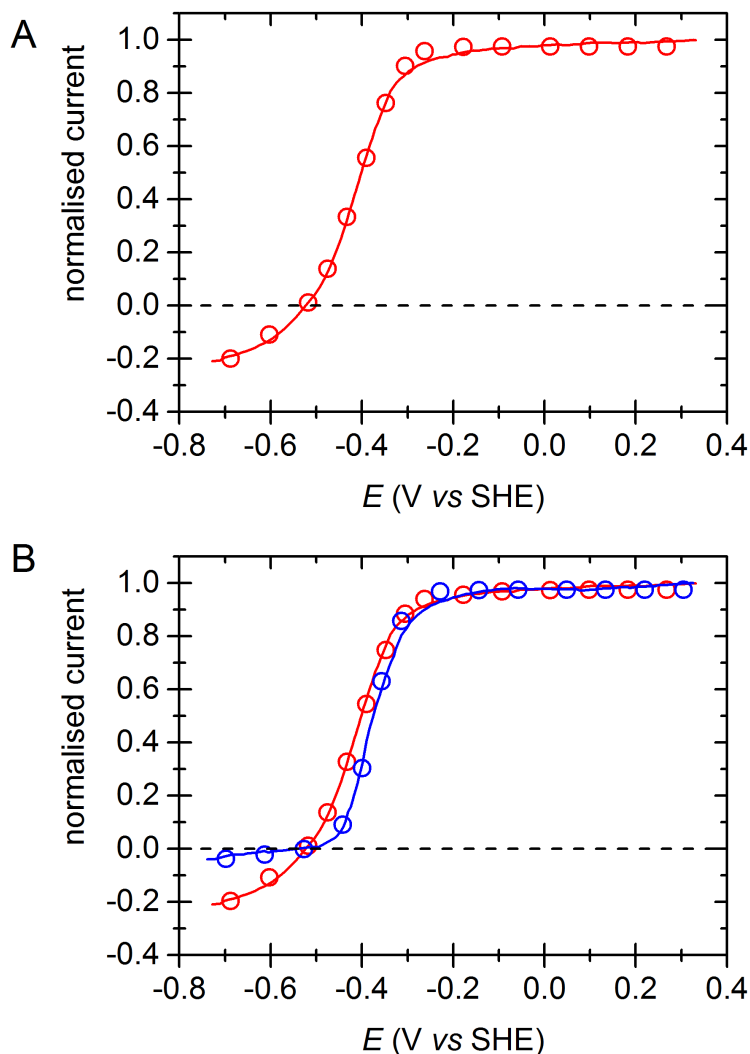


**Figure 4.3:** Fit of Equation 4.14 to a voltammogram of *EcHyd2* scanned from high (+0.270 V) to low (−0.560 V) potential. The hydrogenase was converted to the inactive Ni-B state by poisoning at +0.270 V for *ca.* 2.5 hours prior to the start of the scan. Conditions: pH 7, 25 °C,  $\omega = 1500$  rpm,  $\nu = 1$  mV s<sup>−1</sup>, 10% H<sub>2</sub> in Ar. The values of the parameters obtained are as follows:  $E_{\text{Ox/R}}^0 = -0.365$  V, (under the conditions of the experiment  $E_{2\text{H}^+/\text{H}_2} = -0.384$  V giving, from Equation 3.5,  $e_2 = +0.22769$ ),  $p = +4.88278$ ,  $\beta d_0 = +6.8508$ ,  $j_{\text{lim}} = +1.61934$ ,  $E_{\text{switch}} = -0.13974$  V and  $b = +0.02016$ . The parameters  $n$  and  $n'$  were fixed at 2 and 1 respectively.

but not to the more oxidised state, C<sub>red1</sub>. The use of protein film electrochemistry means that this very specific potential dependence can be seen clearly during voltammetry, as detailed in a paper by Wang *et al.*<sup>141</sup>

In order to model the inhibitory effect of cyanate upon CODH, data from Wang *et al.*<sup>141</sup> were digitised and then normalised so that the maximum oxidation current was equal to one in each case (Figure 4.4). The voltammogram obtained in the absence of any inhibitors was fitted to the basic model outlined in Chapter 3 and described by Equation 3.12 (Figure 4.4A). This allowed the values of the parameters  $E_{\text{Ox/R}}^0$ ,  $p$ ,  $\beta d_0$  and  $j_{\text{lim}}$  to be found, the values of which should not be altered to a large extent upon the introduction of cyanate. The values obtained were then substituted into Equation 4.16, in which State II is the more reduced state, and this equation was then used to fit the data obtained from both uninhibited and inhibited enzyme (Figure 4.4B).

When values of  $E_{\text{Ox/R}}^0$ ,  $p$ ,  $\beta d_0$  and  $j_{\text{lim}}$ , as found from the basic model, are substituted into Equation 4.16 and used to fit the data obtained from CODH in the



**Figure 4.4:** Normalised voltammograms for  $\text{CO}_2/\text{CO}$  interconversion catalysed by CODH  $I_{Ch}$  from *Carboxydothamus hydrogenoformans*. (A) Voltammogram obtained in the absence of KOCN (red line) and the corresponding fit to Equation 3.12 (red circles). The values of the parameters obtained are as follows:  $E_{\text{Ox/R}}^0 = -0.504$  V, (under the conditions of the experiment  $E_{\text{CO}_2/\text{CO}} = -0.523$  V giving  $e_2 = +0.22769$  from Equation 3.4),  $p = +1.75725$ ,  $\beta d_0 = +6.76555$ ,  $j_{lim} = +0.97429$ . The parameter  $n$  was fixed with a value of 2. (B) Voltammograms obtained in the absence (red line) and presence (blue line) of KOCN (6.67 mM) and the corresponding fits to Equation 4.16 (circles). Values of  $E_{\text{Ox/R}}^0$ ,  $p$ ,  $\beta d_0$ ,  $j_{lim}$  and  $n$  were fixed as found in (A). The values of  $E_{\text{switch}}$  and  $b$  obtained are  $E_{\text{switch}} = -0.113$  V,  $b = +0.98106$  and  $E_{\text{switch}} = -0.400$  V,  $b = +0.18146$  for the uninhibited and inhibited forms of CODH respectively. The parameter  $n'$  was fixed at 1. Conditions: pH 7, 25 °C,  $\omega = 3500$  rpm,  $\nu = 1$  mV s $^{-1}$ , 50%  $\text{CO}_2$ , 50% CO.

absence of any inhibitor, a value of  $b$  close to 1 is obtained (as expected due to the good fit already achieved by Equation 3.12). This is indicative of a catalyst which does not undergo any change in inherent activity over the potential range studied. However, when the same treatment is applied to data obtained from CODH in the presence of cyanate a value of  $b = 0.18$  is found. In other words, the presence of 6.67 mM of  $\text{NCO}^-$  has selectively decreased the catalytic activity of the reduced state of the active site by *ca.* 82 %. The value of  $E_{\text{switch}}$  found ( $-0.400$  V) is more positive than  $E_{\text{CO}_2/\text{CO}}$  under the conditions of the experiment ( $-0.523$  V). This results in the additional effect that the presence of  $\text{NCO}^-$  causes an overpotential requirement for CO oxidation.

### 4.3.3 The Effect of Mutagenesis

Studies which make use of Site Directed Mutagenesis (SDM) have been made on many enzymes including hydrogenases. This technique enables a specific amino acid within a protein to be exchanged for another. The functionality of the variant enzyme can then be tested and compared to the native enzyme and in this way knowledge regarding the role of individual amino acids within a protein can be determined.

One such study was carried out by Hamdan *et al.*<sup>6</sup> who compared the activity of the native and some genetically-modified variants of the [NiFe]-hydrogenase from *Desulfovibrio fructosovorans*. The variants compared were a valine to methionine single site mutation (V74M) and a leucine to phenylalanine and valine to isoleucine double mutation (L122F V74I). The replacement sites, valine-74 and leucine-122, are situated within the large subunit but close to the interface with the small subunit, approximately 5 Å and 10 Å from the Ni atom at the active site respectively. The mutations were designed to influence the transport of  $\text{H}_2$  within the enzyme without modifying the active site.

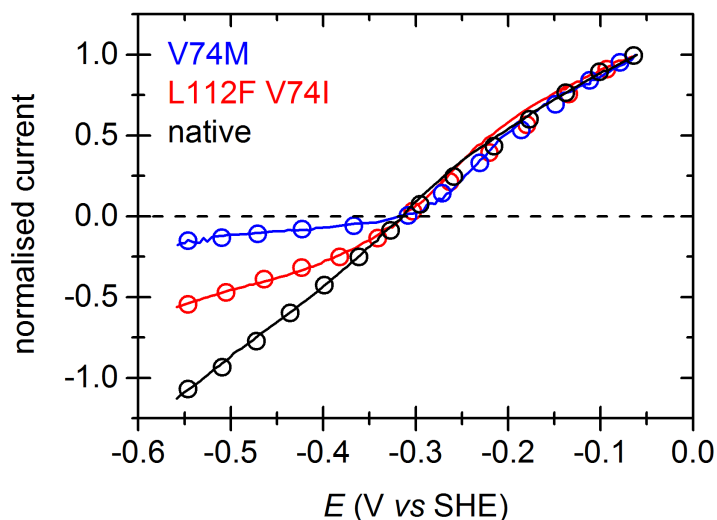
Redox titrations of the active sites of the V74M variant and the native enzyme monitored by fourier transform infrared spectroscopy (FTIR) showed that the reduc-

tion potentials and FTIR signatures of the various active-site states in the variant were similar to the native enzyme.<sup>6</sup> Leucine-122 is further from the active site than valine-74 and so the authors suggested that the L122F mutation would also have no significant effect on the redox behaviour of the active site. However, the variants did vary greatly in electrocatalytic properties when compared to the native enzyme. The native enzyme has very little bias towards either  $\text{H}_2$  oxidation or  $\text{H}^+$  reduction activity whilst the L122F V74I variant has relatively low  $\text{H}^+$  reduction activity and the V74M variant has almost none. Hamdan and colleagues proposed, on the bases of isotope exchange experiments, that in both the native enzyme and the variants,  $\text{H}^+$  reduction is limited by  $\text{H}_2$  diffusion away from the active site, an effect which is magnified in the variants because the gas channel is obstructed.

An alternative viewpoint offered by the model proposed in this chapter is that it is a redox site other than the active-site that determines the overall activity of the enzyme and that this site is somehow influenced by the mutations. The distance between the mutations and the distal FeS cluster (*ca.* 30 Å) indicates that it is unlikely that the mutations affect  $E_{\text{Ox/R}}^0$ , the potential at which electrons enter or leave the enzyme, and so in turn  $e_1$ , the driving force for interfacial electron transfer, would be unaffected by the mutations. Another possibility is that the mutations, which lie at the interface between the large and small subunits, influence the potential of the proximal FeS cluster, the cluster closest to the active site. The prevailing oxidation level of the proximal cluster may be a switch that controls catalytic activity and the mutations may affect the value of  $E_{\text{switch}}$  for this cluster.

In order to test whether the model could be used to fit the voltammetric data obtained from the native and V74M and L122F V74I variant hydrogenases presented in the paper by Hamdan *et al.*<sup>6</sup> the data were digitised and then normalised so that the maximum oxidation current was equal to 1 in each case (Figure 4.5). This allowed easy comparison between the different sets of data. Each voltammogram was then fitted to Equation 4.16, in which State II is the more reduced state. The values of  $E_{\text{Ox/R}}^0$ ,  $p$ ,  $\beta d_0$  and  $j_{\text{lim}}$  were the same in each case, and fixed at the values

found by fitting the voltammogram of the native enzyme to Equation 3.12. As shown in Figure 4.5, the model can be used to describe the changing voltammetric response with potential in each case by changing only the values of  $E_{\text{switch}}$  and  $b$ .



**Figure 4.5:** Normalised voltammograms (lines) of the native and V74M and L112F V74I variants of the [NiFe]-hydrogenase from *Desulfovibrio fructosovorans* as indicated in the figure. Data were obtained by digitising the data shown in Reference 6. Circles represent the fit of each voltammogram to Equation 4.16. Values for  $E_{\text{Ox/R}}^0$ ,  $p$ ,  $\beta d_0$  and  $j_{\text{lim}}$  were found by fitting the data from the native enzyme to Equation 3.12 and are as follows:  $E_{\text{Ox/R}}^0 = -0.311$  V, ( $E_{2\text{H}^+/\text{H}_2}$  under the conditions of the experiment is  $-0.310$  V giving, from Equation 3.5,  $e_2 = +1.0769$ ),  $p = +1$ ,  $\beta d_0 = +9.2397$ ,  $j_{\text{lim}} = +1.07584$ . The parameter  $n$  was fixed with a value of 2. Using these values, the values of  $E_{\text{switch}}$  and  $b$  found from the fit to Equation 4.16 are as follows: Native enzyme,  $E_{\text{switch}} = -0.125$  V,  $b = +1.0478$ ; V74M variant,  $E_{\text{switch}} = -0.302$  V,  $b = +0.1477$ ; L122F V74I variant,  $E_{\text{switch}} = -0.381$  V,  $b = +0.5339$ . The parameter  $n'$  was fixed at 1 in all cases. Conditions: pH 5.5, 40 °C,  $\omega = 3000$  rpm,  $\nu = 10$  mV s $^{-1}$ , 10% H $_2$ .

Applying Equation 4.16 to the voltammogram obtained from the native hydrogenase gives a value of  $b$  close to 1, consistent with the inherent catalytic activity being uniform across the potential range. When  $b$  is close to 1, the value for  $E_{\text{switch}}$  found by the model, in this case  $-0.125$  V, becomes irrelevant. The values of  $b$  found from the voltammograms of the V74M and L122F V74I variants were  $+0.1477$  and  $+0.5339$  respectively, indicating that the reduced state has an inherent catalytic activity of 15% and 53% that of the oxidised state in each case. The values of

$E_{\text{switch}}$  found from the fits to the voltammograms obtained from the variant hydro-genases ( $E_{\text{switch}} = -0.302$  V and  $-0.381$  V for the V74M and L122F V74I variants respectively) are close to  $E_{2\text{H}^+/\text{H}_2}$  ( $-0.310$  V under the conditions of the experiment) meaning that, in both cases, the interconversion to the less active state occurs close to the thermodynamic change in the net direction of the reaction. As indicated in Figures 4.1B and 4.1C, this gives rise to a catalyst which is biased towards carrying out catalysis in one direction over the other. This is particularly pronounced in the data for the V74M variant due to the small value for  $b$ .

## 4.4 A Comparison between Enzymes and the Passivation of Metal Surface Catalysts

For a metallic surface electrocatalyst, the relay centre is the surface of the catalyst itself and so  $E_{\text{Ox/R}}^0$  is always equal to the applied potential  $E$ . In this limiting case,  $e_1$ , as described by Equation 3.3 in Chapter 3, now equals 1 and the potential dependence of the electrocatalytic reaction is now expressed in terms of  $k_{2a}$  and  $k_{2c}$  where  $e_2$  becomes:

$$e_2 = \frac{k_{2c}}{k_{2a}} = \exp \left[ \frac{-nF}{RT} (E - E_{\text{eqm}}) \right] \quad (4.17)$$

In this case, where the metallic surface is the catalyst, the term  $\beta d_0 = 0$  and Equation 3.7 is used in place of Equation 3.12.  $[\text{Ox}]$  and  $[\text{Red}]$ , as represented in Scheme 4.1 in this chapter and in Scheme 3.2 in Chapter 3, now refer to the concentration of oxidised and reduced forms of the substrate respectively, as opposed to oxidised and reduced forms of the electrochemical control centre. The rate constants  $k_{1a}$  and  $k_{1c}$ , which previously described the rates of interfacial electron transfer, are no longer required and the electrocatalytic rates are now given by a simple Butler-Volmer expression. The parameter  $p$  is no longer described by Equation 3.9 but is needed to retain control of the waveform: It will now be replaced by  $p'$ . Since



$e_1 = 1$ , Equation 3.7 can now be written as;

$$j = j_{\text{lim:metal}} \frac{e_2^{-\alpha_c} - e_2^{1-\alpha_c}}{2 + p'(e_2^{-\alpha_c} + e_2^{1-\alpha_c})} \quad (4.18)$$

where;

$$j_{\text{lim:metal}} = nF\Gamma_m k^{0'} \quad (4.19)$$

and  $k^{0'}$  is defined as the standard rate constant for the catalytic reaction.

Assuming that the electrocatalyst may be in one of two states (State I or State II) which differ in inherent catalytic activity, then by applying the extended model to the case of a metallic surface electrocatalyst,  $j_{\text{lim:metal}}$  can be described by  $j_{\text{lim:metal}}^{\Gamma_I + \Gamma_{II}}$  where;

$$j_{\text{lim:metal}}^{\Gamma_I + \Gamma_{II}} = j_{\text{lim:metal}} \left( \frac{1}{1 + e_3} + \frac{be_3}{1 + e_3} \right) \quad (4.20)$$

or

$$j_{\text{lim:metal}}^{\Gamma_I + \Gamma_{II}} = j_{\text{lim:metal}} \left( \frac{b}{1 + e_3} + \frac{e_3}{1 + e_3} \right) \quad (4.21)$$

depending upon whether an inherent change in catalytic activity is being measured in relatively oxidising or reducing conditions, respectively. Equation 4.18 now becomes

$$j = j_{\text{lim:metal}} \left( \frac{1}{1 + e_3} + \frac{be_3}{1 + e_3} \right) \frac{e_2^{-\alpha_c} - e_2^{1-\alpha_c}}{2 + p'(e_2^{-\alpha_c} + e_2^{1-\alpha_c})} \quad (4.22)$$

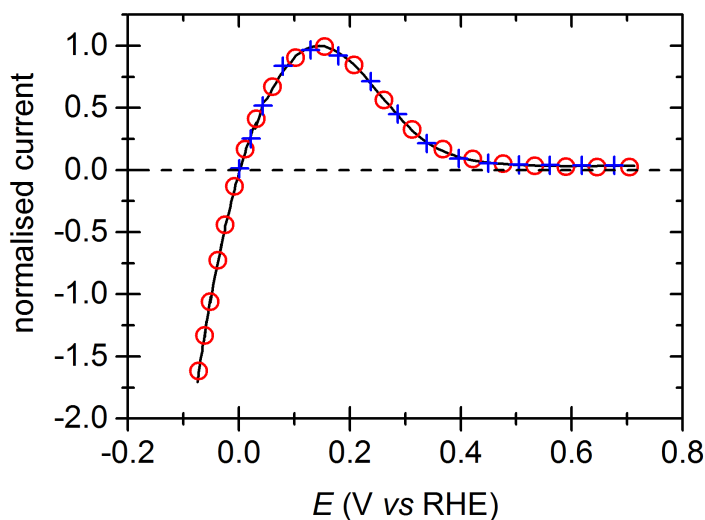
or

$$j = j_{\text{lim:metal}} \left( \frac{b}{1 + e_3} + \frac{e_3}{1 + e_3} \right) \frac{e_2^{-\alpha_c} - e_2^{1-\alpha_c}}{2 + p'(e_2^{-\alpha_c} + e_2^{1-\alpha_c})} \quad (4.23)$$

respectively.

In order to test whether this adaption to the extended model could be used to fit the voltammetry obtained from a metallic surface electrocatalyst, a voltammogram of a single-crystal ruthenium electrode, Ru(0001), displaying  $\text{H}^+$  reduction and  $\text{H}_2$  oxidation activity in sulfuric acid, was digitised from a paper by Inoue *et al.*<sup>145</sup> Before analysis the data were normalised so that the maximum oxidation current was equal to 1. Similar to the interconversion of active hydrogenase into the inactive

Ni-B or  $\text{H}_{\text{ox}}^{\text{inact}}$  states at oxidising potentials, passivation of the Ru surface occurs in which inactive  $\text{Ru}^{n+}\text{-OH}$  surface species are generated blocking catalysis. In voltammetry this is observed as a decrease in current with increasing driving force for  $\text{H}_2$  oxidation. Similar oxidative inactivation is observed for Pt, likewise resulting in inactive  $\text{Pt}^{n+}\text{-OH}$  species.<sup>146</sup> Figure 4.6 shows the fit of Equation 4.22 to the digitised data.



**Figure 4.6:** The fit of Equation 4.22 to the digitised and normalised data from Reference 145 showing the  $\text{H}_2$  oxidation and  $\text{H}^+$  reduction behaviour of Ru(0001). Fits were obtained using  $n = 1$  and  $n = 2$  (above  $-0.025$  V only), represented with red circles and blue crosses respectively. The values of the parameters obtained from the  $n = 1$  fit are as follows:  $j_{\text{lim:metal}} = +6.13511$ ,  $p' = +0.04732$ ,  $n' = 0.39662$ ,  $E_{\text{switch}} = -0.090$  V,  $b = +0.00016$ . The corresponding values for the  $n = 2$  fit are as follows:  $j_{\text{lim:metal}} = +0.49882$ ,  $p' = +0.47882$ ,  $n' = 0.60361$ ,  $E_{\text{switch}} = +0.270$  V,  $b = +0.03562$ . Conditions:  $\text{H}_2$  saturated  $0.05$  M  $\text{H}_2\text{SO}_4$  solution,  $40^\circ\text{C}$ ,  $\omega = 900$  rpm,  $\nu = 20$  mV s<sup>-1</sup>.

Unlike the fits of Equations 4.14 and 4.16 to the data obtained from immobilised enzymes, shown in Figures 4.3, 4.4 and 4.5, a unique fit is not found. Good fits were obtained using  $n = 1$  across the entire potential range, however a good fit using  $n = 2$  (corresponding to the stoichiometric reaction) could only be obtained if the data recorded at potentials below  $-0.025$  V was not included in the fits. In both cases interconversion between the active and inactive catalytic states required a sub-1 electron process: When  $n = 1$ , the best fit was found when  $n' = 0.39662$  and  $E_{\text{switch}} = -0.090$  V and when  $n = 2$ , values of  $n' = 0.60361$  and  $E_{\text{switch}} = +0.270$  V

gave the best fits. The sub-1 values of  $n'$  highlight the complexity of forming a Ru-oxide layer where the neighbouring catalytic sites interact strongly with each other and offer the possibility for  $\text{OH}^-$  bridging ligands to form over multiple sites. This is in contrast with the oxidation of [NiFe]-hydrogenase active sites to form the Ni-B state, where there is a 1:1 ratio of  $\text{OH}^-$  ligands to active site entities, and where the surrounding protein shields each active site from its neighbours. The values of  $E_{\text{switch}}$  are highly sensitive to the value of  $n$  chosen for the fits because it is the value of  $n$  (and  $n'$ ) that determines the steepness of the slope of increasing (or decreasing) current. In both cases the parameter  $b$  is close to 0 indicating a complete inactivation of the Ru surface towards  $\text{H}_2$  oxidation.

## 4.5 Conclusions and Perspectives

The model proposed in this chapter, an extension of the model proposed in Chapter 3, accounts for many of the observations made during voltammetry experiments of redox enzymes immobilised onto an electrode. The complex patterns seen in the waveshape of a voltammogram may arise from potential-dependent variations in the absolute activity of a catalyst and, in the extreme case, complete inactivation of the catalyst within a certain potential window. As demonstrated here using data from hydrogenase and CODH, these variations may arise from an enhanced inhibitor binding or product inhibition of a specific redox state which dominates within a certain potential window.

Potential-dependent changes in the inherent activity of a catalyst may also arise from changes in affinity for its substrate, reflected by a change in the value of the Michaelis constant,  $K_{\text{M}}$ , over a potential range (see Appendix E for a discussion about the meaning of  $K_{\text{M}}$ ). Although not dealt with in this chapter, where substrate binding affinities have been de-emphasised as it is assumed that the catalytic site is always saturated, if the substrate concentration was close to or lower than  $K_{\text{M}}$ , then a change in  $K_{\text{M}}$  with potential would be observed as a change in the parameter  $b$ .

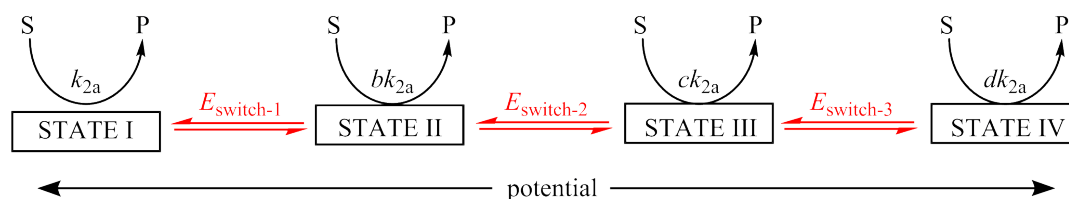
The extended model proposed in this chapter has shown that catalytic bias,

the ability of a catalysis to work preferentially in one direction, can stem from two sources. Firstly, it may arise due to a difference between the reduction potentials of the substrate-product couple undergoing conversion and the electrochemical control centre - the point at which electrons enter or exit the catalyst, a large difference between these two potentials leading to irreversibility (as proposed to be the case for the [NiFe]-hydrogenase *EcHyd1* from *Escherichia coli* in Chapter 3). Secondly, a potential-dependent transformation between catalytic states of differing absolute activities will lead to a potential-dependent variation in the observed activity of the catalyst. If the transformation potential,  $E_{\text{switch}}$ , lies close to the equilibrium potential of the substrate, a catalytic bias may result. The selective inhibition of a catalyst in a specific state may enhance such a catalytic bias as exemplified by CODH and the selective inhibition of the  $C_{\text{red2}}$  state by  $\text{NCO}^-$ . Although in this case it is an exogenous ligand which causes the inactivation, an obvious extrapolation includes the possibility that a suitably positioned endogenous ligand could also bind selectively to one particular redox state. Which one of these two scenarios is responsible for a particular instance of catalytic bias would not necessarily be clear from the fits of the model to experimental data and accompanying experimental results or knowledge would be required to gain a complete picture. The possible effect of an inhibitor on electron entry/exit into the catalyst and on the electron relay pathway, and the effect of an amino acid mutation near the substrate binding site on the wider enzyme function and structure, are examples of things that may need to be considered and explored, for example through spectroscopic titrations and structural studies, in order to find a definitive answer as to why a catalytic bias exists in a particular case.

A comparison between the anaerobic oxidative inactivation which occurs when [NiFe]-hydrogenases are subjected to high potential and the passivation which occurs at metallic surfaces has been made. In the case of the hydrogenase, the inactivation is due to the well characterised formation of the Ni-B state, an oxidation of  $\text{Ni}^{2+}$  to  $\text{Ni}^{3+}$  accompanied by one  $\text{OH}^-$  ligand bridging the Ni and Fe atoms within the active

site. On the other hand, passivation on metal surfaces, although also accompanied by binding of  $\text{OH}^-$  ligands, is much less well defined and the oxidation state of the surface atoms is often unknown as is the exact position of the  $\text{OH}^-$  ligands. It is possible that a mixture of oxidation states may result accounting for the sub-1 values of  $n'$  which are found from the fits to the voltammogram obtained from a Ru(0001) electrode.

In this chapter the most simple case is considered, a catalytic site which can be in one of two states. Only relatively few parameters are needed to reproduce the voltammetry observed with a number of surface-immobilised catalysts. However, the principles upon which this model is grounded can be used to account for a catalytic site that interconverts between any number of states, the potential dependent interconversion between each state being described by a different  $E_{\text{switch}}$  value and the relative activity of each state being described by another multiplication factor, as depicted in Scheme 4.2.



**Scheme 4.2:** A representation of a catalyst, capable of converting substrate, S, into product, P, which is able to interconvert between four states, referred to as State I, State II, State III and State IV. Interconversion between each state is potential-dependent and can be described by the value of  $E_{\text{switch}-x}$ . The inherent catalytic activity of each state can be described as a multiple of  $k_{2a}$  as indicated by the multiplication factors  $b$ ,  $c$  and  $d$  in the scheme.

Finally, this model allows us to theorise on the hypothetical waveshapes which would result from the electrocatalytic voltammetry of other immobilised catalysts, even if, in practice, the catalyst does not interact with an electrode. Nitrogenase, the enzyme responsible for reducing  $\text{N}_2$  to  $\text{NH}_3$ , requires reducing conditions and ATP for its catalytic activity, but the potentials involved are far more reducing than should be required to achieve the  $\text{N}_2 \rightarrow 2\text{NH}_3 + \text{H}_2$  conversion.<sup>147</sup> According to the model presented here, this large overpotential requirement may represent the free

energy required to maintain nitrogenase in an active state, rather than the energy required to drive each individual step of the eight-electron cycle. It may be the case that the overpotential requirement for many electrocatalytic reactions is simply the potential required to keep the catalyst in its active state.

## Chapter 5

# The Unusual Reaction of [NiFe]-Hydrogenases with Cyanide





## Abstract

This chapter explores an observation originally made in the 1940s, that cyanide inhibits microbial  $H_2$  oxidation. Here Protein Film Electrochemistry (PFE) provides an important new insight into the reaction taking place between exogenous cyanide and the [NiFe]-hydrogenases *EcHyd1* and *EcHyd2* from *Escherichia coli*.

Protein Film Electrochemistry, in which the catalytic activity of the enzyme is recorded as a direct and continuous function of the electrode potential, makes it possible to induce and observe reactions that alter the activity of an enzyme and to measure their rates and the characteristic potentials needed for them to occur. A detailed and quantitative description of enzyme activity is thus obtained that complements information provided by other methods. As recorded by PFE, under mildly oxidising conditions cyanide inhibits the electrocatalytic oxidation of hydrogen. Using PFE in conjunction with Electron Paramagnetic Resonance spectroscopy and Attenuated Total Reflectance Infrared spectroelectrochemistry, it is demonstrated that the final enzyme product is the inactive Ni-B state. Thus, cyanide inhibition is easily reversed because it is simply the reductive activation of Ni-B. This chapter addresses the interesting mechanism by which cyanide promotes the formation of Ni-B.

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## 5.1 Introduction

This chapter focuses on the widely unreported affect of cyanide on the catalytic capabilities of [NiFe]-hydrogenases. The majority of work in this chapter makes use of the technique of Protein Film Electrochemistry (PFE) to give a unique insight into the reactions taking place. This technique allows catalytic activity, observable as current, to be measured as a direct and continuous function of electrode potential, allowing a quantitative and detailed description of enzyme activity to be obtained (see Chapter 2). Protein Film Electrochemistry permits direct potential control of the enzyme film, allowing the effect of substrate and inhibitor to be determined at a precise potential, a crucial factor in the study of potential dependent reactions.

The PFE results are complemented by Electron Paramagnetic Resonance and Attenuated Total Reflectance Infrared spectroscopy (ATR-IR) studies that provide additional details of the effect of exogenous  $\text{CN}^-$  on the [NiFe]-hydrogenase *EcHyd2* from *Escherichia coli*.

The ATR-IR experiments described in this chapter were undertaken by Min-Wen Chung working under the supervision of Prof. Kylie Vincent, University of Oxford.

### 5.1.1 Previous Studies of the Effect of Cyanide on [NiFe]-Hydrogenases

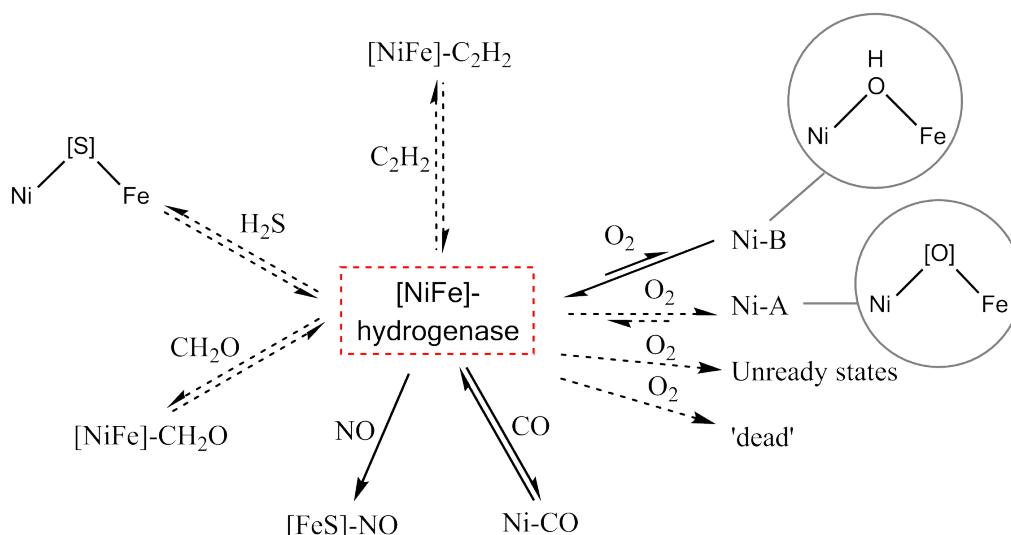
Previous studies investigating the affect of exogenous  $\text{CN}^-$  on [NiFe]-hydrogenases are sparse: Lee *et al.*<sup>148</sup> first reported the inhibitory effect of cyanide on the  $\text{H}_2$  oxidation activity of whole cell samples of *Azotobacter vinelandii* in 1942. Hyndman *et al.*<sup>149</sup> observed similar results in 1952 with cell-free extracts of *Azotobacter vinelandii* hydrogenase. They found that cyanide inhibited this hydrogenase to a greater extent when exposure was made in air, rather than when under an atmosphere of  $\text{H}_2$ .<sup>149</sup>

Seefeldt and Arp<sup>150</sup> published the first study on the effect of cyanide on purified hydrogenase from *Azotobacter vinelandii* in 1989. Using spectrophotometric assay methods, in which  $\text{H}_2$ -oxidation activity was monitored using methylene blue as an

electron acceptor and using  $O_2$  as an oxidant, they found cyanide to irreversibly inhibit enzyme under oxidising conditions. Addition of the reductant sodium dithionite during the course of the experiment immediately halted the inactivation process, but did not result in the reactivation of the enzyme which had previously been inactivated.<sup>150</sup>

### 5.1.2 Other Known Inhibitors of [NiFe]-Hydrogenases

Many studies have been undertaken which investigate the inhibitory effect of various small molecules on the [NiFe]-hydrogenases. By far the most thoroughly studied inhibitor is  $O_2$ , however there are many other known examples, as highlighted in Scheme 5.1, and discussed below.



**Scheme 5.1:** Summary of the known inhibitors of [NiFe]-hydrogenases as detailed in Section 5.1.2. Dotted arrows indicate inhibition mechanisms that only affect  $O_2$ -sensitive [NiFe]-hydrogenases. The known bridging positions of the oxygen ligands present in the Ni-A and Ni-B states are shown within grey circles. Represented by [O] in the figure, the exact nature of the bridging ligand in the Ni-A state is unclear but is commonly considered to be a  $OOH^-$  ligand.  $O_2$  attack of the  $O_2$ -sensitive [NiFe]-hydrogenases also leads to the formation of other Unready states and some irreversibly inactivated 'dead' enzyme, the structures of which are currently unknown. [S] represents either a  $S^{2-}$  or  $HS^-$  species that bridges the metal ions at the active site. CO is known to bind to the Ni atom.  $C_2H_2$  and  $CH_2O$  are both thought to attack the active site but the mechanisms are unclear. NO is thought to target the FeS clusters through two different pathways, one reversible and one irreversible.

### 5.1.2.1 Inhibition by Oxygen

The effect of  $O_2$  upon a [NiFe]-hydrogenase can vary depending upon the prevailing conditions at the time of exposure as well as the [NiFe]-hydrogenase in question. Under electron-rich conditions,  $O_2$ -sensitive [NiFe]-hydrogenases will form the quickly reactivating Ni-B state,<sup>110</sup> a  $Ni^{3+}$  state in which a  $OH^-$  ligand bridges the Ni and Fe atoms at the active site.<sup>26,39</sup> Under electron poor conditions, a number of slowly reactivating ‘Unready’ states may form.<sup>67,69</sup> This term is used to describe a number of inactive states that can be reactivated but only very slowly, in some cases needing over 24 hours for full reactivation. The rate of reactivation of these states may be, but is not necessarily, potential dependent. The most well characterised Unready state, spectroscopically defined by EPR spectroscopy by its  $g$ -values and commonly referred to as Ni-A, is thought to have a bridging  $OOH^-$  ligand at its active site.<sup>24,26</sup>

The ability of  $O_2$ -tolerant hydrogenases to carry out sustained catalysis in the presence of  $O_2$  is now known to be related to the ability of these enzymes to avoid the formation of Unready states, instead quickly forming the Ni-B state.<sup>4</sup> There are two important characteristics of the FeS-cluster relay chain of  $O_2$ -tolerant hydrogenases which enable the exclusive formation of Ni-B: (i) Instead of the [4Fe4S]-cluster found proximally to the active site in  $O_2$ -sensitive hydrogenases, the  $O_2$ -tolerant hydrogenases incorporate a [4Fe3S]-cluster capable of undergoing two redox transitions within a narrow potential window at high potential.<sup>17,31,33,34</sup> This enables the cluster to direct two electrons to the active site upon  $O_2$  attack (see Section 1.4.3 for more details on this unusual [4Fe3S]-cluster);<sup>4,32,69</sup> (ii) The reduction potential of the medial [3Fe4S]-cluster in  $O_2$ -tolerant hydrogenases is considerably higher than the reduction potentials of the equivalent cluster in  $O_2$ -sensitive hydrogenases.<sup>34</sup> This increases the likelihood of this cluster being in a reduced state and so able to transfer an electron to the active site when required.<sup>4</sup> Together the proximal cluster and the medial cluster in  $O_2$ -tolerant hydrogenases are able to transfer three electrons to the active site upon  $O_2$  attack. This is the maximum number of electrons

required to ensure that, no matter what state the catalytic site is in at the time of attack, *i.e.* Ni-R, Ni-C or Ni-SI (see Section 1.6 and 1.7 for details on the catalytic states involved in catalysis), the quickly reactivating Ni-B state can always be formed.<sup>4</sup> Once in the Ni-B state, a one electron reduction fully reduces the original O<sub>2</sub> molecule into H<sub>2</sub>O.<sup>18,36</sup>

Exposure to O<sub>2</sub> may also lead to some irreversibly inactivated states, or ‘dead’ states.<sup>151</sup> It has been suggested that this may be due to the oxidation of the cysteine-sulfur atoms near to the active site<sup>110</sup> or due to damage of the FeS-relay chain.<sup>151</sup>

At high potentials, the Ni-B state is also formed anaerobically, as indicated by the decrease in current seen within the high potential region of cyclic voltammograms of both O<sub>2</sub>-tolerant and O<sub>2</sub>-sensitive [NiFe]-hydrogenases,<sup>132</sup> as can be seen in the cyclic voltammogram of *EcHyd2* shown in Figure 4.2 in Chapter 4.

#### 5.1.2.2 Inhibition by Other Small Molecules

Carbon monoxide (CO) acts as a competitive inhibitor of H<sub>2</sub>-cycling catalysis in O<sub>2</sub>-sensitive,<sup>152</sup> and to a much lesser extent O<sub>2</sub>-tolerant,<sup>122,153</sup> [NiFe]-hydrogenases, binding as a terminal ligand to the Ni atom at the active site.<sup>76,154</sup> Upon the removal of CO gas from the atmosphere full activity is restored.<sup>153</sup> A site directed mutagenesis study, in which two cysteine residues that usually ligate to the proximal FeS-cluster within the O<sub>2</sub>-tolerant [NiFe]-hydrogenase *EcHyd1* from *Escherichia coli* were exchanged with glycines, resulted in O<sub>2</sub>-sensitive enzymes which had only very small changes in sensitivity to CO relative to the native enzyme, thus indicating that O<sub>2</sub>-tolerance and insensitivity to CO are not necessarily related.<sup>69</sup> A second study on *EcHyd1*, in which a proline residue at the medial [3Fe4S]-cluster was exchanged for a cysteine, resulted in the formation of an O<sub>2</sub>-sensitive enzyme that was largely CO-tolerant,<sup>4</sup> adding further weight to the argument that O<sub>2</sub>-sensitivity is not a direct indicator of CO-sensitivity.

Sulfide reversibly inhibits O<sub>2</sub>-sensitive [NiFe]-hydrogenases but PFE studies have shown that the product of the inhibition is stable only at high potentials.<sup>155</sup> It

has also been shown that sulfide cannot react with the Ni-B form of the enzyme, the conclusion being that the site of attack is the active site rather than an FeS-cluster.<sup>155</sup> Crystallographic data suggests that sulfide binds in a bridging position between the Ni and Fe atom at the active site, either as  $S^{2-}$  or as  $HS^-$  (analogous to the  $OH^-$  bridging ligand in the Ni-B species).<sup>156</sup> An increase in pH leads to a decrease in the rate of inactivation, consistent with the sulfide species entering the enzyme as  $H_2S$  (a charged  $HS^-$  species may be less able to pass through the hydrophobic gas channels which lead to the active site).<sup>157,158</sup> Experiments on the  $O_2$ -tolerant [NiFe]-hydrogenases from *Ralstonia entrophae*, *Ralstonia metallidurans* and *Escherichia coli* (*EcHyd1*) have shown that the hydrogenases from *Ralstonia* sp. do not react with sulfur<sup>157,158</sup> whilst inhibition can only be observed in *EcHyd1* when sulfur is introduced under low  $H_2$  conditions.<sup>158</sup>

Nitric oxide (NO) has been found to irreversibly inhibit the as-isolated form of the [NiFe]-hydrogenase *Alcaligenes (Ralstonia) entrophae* H16 under non-turnover conditions.<sup>159</sup> It was found that under  $H_2$ -oxidising conditions, approximately 50% of the activity could be recovered after removal of NO.<sup>159</sup> This effect of NO inhibition was later also observed on the [NiFe]-hydrogenase from *Azotobacter vinelandii*.<sup>160</sup> In both cases, and in later studies on the  $O_2$ -sensitive hydrogenase from *Allochromatium vinosum* and the  $O_2$ -tolerant hydrogenase from *Ralstonia metallidurans* CH34,<sup>158</sup> there was no element of competition found between NO and  $H_2$ <sup>158–160</sup> suggesting that NO does not target the active site, but instead targets the FeS clusters, the reversible and irreversible components of the inhibition being related to two distinct interactions of NO with the FeS centres.<sup>160</sup>

Acetylene ( $C_2H_2$ ) inhibition of [NiFe]-hydrogenase function was first reported by Smith and co-workers in 1976.<sup>161</sup> Later studies reported that this inhibition was reversible and that  $H_2$  and CO protected the  $O_2$ -sensitive [NiFe]-hydrogenase from *Azobacter vinelandii* from  $C_2H_2$  inhibition.<sup>162,163</sup> It was also reported that only the active form of this enzyme (formed by reductive activation of aerobically inactivated enzyme) was sensitive to  $C_2H_2$  and that as-isolated, aerobically prepared, inac-

tive hydrogenase was not affected by  $C_2H_2$ .<sup>163</sup> Electrochemistry studies on the  $O_2$ -sensitive hydrogenases from *Azobacter vinelandii*, *Desulfovibrio gigas*, *Desulfovibrio vulgaris* and *Escherichia coli* (*EcHyd2*) found that  $C_2H_2$  inhibits the active form of each of these enzymes.<sup>158</sup> This study also reinforced the conclusion that the presence of  $H_2$  and CO protects these enzymes from  $C_2H_2$  inhibition, an observation consistent with  $C_2H_2$  targeting the active site.<sup>158</sup> Both spectroscopic and electrochemistry studies have been unable to detect any inhibition by  $C_2H_2$  of [FeFe]-hydrogenases, indicating that  $C_2H_2$  may bind to the Ni atom at the active site of [NiFe]-hydrogenases.<sup>158,164</sup> Although early studies suggested that the  $O_2$ -tolerant [NiFe]-hydrogenases from *Alcaligenes (Ralstonia) entrophia* H16 could be inhibited by  $C_2H_2$ ,<sup>162</sup> later electrochemical studies investigating the affect of  $C_2H_2$  on the  $O_2$ -tolerant [NiFe]-hydrogenases from *Ralstonia metallidurans* and *Escherichia coli* (*EcHyd1*) found no affect of  $C_2H_2$  on these enzymes.<sup>158</sup>

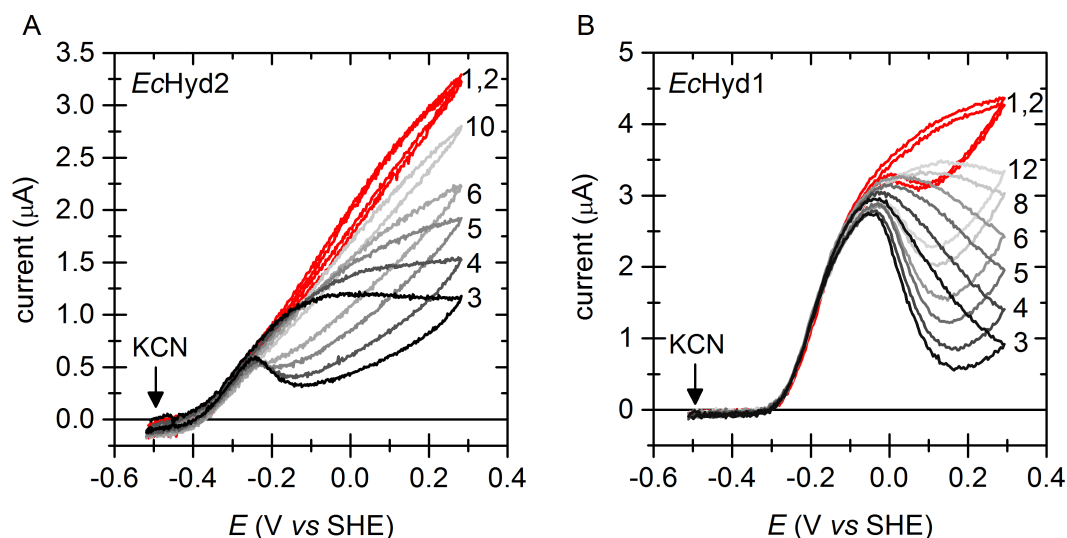
Finally, electrochemical studies on the effect of formaldehyde ( $CH_2O$ ) on the  $O_2$ -sensitive hydrogenase *EcHyd2* from *Escherichia coli* have shown that  $CH_2O$  does have a small, reversible effect on  $H^+$  reduction activity, however the sensitivity to  $CH_2O$  is far less than that found in [FeFe]-hydrogenases.<sup>165</sup> Furthermore,  $CH_2O$  has no effect on the  $H_2$  oxidation activity of *EcHyd2*, again in contrast to the  $CH_2O$  inhibition of [FeFe]-hydrogenases.<sup>165</sup>

## 5.2 Protein Film Electrochemistry Studies

### 5.2.1 Cyclic Voltammetry Studies of the Effect of Cyanide on *EcHyd1* and *EcHyd2*

The use of Protein Film Electrochemistry (PFE) coupled with cyclic voltammetry allows any potential dependent effect that an inhibitor may have upon a redox enzyme to be easily visualised. The affect of cyanide on the [NiFe]-hydrogenases *EcHyd2* and *EcHyd1* from *Escherichia coli* over a potential range of 810 mV was assessed (Figure 5.1). In each case, two scans were completed in the absence of

cyanide (shown in red), before 0.15 mL of 20 mM KCN solution (made by dissolving KCN in buffer identical to that present in the electrochemical cell) was injected into the cell solution (2 mL initial volume), leading to a concentration of 1.4 mM KCN in the cell immediately following the injection at the start of scan three.



**Figure 5.1:** The effect of cyanide on (A) *EcHyd2* and (B) *EcHyd1* when introduced at  $-520$  mV (as indicated by an arrow) during a set of cyclic voltammetry scans. Scans prior to the introduction of cyanide are shown in red and those after are shown in shades of grey. In both cases cyanide was introduced at the start of the third scan (the potential was swept from low to high between  $-520$  mV and  $+290$  mV), to give a final concentration of 1.4 mM KCN in the cell immediately after the injection. The scan number is indicated alongside each plot. In order to minimise the rate at which HCN is flushed from the cell, the electrode was rotated at 100 rpm and the gas flow was turned off. Conditions: pH 7,  $25$  °C,  $\nu = 4$  mV s $^{-1}$ . The mixed hydrogenase buffer was equilibrated to 100% H $_2$  prior to the start of each experiment. Figure adapted from Reference 166.

The introduction of cyanide does not have an effect on the H $^+$  reduction or H $_2$  oxidation activity of *EcHyd2* below approximately  $-250$  mV (Figure 5.1A). However, as the potential is swept to values above  $-250$  mV, the presence of cyanide results in a decrease in current compared to when no cyanide is present. The inactivation that is demonstrated by the drop in current continues throughout the cyclic voltammogram until a sufficiently low potential is reached during the reductive sweep (*ca.*  $-120$  mV) at which point reactivation of the enzyme commences, as seen by a sharp increase in current with decreasing driving force until, eventually, the activity of the enzyme returns to its uninhibited value.

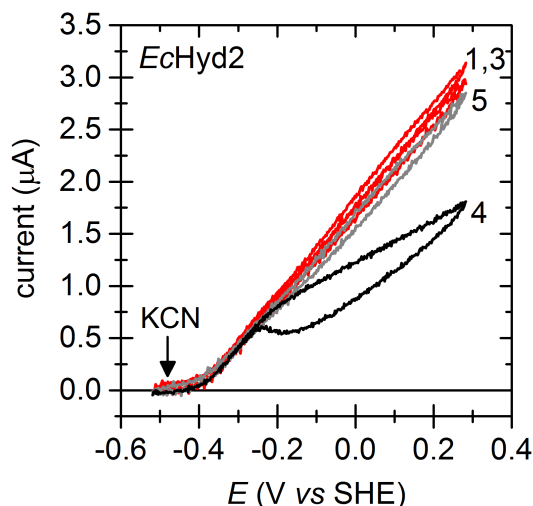


The inhibitory effect of cyanide is not specific to *EcHyd2* and Figure 5.1B shows an analogous experiment on *EcHyd1*. In this case a higher potential (driving force) is necessary for cyanide to have an inhibitory effect, with a decrease in current being observed at potentials over  $-100$  mV. Like *EcHyd2*, the inactivation is easily reversed and reactivation is observed during the reductive sweep starting at potentials below approximately  $+160$  mV.

The  $pK_a$  value of HCN is 9.21,<sup>167</sup> thus essentially all the cyanide in the cell at pH 7 will be in the HCN form. Gaseous HCN will be quickly flushed from the cell and the  $CN^-/HCN$  equilibrium will thus shift to replace the lost HCN and cyanide will be gradually depleted. This accounts for the diminishing effect of cyanide seen over successive scans (Figure 5.1). The rate at which HCN is removed from the electrochemical cell can be limited by stopping the flow of  $H_2$  through the cell and by employing a slow electrode rotation rate (100 rpm), as done in the experiments shown in Figure 5.1. However, usually PFE experiments are carried out under a continuous flow of gas (*e.g.*  $H_2$ ) and with the working electrode rotating fast enough to ensure that mass transfer limiting conditions are avoided (Section 2.1.3.4). Under the conditions of the experiments shown in Figure 5.1 the catalytic reaction will gradually become substrate limited, however the scans obtained prior to the injection of KCN solution show minimal evidence of this. The Michaelis constants,  $K_M^{H_2}$ , for *EcHyd1* and *EcHyd2* have been calculated to be  $9 \pm 1$   $\mu$ M and  $17 \pm 4$   $\mu$ M respectively at  $-175$  mV at  $30$  °C at pH 6.<sup>122,142</sup> These values are sufficiently low that partial depletion of the substrate is not an issue and both enzymes are able to tolerate these conditions without a significant effect on activity over the short duration of the experiment.

A comparative experiment showing the effect of cyanide on *EcHyd2* when gas is flowing through the cell and the electrode rotation rate is set to 3500 rpm is shown in Figure 5.2. Appendix F details ultraviolet-visible spectroscopic studies which measure the rate of decrease in  $CN^-$  concentration under these conditions.

Similar to the data shown in Figure 5.1A, Figure 5.2 shows that the introduction



**Figure 5.2:** The effect of cyanide on *EcHvd2* when introduced at  $-520$  mV during a set of cyclic voltammetry scans where the working electrode was rotated at 3500 rpm and the gas flow (100%  $H_2$ ) was set at a rate of 500 sccm. Scans prior to the introduction of cyanide are shown in red and those after are shown in shades of grey. Cyanide was introduced at the start of scan 4 at  $-520$  mV as indicated by the arrow and the potential was swept from low ( $-520$  mV) to high ( $+290$  mV). The scan number is indicated alongside each plot. Conditions: pH 7 mixed hydrogenase buffer,  $25^\circ C$ ,  $\nu = 4 \text{ mV s}^{-1}$ . The final concentration of KCN in the cell solution immediately after the injection was 1.4 mM.

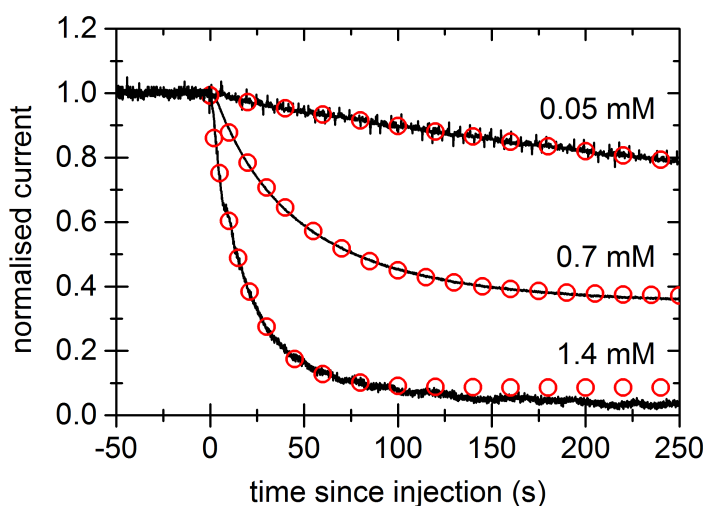
of cyanide to *EcHvd2* under conditions with a gas flow and an electrode rotation rate of 3500 rpm, leads to a reduction in current at potentials above approximately  $-250$  mV. A reactivation of the inactive enzyme can again be detected as the potential is lowered below *ca.*  $-120$  mV. However, the decrease in current relative to the scans obtained when no cyanide is present is less, reflecting the lower concentration of cyanide in the cell due to HCN flushing out at a higher rate.

## 5.2.2 Elucidating the Mechanism of the Reaction between Cyanide and *EcHvd2*

### 5.2.2.1 The Effect of Cyanide and Hydrogen Concentration

In a chronoamperometry experiment the potential is held at a constant value allowing current to be recorded as a function of time. This approach deconvolutes the potential and time domains, allowing rates of inactivation and reactivation to be

determined. Figure 5.3 shows experiments carried out at +394 mV with *EcHyd2*. In these experiments the current was left to stabilise for approximately 200 s, before a 0.15 mL aliquot of KCN solution with an initial concentration of 0.7 mM, 10 mM or 20 mM was injected into the cell solution (2 mL initial volume) resulting in a concentration of 0.05 mM, 0.7 mM or 1.4 mM KCN in the cell solution immediately after the injection, respectively. In order to minimise the rate at which HCN was flushed out of the cell, the gas flow through the cell was turned off and the rotation rate was set to 100 rpm for the duration of the experiment. The buffer solution in the cell and the buffer used to make up the cyanide solution were both equilibrated to the conditions of the experiment beforehand. The data have been normalised to account for film loss (see Section 2.3.1.5).



**Figure 5.3:** The effect of cyanide on *EcHyd2* when introduced at +394 mV during a chronoamperometry scan. An aliquot of cyanide was injected into the electrochemical cell at  $t = 0$  s resulting in a final concentration of 0.05 mM, 0.7 mM and 1.4 mM KCN, as indicated. The current has been normalised to account for film loss. In each case, the first ‘fast’ phase of inactivation has been fitted to a single exponential (red circles). Conditions: pH 7, 25 °C,  $\omega = 100$  rpm. The headspace of the cell and the mixed hydrogenase working buffer solution were equilibrated to 100%  $\text{H}_2$  prior to the start of each experiment. The gas flow was then turned off immediately before the start of each experiment. Figure adapted from Reference 166.

The introduction of cyanide leads to a reduction in current in all cases, indicating a decrease in the activity of *EcHyd2*, with the rate of inactivation increasing with cyanide concentration. Over time a limiting current is established. The initial, ‘fast’

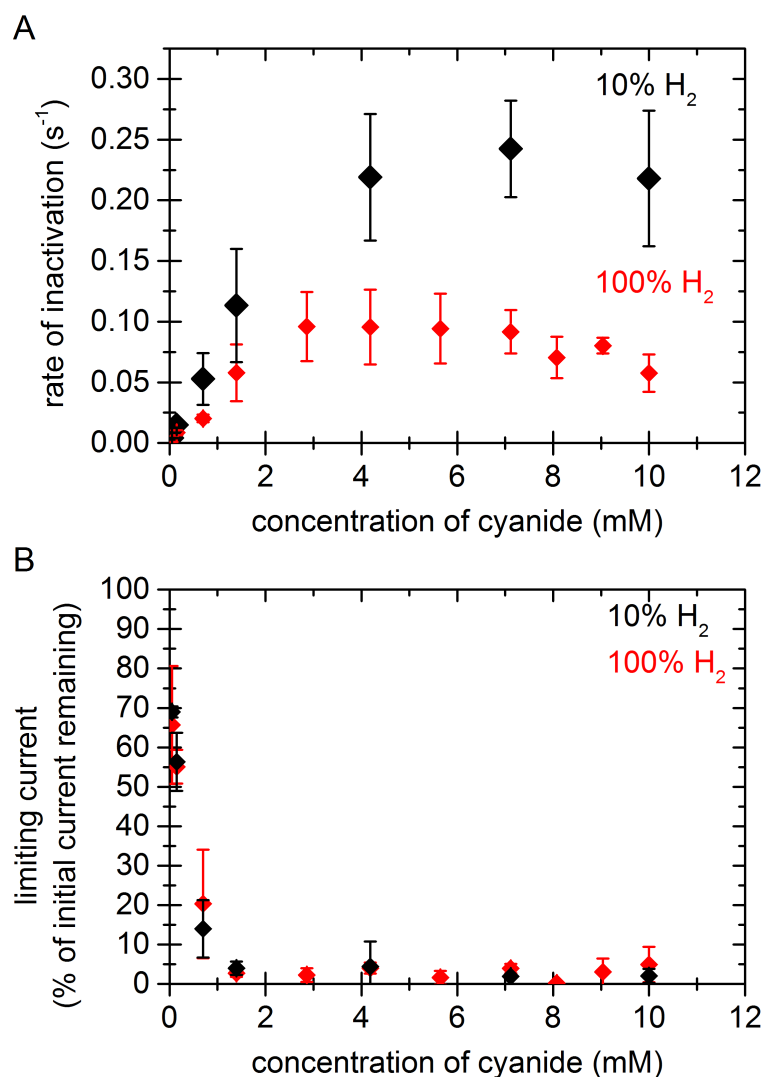
phase of inactivation, accounting for at least the first three half lives and 80% of the decrease in current, can be fitted to a single exponential (represented as red circles in Figure 5.3) showing that the reaction is first order with respect to cyanide. The fit allowed the rate of the inactivation to be determined in each case. The fits in Figure 5.3 give rates of  $0.00251 \text{ s}^{-1} \pm 6.714 \times 10^{-5}$  (0.05 mM),  $0.02033 \text{ s}^{-1} \pm 3.386 \times 10^{-5}$  (0.7 mM) and  $0.0524 \text{ s}^{-1} \pm 2.314 \times 10^{-4}$  (1.4 mM).

Similar experiments, in which a number of different cyanide concentrations were used, were carried out under atmospheres of both 100% and 10%  $\text{H}_2$  (Figure 5.4). The potential used in each experiment was adjusted to take account of the changing value of  $E_{2\text{H}^+/\text{H}_2}$  with  $\text{H}_2$  concentration (cyanide was introduced at a potential +807 mV above  $E_{2\text{H}^+/\text{H}_2}$  in all cases).

The rate at which *EcHyd2* is inactivated by cyanide is dependent upon the concentration of both cyanide and  $\text{H}_2$  in the atmosphere. In general, a higher concentration of cyanide results in a faster rate of inactivation, however a slight decrease in the rate of inactivation can be observed for the highest concentrations of cyanide used in this study. At all cyanide concentrations used, a faster rate of inactivation occurs under an atmosphere of 10%  $\text{H}_2$  in  $\text{N}_2$  compared to 100%  $\text{H}_2$ . The limiting current reached is similar under both atmospheres and at concentrations above approximately 1 mM KCN the limiting current remains at about 3% of the initial current.

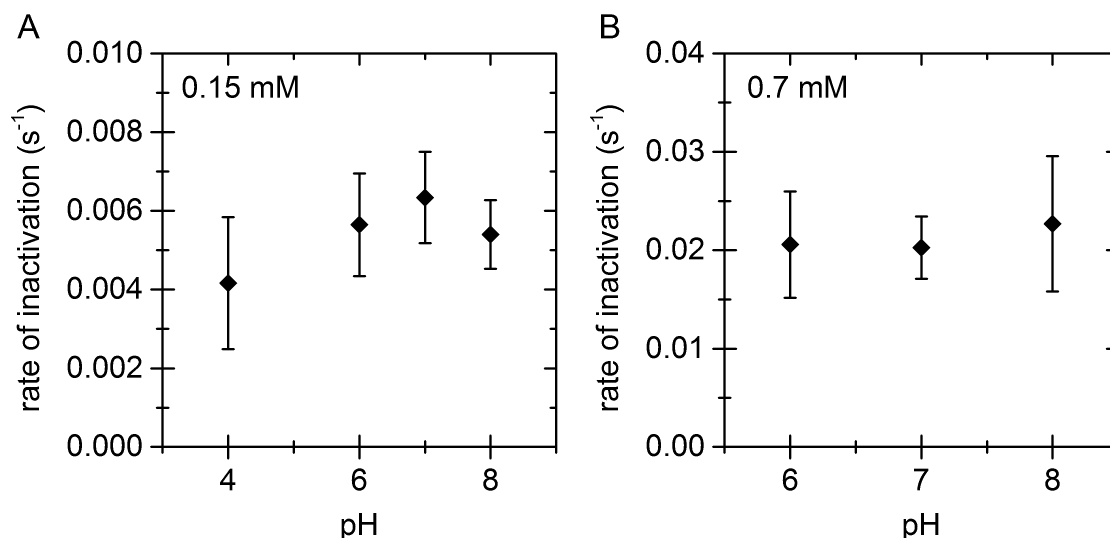
### 5.2.2.2 The Effect of pH

Through undertaking experiments identical to those shown in Figure 5.3, but in buffer titrated to different pH values, the effect of pH on the rate and extent of the inactivation of *EcHyd2* due to cyanide could be investigated (Figure 5.5). Experiments were undertaken in which the concentration of cyanide directly after the injection of KCN solution into the cell was either 0.15 mM (Figure 5.5A) or 0.7 mM (Figure 5.5B). The potential used in each experiment was adjusted to take account of the changing value of  $E_{2\text{H}^+/\text{H}_2}$  with pH, and set as +807 mV above



**Figure 5.4:** A summary of the effect of cyanide on *EcHvd2* when introduced at 807 mV above  $E_{2\text{H}^+/\text{H}_2}$  during a chronoamperometry experiment. (A) The relationship between the concentration of cyanide directly after injection of KCN solution into the cell and the rate of inactivation under an atmosphere of 100% and 10%  $\text{H}_2$  in  $\text{N}_2$ . Rates were found by fitting at least the first three half lives of the inactivation current to a single exponential, as exemplified in Figure 5.3. (B) The relationship between the concentration of cyanide directly after injection of KCN solution into the cell and the limiting current reached after inactivation (measured 400 s after the injection of cyanide) under an atmosphere of 100% and 10%  $\text{H}_2$  in  $\text{N}_2$ . Conditions: pH 7, 25  $^\circ\text{C}$ ,  $\omega = 100$  rpm. The headspace of the cell and the mixed hydrogenase working buffer solution were equilibrated to 100% or 10%  $\text{H}_2$  prior to the start of each experiment. The gas flow was then turned off immediately before the start of each experiment.

$E_{2H^+/H_2}$  in each case.

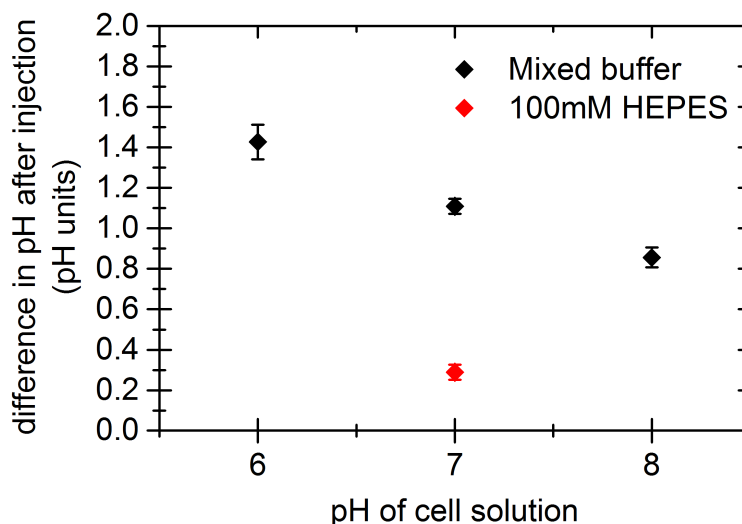


**Figure 5.5:** A summary of the pH dependence of the effect of cyanide on *EcHyd2* when introduced at 807 mV above  $E_{2H^+/H_2}$  during a chronoamperometry experiment. (A) Concentration of cyanide directly after injection of KCN solution = 0.15 mM; (B) Concentration of cyanide directly after injection of KCN solution = 0.7 mM. Rates were found by fitting at least the first three half lives of the inactivation current to a single exponential. Error bars indicate the standard deviation of three repeats. Conditions: 25 °C,  $\omega$  = 100 rpm. The headspace of the cell and the mixed hydrogenase working buffer solution were equilibrated to 100%  $H_2$  prior to the start of each experiment. The gas flow was then turned off immediately before the start of each experiment.

Upon increasing the pH from 4 to 8, there is a significant increase in the amount of  $CN^-$  relative to HCN in solution (the  $pK_a$  value of HCN is 9.21<sup>167</sup> corresponding to the concentration of  $CN^-$  being 0.0006% and 6% that of HCN at pH 4 and pH 8 respectively). However, within this pH range the rate of inactivation upon the introduction of cyanide into the cell solution was not significantly altered.

### 5.2.2.3 The Effect of Buffer Composition

In order to ensure that the effect observed upon cyanide introduction was not due to a change in the pH, the pH of the cell solution before and after KCN addition was monitored. Two different buffer compositions, ‘mixed hydrogenase buffer’ (see Section 2.3.4) and 100 mM HEPES buffer, were used, and in both cases the change in pH upon introduction of the highest KCN concentration used in this study, giving a final concentration of 10 mM, was monitored (Figure 5.6).



**Figure 5.6:** The change in pH of the cell solution upon injecting 0.15 mL KCN solution (initial concentration 143 mM) into 2 mL cell solution made up of either mixed hydrogenase buffer initially titrated to pH 6, 7 or 8 (black) or 100 mM HEPES buffer initially titrated to pH 7 (red). The injection resulted in 10 mM of cyanide in the solution directly after the injection. All pH values were measured at 25 °C. Error bars reflect the standard deviation of three repeats.

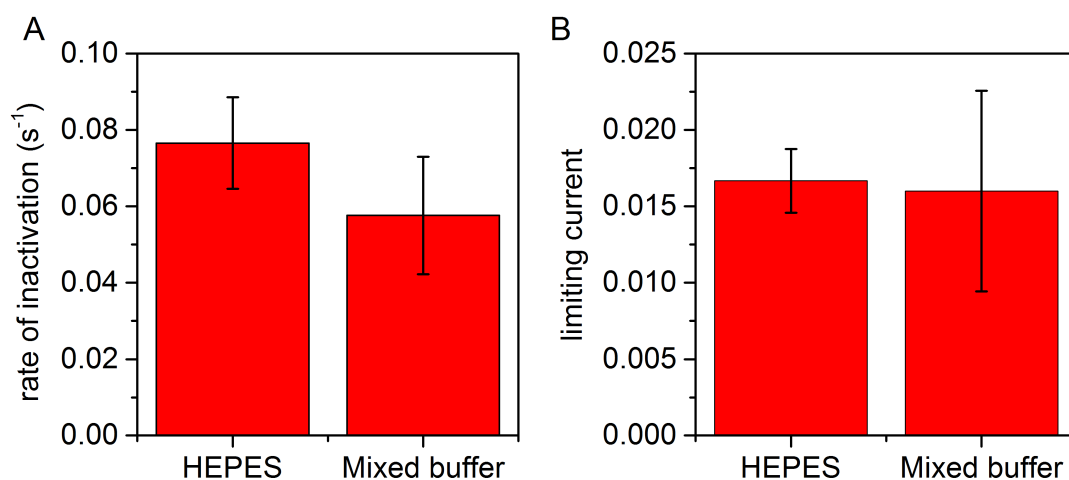
Addition of a high concentration of KCN into mixed hydrogenase buffer does lead to a substantial increase in pH, a change which decreases in magnitude with increasing pH of the buffer. Addition of the same concentration of KCN into 100 mM HEPES buffer has a significantly smaller effect.

The rate and extent of inhibition of *EcHvd2* upon addition of 0.15 mL, 143 mM KCN solution into 2 mL cell solution, resulting in 10 mM of cyanide in the solution directly after the injection, was monitored in mixed hydrogenase buffer and 100 mM HEPES buffer which in both cases had been initially titrated to pH 7 (Figure 5.7).

No significant difference in the rate or extent of inactivation of *EcHvd2* upon the introduction of cyanide can be observed between experiments in mixed hydrogenase buffer and those in 100 mM HEPES buffer. This indicates that the pH change observed in Figure 5.6 is not having a significant role in the inactivation observed.

#### 5.2.2.4 The Effect of Potential

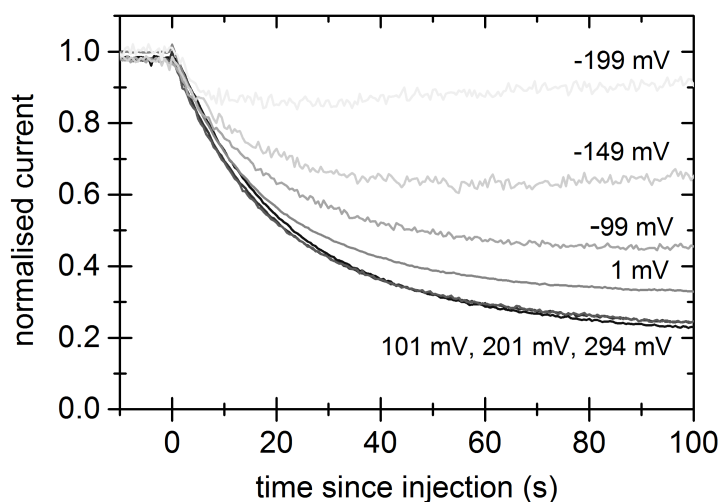
From Figure 5.1 it is clear that potential does have an effect on the observed behaviour of *EcHvd1* and *EcHvd2* in the presence of cyanide. To distinguish whether



**Figure 5.7:** The difference in (A) the rate of inactivation and; (B) the limiting current (measured 400 s after the injection and plotted as a percentage of the initial current measured before the introduction of cyanide), upon the injection of 0.15 mL KCN solution (initial concentration 143 mM) into 2 mL cell solution made up of either mixed hydrogenase buffer or 100 mM HEPES buffer as indicated. A concentration of 10 mM KCN was in the cell immediately following the injection. The rate was found by fitting a single exponential to the data obtained in the first 100 s after the injection. Error bars reflect the standard deviation from at least four repeats. Conditions: pH 7, 25 °C,  $\omega = 100$  rpm. The headspace of the cell and the working buffer were equilibrated to 100%  $H_2$  prior to the start of each experiment. The gas flow was then turned off immediately before the start of each experiment.



it is a potential dependent inactivation or reactivation process which is observed, chronoamperometry experiments were completed in which KCN solution was introduced into the cell at different potentials (Figure 5.8). In all cases, the current was left to stabilise for approximately 200 s before 0.15 mL of 20 mM KCN solution was injected into 2 mL cell solution, resulting in a final concentration of 1.4 mM KCN. In order to achieve an accurate value for the initial rate of inactivation, it was important that the KCN solution was mixed thoroughly and quickly after the injection into the cell. To ensure this, a high rotation rate of 3500 rpm was used under a gas flow of 100%  $\text{H}_2$  at 500 sccm.



**Figure 5.8:** The effect of cyanide on *EcHyd2* when introduced during a chronoamperometry experiment at various potentials as indicated. In each case, an aliquot of cyanide was injected into the electrochemical cell containing mixed hydrogenase working buffer at  $t = 0$  s resulting in a final concentration of 1.4 mM KCN. The data have been normalised to account for film loss. Conditions: pH 7, 25 °C,  $\omega = 3500$  rpm, 100%  $\text{H}_2$  at 500 sccm. Figure adapted from Reference 166.

Figure 5.8 shows the changing affect of cyanide on *EcHyd2* with potential; in each experiment, the initial rate of inactivation upon injection of KCN solution is similar, and the inactivation traces overlay for all potentials over the first 5 s immediately following the injection (the traces shown give an average initial rate of inactivation of  $0.0290 \text{ s}^{-1}$  with a standard deviation of  $0.00468 \text{ s}^{-1}$  as found from the slope of a linear fit to the first 5 s of data immediately following the injection). After this point, reactivation (seen as an increase in current with time) is evident

in the experiments carried out at  $-149$  mV and  $-199$  mV as this process starts to become dominating. At higher potentials reactivation is not visible. A comparison of experiments carried out at  $-99$  mV,  $+1$  mV and  $+101$  mV show differences in the limiting current reached in each case, indicative of a difference in the position of the equilibrium between the inactive and active species at each potential. Increasing the potential above  $+101$  mV does not change the magnitude of the limiting current any further and the position of the equilibrium is therefore potential independent in this potential region.

#### 5.2.2.5 The Reactivation of the Cyanide Inhibited State

The parameter  $E_{\text{switch}}$  has previously been used to compare different potential dependent inactive states and the ease at which these states can be reactivated in different hydrogenases.<sup>122,155</sup>  $E_{\text{switch}}$  is defined as the midpoint potential for the reactivation of an inactive state, found in PFE experiments from the minima of the first derivative of a slow reductive voltammetric sweep directly following inactivation.<sup>67,132</sup>

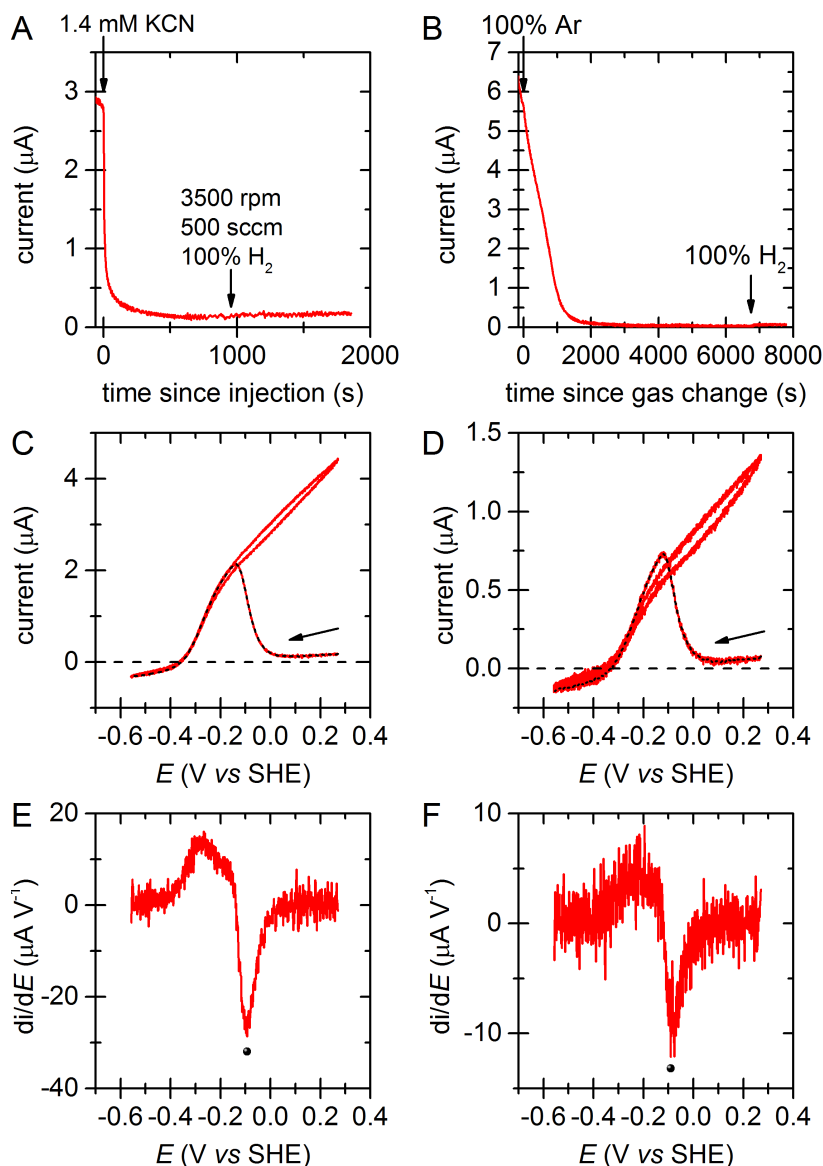
In order to achieve an accurate value for  $E_{\text{switch}}$  it is important that the enzyme is first inactivated, and then reactivated, to the maximum extent in both cases. To this end, the inactivation (depicted in Figure 5.9A) was achieved under a low  $\text{H}_2$  concentration (10%  $\text{H}_2$  in Ar) with a low rotation rate (100 rpm) and with the gas flow turned off. The potential was held at  $+453$  mV for approximately 200 s before 0.15 mL of 20 mM KCN solution was injected into 2 mL cell solution giving a final concentration of 1.4 mM KCN in the cell. This potential was maintained for *ca.* 1000 s, ensuring a limiting current had been reached, before the gas flow was turned back on, at 100%  $\text{H}_2$  (500 sccm), and the rotation rate set to 3500 rpm. To ensure full equilibration of the atmosphere with the cell solution and removal of cyanide from the cell the chronoamperometry step was continued for at least a further 800 s before a cyclic voltammetry scan was started from high ( $+282$  mV) to low ( $-547$  mV) potential at a scan rate of  $1 \text{ mV s}^{-1}$  (Figure 5.9C). The use of a slow scan rate

was necessary to ensure an accurate value of  $E_{\text{switch}}$  was obtained. An atmosphere of 100%  $\text{H}_2$  ensured that the maximum extent of reactivation was achieved.

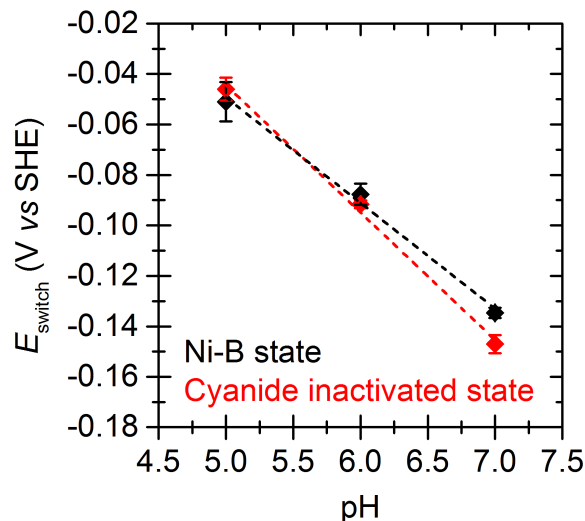
The values of  $E_{\text{switch}}$  obtained from the cyanide inactivated species were then compared to the  $E_{\text{switch}}$  values obtained from enzyme which had been anaerobically inactivated at high potential, in the absence of cyanide or any other known inhibitor, forming the well characterised Ni-B state in which a  $\text{OH}^-$  ligand bridges the Ni and Fe atoms of the active site (Section 1.6). In order to find accurate values of  $E_{\text{switch}}$  for the Ni-B state the potential was held at +453 mV for approximately 200 s under 100%  $\text{H}_2$  before exchanging the  $\text{H}_2$  atmosphere completely with Ar (Figure 5.9B). It has been shown that  $\text{H}_2$  protects the active site from forming the Ni-B state<sup>132</sup> and this atmosphere exchange was necessary to speed up the complete conversion of active enzyme into the inactive Ni-B state. After complete inactivation, the atmosphere was changed back to 100%  $\text{H}_2$  before the start of a high (+282 mV) to low (−547 mV) potential cyclic voltammetry scan (Figure 5.9D), the high concentration of  $\text{H}_2$  ensuring the greatest extent of reactivation of the Ni-B state and so the most accurate determination of  $E_{\text{switch}}$ . In order to compare the value of  $E_{\text{switch}}$  across different experiments it was imperative that the scan rate was the same in all cases ( $\nu = 1 \text{ mV s}^{-1}$ ).

The minima of the first derivative of the initial high to low potential sweeps shown in Figures 5.9C and D give values of  $E_{\text{switch}} = -93 \text{ mV}$  and  $E_{\text{switch}} = -91 \text{ mV}$  respectively (indicated by black spheres in Figures 5.9E and F respectively). In order to assess whether the  $E_{\text{switch}}$  profile of the cyanide inhibited state and the Ni-B state varied differently with pH, experiments analogous to those described above were completed in mixed hydrogenase buffer titrated to pH 5, 6 and 7 (Figure 5.10). In experiments with cyanide, the chronoamperometry step was completed at +778 mV above  $E_{2\text{H}^+/\text{H}_2}$  (in 10%  $\text{H}_2$  at the appropriate pH) in each case, *i.e.* the applied potential for the chronoamperometry step was +512 mV, +453 mV and +394 mV for pH 5, 6 and 7 respectively.

No significant difference is observed between the  $E_{\text{switch}}$  values obtained from



**Figure 5.9:** The experimental procedure and analysis used to obtain an  $E_{\text{switch}}$  value for the reactivation of the cyanide inhibited (panels A, C and E) and Ni-B (panels B, D and F) states. (A) Chronoamperometry trace ( $E = +453$  mV) showing inactivation due to cyanide. Prior to the start of the experiment the cell solution was equilibrated with 10%  $\text{H}_2$  in Ar. At  $t = 0$  s, KCN solution was injected into the cell resulting in a final concentration of 1.4 mM KCN (100 rpm, no gas flow). After *ca.* 1000 s, the rotation rate was set to 3500 rpm and the gas flow set to 100%  $\text{H}_2$  at 500 sccm for *ca.* 800 s. (B) Chronoamperometry ( $E = +453$  mV) trace showing inactivation due to Ni-B formation. The cell headspace and working buffer solution were equilibrated with 100%  $\text{H}_2$  before exchanging to 100% Ar at  $t = 0$  s. After *ca.* 7000 s the gas flow was returned to 100%  $\text{H}_2$  for *ca.* 1000 s to ensure full gas equilibration. Total gas flow = 500 sccm and  $\omega = 3500$  rpm throughout. (C) and (D) Voltammograms showing a scan from high (+282 mV) to low (-547 mV) potential made directly after the inactivation procedures shown in panels A and B followed by a full cyclic voltammogram (100%  $\text{H}_2$  at 500 sccm,  $\omega = 3500$  rpm,  $\nu = 1$  mV s $^{-1}$ ). The arrows indicate the direction of the scan. In order to get an accurate value for  $E_{\text{switch}}$ , the first reductive sweep was smoothed using a Savitzky-Golay function with 10 points of window (black dashed line). (E) and (F) The first derivative of the smoothed data shown in (C) and (D) respectively. The potential corresponding to  $E_{\text{switch}}$  is shown as a black sphere in each case. Conditions in all panels: mixed hydrogenase buffer titrated to pH 6, 25  $^{\circ}\text{C}$ .



**Figure 5.10:** A summary of the values of  $E_{\text{switch}}$  obtained from the reactivation of the cyanide inactivated state (red) and Ni-B state (black) in mixed hydrogenase buffer titrated to pH 5, 6 and 7 prior to the experiment. Error bars reflect the standard deviation from three repeats. A line of best fit has been applied to each dataset (dashed line) giving a gradient of  $-51$  mV and  $-42$  mV for the cyanide inactivated state and Ni-B state respectively. Figure adapted from Reference 166.

*EcHyd2* inactivated *via* the two different procedures at pH 5, 6 and 7 (Figure 5.10). The error bars for the reactivation of the two states, found from the standard deviation of three repeats, overlap at pH 5 and pH 6. In both cases,  $E_{\text{switch}}$  decreases with increasing pH. A line of best fit gives gradients of  $-51$  mV and  $-42$  mV per pH unit for the cyanide inactivated and Ni-B states respectively.

### 5.3 Electron Paramagnetic Resonance Studies

Protein Film Electrochemistry, although very useful for studying the rates of inactivation and reactivation of an electrocatalyst and the affect that the ambient conditions have upon these rates, is not able to give structural insight into the different states involved in a process. In order to obtain this information it is necessary to complement electrochemistry studies with those obtained through spectroscopic methods. This section details Electron Paramagnetic Resonance (EPR) studies undertaken to (i) characterise the inactive state formed as a result of cyanide inactivation of *EcHyd2*, and (ii) attempt to isolate any intermediates formed during

this reaction.

### 5.3.1 EPR Characterisation of the Inactive State Formed upon the Reaction of *EcHyd2* and Cyanide

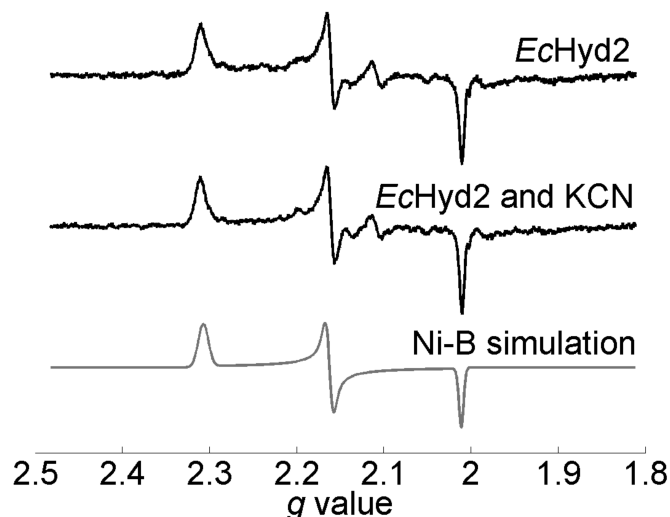
Samples of anaerobically oxidised *EcHyd2* were compared in the presence and absence of cyanide. Prior to making the samples, the enzyme was activated anaerobically under a flow of  $\text{H}_2$  as described in Section 2.3.1.4. Once fully activated,  $\text{H}_2$  was flushed from the cell with a flow of Ar for *ca.* 3 hours.

To oxidise *EcHyd2* anaerobically in the absence of cyanide, aliquots of potassium ferricyanide ( $\text{K}_3[\text{Fe}(\text{CN})_6]$ ) solution were injected into the cell until a suitable potential, at which *EcHyd2* would be oxidised to form the Ni-B state, was reached as monitored by zero current potentiometry (the sample shown in Figure 5.11 was taken at a potential of +413 mV). Once the potential of the enzyme solution had stabilised it was quickly transferred into an EPR tube and immediately frozen by immersing the tube into an isopropanol slush (isopropanol stirred over liquid  $\text{N}_2$ ).

In order to make a comparable sample in the presence of cyanide, a solution of *EcHyd2* was prepared as above, however an aliquot of KCN solution (resulting in a final concentration of 5.1 mM) was injected into the cell prior to the injection of  $\text{K}_3[\text{Fe}(\text{CN})_6]$  (the sample shown in Figure 5.11 was frozen when at a potential of +416 mV). In order to avoid HCN flushing from the cell, the Ar gas flow was turned off just before the injection of KCN solution. The enzyme solution was then transferred into an EPR tube and frozen as above.

At 80 K, only EPR signals arising from the Ni atom within the active site are observable. Other EPR active signals arising from the medial  $[\text{3Fe-4S}]^+$  cluster relax rapidly at this temperature and are not detected.<sup>122</sup> The two spectra shown in Figure 5.11, made with and without cyanide, are almost identical. The major signal in each spectra can be attributed to the Ni-B state ( $g_x = 2.31$ ,  $g_y = 2.16$  and  $g_z = 2.01$ ),<sup>122</sup> a simulation of which is also shown.

In each spectrum a small rhombic signal with  $g_x = 2.24$ ,  $g_y = 2.10$  and  $g_z = 2.05$



**Figure 5.11:** X-band continuous wave EPR spectra of *EcHyd2* (black lines) and a simulation of the Ni signals obtained from the Ni-B component (grey line). Samples were made with KCN (final concentration of 5.1 mM) and without KCN as indicated.  $K_3[Fe(CN)_6]$  was used to drive the potential to +413 mV (*EcHyd2* only) and +416 mV (*EcHyd2* and KCN) in each case. The resonator background, measured under identical conditions, has been subtracted from each spectra. Conditions: 100 mM HEPES buffer titrated to pH 7; temperature, 80 K; microwave power, 2 mW; microwave frequency, 9.381 GHz; field modulation amplitude, 0.5 mT. *EcHyd2* concentration = 2.6 mg/mL.

can be observed. This has been observed previously in *EcHyd2*<sup>168</sup> and a species with the same *g* values has been observed in the bacterium *Ralstonia eutropha* RH.<sup>169</sup> In *Ralstonia eutropha* RH this species was formed upon illumination of the Ni-C species at 30 K and so was deemed to be a light sensitive Ni-L state (see Section 1.6 and Chapter 6 for a more detailed description of Ni-L states). However, investigations into the light sensitivity of these signals in *EcHyd2* found that the corresponding species was not light sensitive and further experiments are necessary to elucidate its nature.<sup>168</sup>

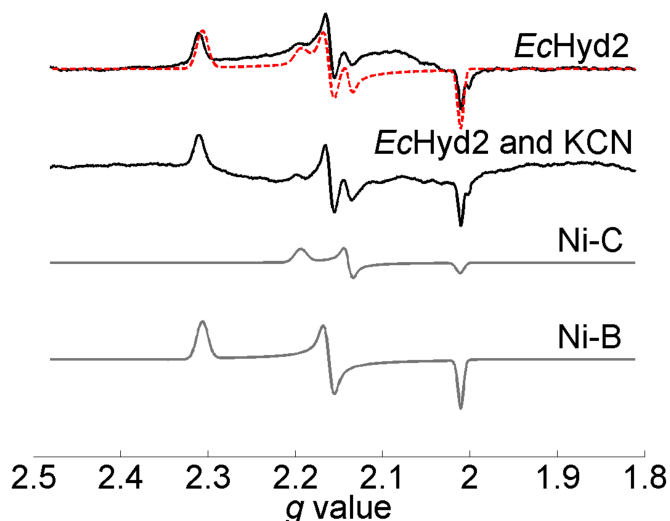
No previously unreported signals can be seen in either spectrum.

### 5.3.2 Freeze-quench EPR Studies Investigating the Reaction of *EcHyd2* and Cyanide

The freeze-quench technique allows reagents to be mixed together and frozen on a sub-second timescale (see Section 2.3.2.2). This technique was used in an attempt

to trap an intermediate species made during the cyanide inactivation of *EcHyd2*, the presence of which could then be investigated using EPR.

A sample of *EcHyd2* (*ca.* 5.2 mg/mL in 100 mM HEPES buffer containing 10% glycerol, 100 mM NaCl and titrated to pH 7) was activated under a flow of  $H_2$  (as described in Section 2.3.1.4). Hydrogen was then flushed from the cell with Ar before the enzyme was loaded into a drive syringe (see Section 2.3.2.2 for a description of the freeze-quench experimental setup). The second drive syringe was filled with  $K_3[Fe(CN)_6]$  dissolved in buffer identical to that of the enzyme solution. Samples with and without cyanide were made. When making the sample with cyanide, KCN was added to the  $K_3[Fe(CN)_6]$  solution. After mixing 4.8 mM  $K_3[Fe(CN)_6]$  and 5.1 mM KCN (if used) were present in the final sample. A comparison of the samples made using this method, both with and without cyanide, are shown in Figure 5.12.



**Figure 5.12:** X-band continuous wave EPR spectra of *EcHyd2* with KCN (final concentration of 5.1 mM) and without KCN as indicated. A final concentration of 4.8 mM  $K_3[Fe(CN)_6]$  was added to each sample in order to achieve a high potential. Simulations of the Ni-C and Ni-B components are shown in grey and a simulation of both signals is shown as a red dotted line overlaid on the *EcHyd2* only spectrum. The resonator background, measured under identical conditions, has been subtracted from each spectra. Conditions: 100 mM HEPES buffer titrated to pH 7; temperature, 80 K; microwave power, 2 mW; microwave frequency, 9.375 GHz; field modulation amplitude, 1 mT. *EcHyd2* concentration = 2.6 mg/mL. Both samples were measured in 4 mm high precision tubes.

The experimental spectra shown in Figure 5.12 are very similar. In both spectra the major rhombic signal present ( $g_x = 2.31$ ,  $g_y = 2.16$  and  $g_z = 2.01$ ) can be



attributed to enzyme in the Ni-B state. Signals previously attributed to the active Ni-C state ( $g_x = 2.19$ ,  $g_y = 2.14$  and  $g_z = 2.01$ )<sup>24,170</sup> can also be seen in both spectra. This is indicative that the timescale between mixing and freezing the sample was too short for an equilibrium to be reached. Although this procedure is too unreliable to provide kinetic information, no previously uncharacterised species appear on this timescale in either spectrum.

Once frozen the enzyme sample had to be ‘packed’, using a needle, into the bottom of an EPR sample tube. Difficulties in achieving a homogenous packing of the sample resulted in the uneven baseline in the spectra shown in Figure 5.12 (compared to those in Figure 5.11).

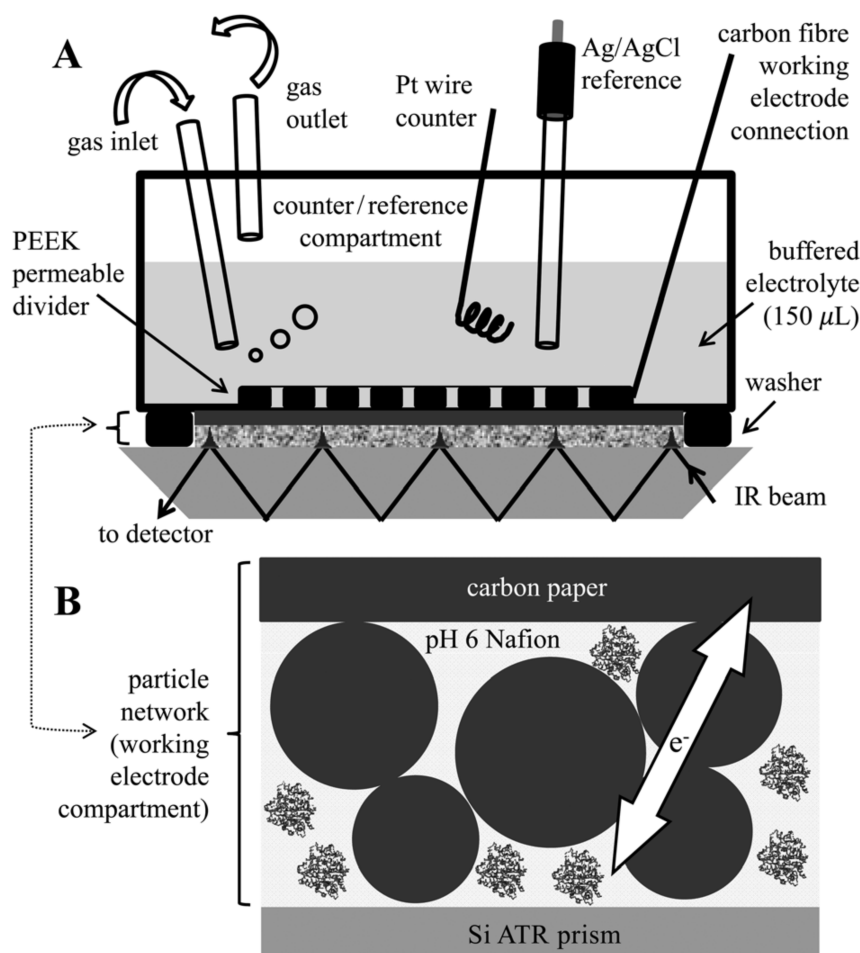
## 5.4 Attenuated Total Reflectance Infrared Spectroelectrochemistry Studies

Electron Paramagnetic Resonance spectroscopy (EPR) is only able to detect paramagnetic states, specifically it will only be able to detect the active site when the Ni atom is in either a +1 or +3 oxidation state. If cyanide reacts with *EcHyd2* to form a Ni<sup>2+</sup> state, or forms via a Ni<sup>2+</sup> intermediate, then these species will not be detectable by EPR.

Infrared spectroscopy is able to detect all states of the active site, regardless of their oxidation and spin state. The presence of intrinsic CO and CN<sup>-</sup> ligands within the active site of hydrogenases means that infrared spectroscopy is a useful method for following electronic and coordination changes in these enzymes such as those brought about by changes to the applied potential or by inhibitor binding.<sup>58,59</sup> The wavenumber positions of bands corresponding to stretching vibrations of the endogenous CO and CN<sup>-</sup> ligands have previously been characterised for a number of hydrogenases for various active site states.<sup>59</sup>

Infrared spectroelectrochemistry studies allow electrochemical control of a sample and simultaneous collection of infrared spectra. Healy and co-workers have

developed an Attenuated Total Reflectance Infrared (ATR-IR) spectroelectrochemical cell in which unmediated, electrochemical control of a hydrogenase is achieved by trapping the enzyme in a carbon particle film that acts as a three-dimensional network working electrode.<sup>171</sup> The high surface area of the carbon particle electrode ensures short diffusion distances and so no soluble redox mediators are necessary in the setup. The use of an all-carbon working electrode instead of a noble metal avoids the possibility of the electrode reacting with solutes such as KCN which can be introduced into the cell during an experiment. A schematic representation of the gas tight ATR-IR cell used in the experiments outlined in this section is shown in Figure 5.13.



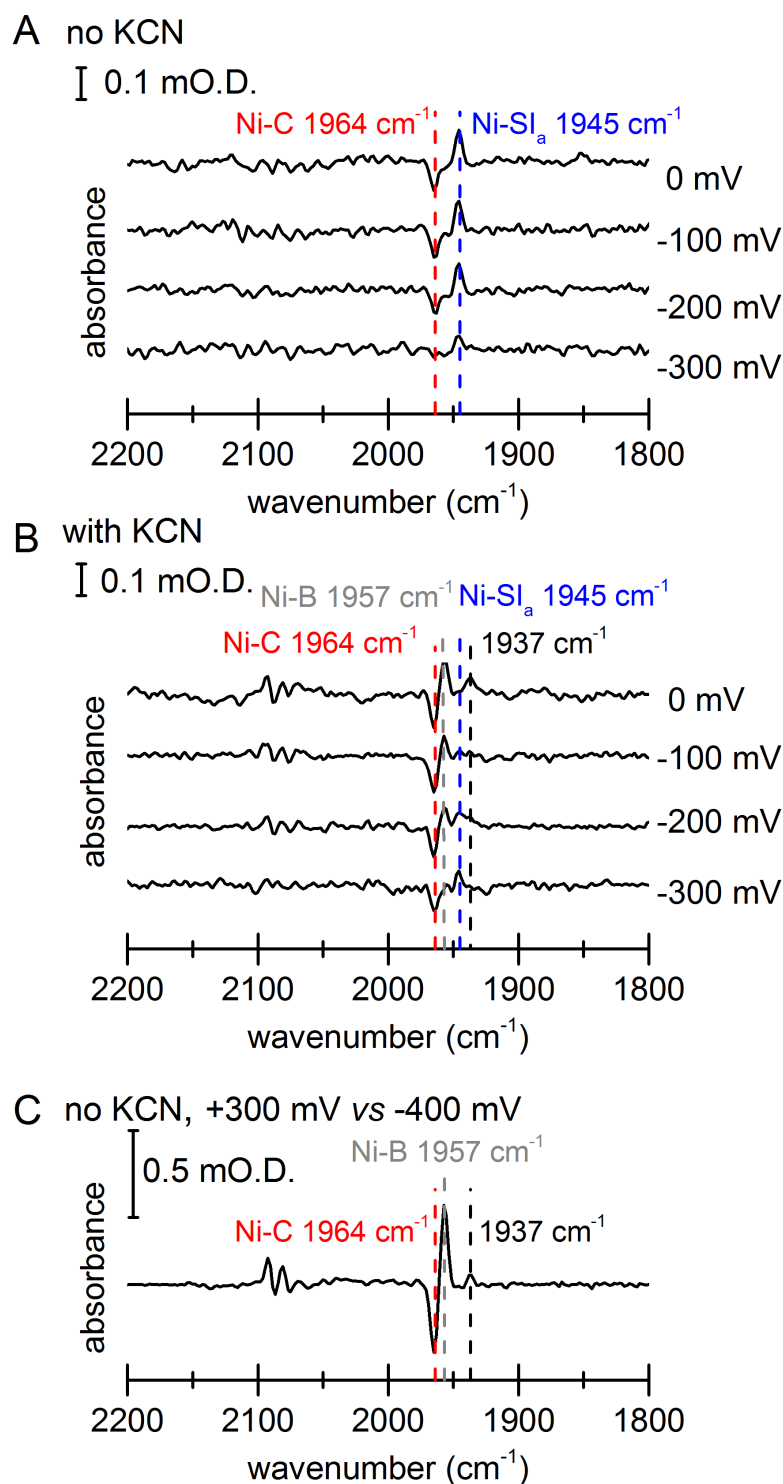
**Figure 5.13:** (A) A schematic representation of the ATR-IR spectroelectrochemical cell. (B) An expanded view of the carbon particle working electrode on the Si ATR prism, comprising a mixture of enzyme (shown as ribbons), electronically conducting carbon black particles (shown as spheres), phosphate buffered Nafion and carbon paper backing. Figure reproduced from Reference 171 with permission from the PCCP Owner Societies.

### 5.4.1 Results

Attenuated Total Reflectance Infrared spectroelectrochemistry (ATR-IR) studies were carried out on *EcHyd2* in the absence and presence of cyanide. Figure 5.14 shows the redox-triggered difference spectra recorded under  $N_2$  with and without KCN present. The potential was stepped from  $-400$  mV to  $0$  mV in  $100$  mV intervals and difference spectra were obtained by subtracting a background spectrum recorded at  $-400$  mV. Only changes in absorbance attributed to the endogenous CO ligand,  $\nu_{CO}$ , are well resolved ( $1900$ - $2000$   $cm^{-1}$  region). The greater intensity of the  $\nu_{CO}$  peaks compared to those resulting from vibrations of the endogenous  $CN^-$  ligands,  $\nu_{CN}$  ( $2050$ - $2150$   $cm^{-1}$  region) mean that  $\nu_{CO}$  features are of greater benefit in identifying states and characterising new species. The spectra presented were recorded 20 minutes after each potential step, at which point the enzyme was found to have reached diffusion-controlled equilibrium with the electrode potential.

As shown in Figure 5.14A, steps from  $-400$  mV to  $0$  mV resulted in a negative band at  $1964$   $cm^{-1}$ , consistent with the loss of the Ni-C state, and growth of a band at  $1945$   $cm^{-1}$ , consistent with the formation of the Ni-SIa state. The band positions for these states correspond most closely to the  $\nu_{CO}$  features observed for the [NiFe]-hydrogenase of *Desulfovibrio vulgaris* as reported by Fichtner *et al.*<sup>172</sup> who reported values of  $\nu_{CO}$  of  $1961$   $cm^{-1}$  and  $1943$   $cm^{-1}$  for the Ni-C and Ni-SIa states respectively.

Figure 5.14B depicts an analogous set of difference spectra recorded after introducing  $10$  mM KCN solution into the cell solution. In the presence of cyanide, a growth band at  $1957$   $cm^{-1}$  is now obvious at  $-200$  mV, accompanied by a weaker band at  $1945$   $cm^{-1}$ . As the potential is raised to  $-100$  mV and then  $0$  mV, the band at  $1957$   $cm^{-1}$  continues to grow, and the  $1945$   $cm^{-1}$  band disappears. The  $1957$   $cm^{-1}$  band corresponds well with  $\nu_{CO}$  reported for the Ni-B state in the *Desulfovibrio vulgaris* hydrogenase ( $1955$   $cm^{-1}$ ).<sup>172</sup> This assignment can be confirmed by comparison to the difference spectra in Figure 5.14C: This data was collected at  $+300$  mV in the absence of cyanide (*i.e.* under conditions which would favour the



**Figure 5.14:** ATR-IR redox-triggered difference spectra of EcHyd2 at a carbon particle electrode (A) without KCN solution present and (B) with 10 mM (final concentration) KCN solution present. In both (A) and (B) background spectra were collected at  $-400$  mV, followed by potential steps in 100 mV intervals. Each spectrum was recorded 20 minutes after the potential step. (C) Redox-triggered difference spectrum ( $+300$  mV *minus*  $-400$  mV) of EcHyd2 with no cyanide present. Conditions:  $\text{N}_2$  saturated 100 mM HEPES buffer titrated to pH 7 containing 100 mM NaCl as added electrolyte. All experiments shown in this figure were carried out by Min-Wen Chung under the supervision of Prof. Kylie Vincent, University of Oxford. Figure reproduced with permission from Reference 166.

anaerobic conversion of active enzyme into the Ni-B state) and shows growth of an intense positive band at  $1957\text{ cm}^{-1}$ . A weak feature is visible at  $1937\text{ cm}^{-1}$  in the spectra of both Figure 5.14B and 5.14C and may correspond to a small portion of the Ni-SIr state.

No further  $\nu_{CO}$  peaks can be identified in the spectra of *EcHyd2* recorded in the presence of cyanide.

## 5.5 Discussion

The results presented herein demonstrate an important reaction between cyanide and [NiFe]-hydrogenases that is revealed in detail when studied by Protein Film Electrochemistry and investigated further by spectroscopy. As observed in Figure 5.1, cyanide accelerates the high potential inactivation which occurs in *EcHyd1* and *EcHyd2*, but has no effect on either enzyme at low potentials. The potential dependence is consistent with cyanide targeting the active site of the enzyme although cyanide must not attack a species which is directly in the catalytic cycle as such an interaction would result in a decrease in  $\text{H}_2$  oxidation current regardless of electrode potential. Instead cyanide must attack a species that is in rapid equilibrium with the active states and can be removed by oxidation as the potential is increased. The higher potential window for cyanide inactivation of *EcHyd1* relative to *EcHyd2* is consistent with the  $\text{Ni}^{2+}$  to  $\text{Ni}^{3+}$  oxidation of the Ni-atom at the active site within *EcHyd1* occurring about 235 mV higher in potential than in *EcHyd2*, as demonstrated by the difference in the value of  $E_{\text{switch}}$  reported for the reactivation of the Ni-B state in these two enzymes.<sup>122</sup> In both *EcHyd1* and *EcHyd2* the inactivation caused by cyanide is reversible, and reactivation is seen during the reductive sweep in the cyclic voltammograms shown in Figure 5.1. Once again the difference in the reductive driving force required to reactivate *EcHyd1* relative to *EcHyd2* is consistent with the difference in  $E_{\text{switch}}$  for the reactivation of the Ni-B state for these two enzymes.

Figures 5.3 and 5.4 reflect the influence that cyanide concentration and  $\text{H}_2$  at-

mosphere has on the rate and extent of inhibition of *EcHyd2* by cyanide. The rate of inhibition increases with increasing cyanide concentration until a plateau in rate is reached at concentrations above *ca.* 5 mM, with a slight decrease in rate seen at cyanide concentrations of 8 mM and above. This small decrease in rate seen when very high concentrations of cyanide are introduced may be due to a secondary, slow inactivation mechanism which occurs only under conditions of high cyanide concentration.<sup>173</sup> A faster rate of inactivation occurs under lower H<sub>2</sub> concentrations, consistent with cyanide competing with H<sub>2</sub> for a binding site. As discussed in Section 1.7, where H<sub>2</sub> binds to the active site is still a matter of much debate, however, the Ni and Fe atoms are only 2.6 Å apart<sup>174</sup> and coordination at one atom may affect the ability of a ligand to coordinate at the other. Furthermore, an exogenous ligand bound to either the Ni or the Fe would disrupt the catalytic cycle by impeding a hydride, made *via* the heterolytic cleavage of H<sub>2</sub>, from being able to form a bridge between the two metal atoms.<sup>175,176</sup>

Upon introduction of cyanide a limiting current is reached after the initial inactivation in all cases (Figure 5.4B), indicating that an equilibrium is set up between active enzyme and the cyanide inactivated state. Increasing the concentration of cyanide introduced into the cell decreases the magnitude of the limiting current observed (representing a move of the position of the equilibrium in favour of the inactivation reaction) until concentrations of over 1 mM are reached, after which the limiting current remains at about 3% of the initial current recorded. This residual current is consistent with a slow (under the conditions of the experiment) reactivation process. The concentration of H<sub>2</sub> in the atmosphere does not have a significant impact upon the limiting current reached.

Experiments investigating the effect of cyanide over a pH range from pH 4 to pH 8 found no significant pH dependence upon the rate of inactivation following cyanide introduction into the cell (Figure 5.5). The  $pK_a$  value of HCN is 9.21<sup>167</sup> and so only a small proportion of the cyanide injected into the cell will be in the CN<sup>-</sup> form at pH values below 8. The instability of the enzyme in conditions above pH 8 meant

that experiments could not be carried out at pH values above this, but the lack of dependence upon pH over the range studied is consistent with cyanide entering the enzyme as HCN, the concentration of which does not alter significantly over the pH range studied (at pH 4, the concentration of  $\text{CN}^-$  in solution will be 0.0006% that of the HCN concentration; at pH 8, the concentration of  $\text{CN}^-$  in solution will be 6% that of the HCN concentration). The presence of hydrophobic gas channels within the protein (see Section 1.4.4) will aid the transport of neutral molecules, such as HCN, from the protein surface to the active site.

Figure 5.8 shows the changing effect of cyanide on *EcHyd2* with potential. It was found that the initial rate of inactivation, over the first 5 s immediately following the introduction of KCN solution into the cell, was similar over a range of *ca.* 500 mV. After the first 5 s, the reactivation reaction is significant at potentials below 0 mV, having more of an effect on the observed current at lower potentials. Three experiments, investigating the effect of cyanide over nearly a 200 mV window from +101 mV to +294 mV, show almost identical inactivation characteristics. At potentials above +100 mV reactivation of the cyanide inactivated state of *EcHyd2* does not take place (Figure 5.1A) and so these experiments are not complicated by the reactivation process. The similarity in the initial inactivation characteristics seen in this set of experiments is consistent with the rate of inactivation being unaffected by potential. The potential dependence observed in the cyclic voltammetry experiments (Figure 5.1A) is instead due to a potential dependent reactivation pathway. A similar potential-independent inactivation—potential-dependent reactivation relationship is observed for the formation/reactivation of the Ni-B state. In electrochemical terms, the formation of the Ni-B state is known to proceed *via* a ‘CE’ inactivation (*i.e.* the first step is a chemical reaction and the second step is an electron transfer). Conversely, the reactivation of the Ni-B state proceeds *via* an ‘EC’ reaction (*i.e.* an electron transfer followed by a chemical reaction).<sup>140,157</sup>

The parameter  $E_{\text{switch}}$  was used to study the reactivation of the cyanide-inhibited state of *EcHyd2* under a range of pH conditions (pH 5 to pH 7). The  $E_{\text{switch}}$  values

found were compared with those found from the reactivation of the Ni-B state, made *via* anaerobic oxidation of the active enzyme. No significant difference in  $E_{\text{switch}}$  was found from the reactivation of the cyanide inhibited state *vs* the Ni-B state over the entire pH range studied (Figure 5.10).

Although Protein Film Electrochemistry is an excellent technique for the study of the catalytic activity of an enzyme and for analysing mechanisms of inactivation and reactivation, it is not able to provide structural insight. In order to access this information for the cyanide inactivated state of *EcHyd2*, further spectroscopic experiments were carried out.

Figure 5.11 compares two samples measured using Electron Paramagnetic Resonance spectroscopy (EPR), one made in the presence of cyanide and one made under inhibitor free conditions, both of which were taken at a potential of *ca.*  $+415 \pm 2$  mV. The spectra observed from each sample are almost identical, and the major signal observed in each case can be attributed to the Ni-B state which has previously been well characterised by EPR.<sup>24,122</sup>

The use of a freeze-quench instrument allowed samples of *EcHyd2* to be mixed with cyanide and oxidised and then frozen on a sub-second timescale, increasing the likelihood of successfully trapping an intermediate species produced during cyanide inactivation. As shown in Figure 5.12, no evidence of a novel species was observed using this method. This may be indicative of three things: (i) the intermediate species for this reaction is not EPR active; (ii) the reaction was not quenched on a sufficiently fast timescale, and; (iii) the concentration of the intermediate at any time is not high enough to be detected by EPR. Importantly, both with and without cyanide present, when *EcHyd2* is exposed to the oxidising agent  $\text{K}_3[\text{Fe}(\text{CN})_6]$ , the well characterised Ni-B state is the only product observed *via* EPR.

Attenuated Total Reflectance Infrared (ATR-IR) spectroelectrochemical experiments (Figure 5.14) show that in the presence of cyanide, more Ni-SIa has converted to Ni-B after 20 minutes.

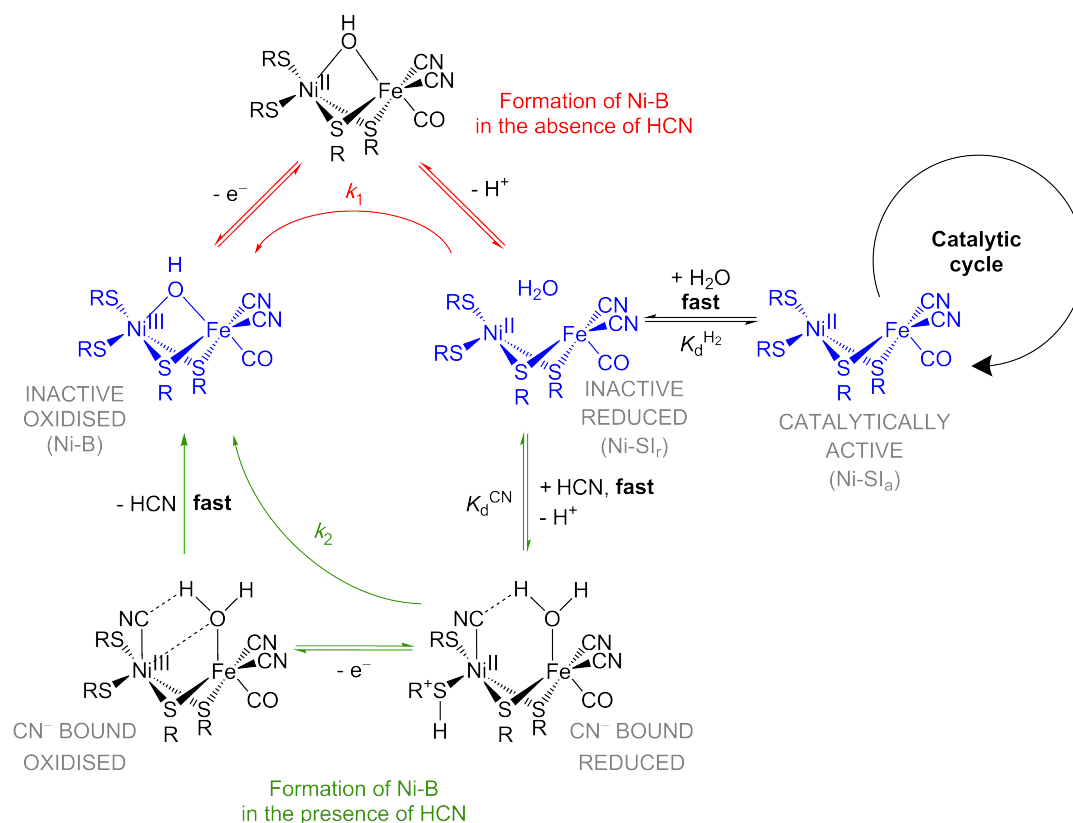
No previously uncharacterised species were detected *via* EPR or ATR-IR.



These results lead to the conclusion that cyanide acts as a strong promoter of Ni-B formation. A possible mechanism for the acceleration of the formation of the Ni-B state by cyanide will be discussed in Section 5.5.1.

### 5.5.1 A Mechanism for the Promotion of Ni-B State Formation by Cyanide

Any mechanistic proposal must account for the observation that the rate of inactivation upon cyanide exposure increases to a limiting value as the cyanide concentration is raised, and decreases as the concentration of  $H_2$  is increased. One such mechanism is outlined in Scheme 5.2.



**Scheme 5.2:** A mechanism by which cyanide facilitates the formation of the Ni-B state showing plausible cyanide intermediates. Well characterised states are shown in blue. Red arrows indicate a pathway leading to the formation of Ni-B state under normal conditions (in the absence of cyanide) whereas green arrows indicate a possible pathway for Ni-B formation which may occur in the presence of cyanide. Figure reproduced with permission from Reference 166.

Although the exact nature of the mechanism leading to the formation of Ni-B

under ‘normal’ conditions (*i.e.* when no cyanide is present) is not established, it has been shown experimentally that it is a CE reaction (*i.e.* first a chemical reaction and then an electron transfer step).<sup>140,157</sup> Under anaerobic conditions the first step is likely to be the transport of a H<sub>2</sub>O molecule to the active site (converting Ni-SIa to Ni-SIr) and then coordination of a OH<sup>-</sup> ligand to either the Fe or the Ni atom (in Scheme 5.2 we have assumed OH<sup>-</sup> coordinates to the Fe atom). This is then followed (given the required driving force) by a rapid electron-transfer in which the Ni<sup>2+</sup> ion is oxidised to Ni<sup>3+</sup>, thus trapping the OH<sup>-</sup> species as a bridging ligand.

When cyanide is present two more species are added to the scheme. The first of these is formed by a chemical step in which CN<sup>-</sup> binds to the H<sub>2</sub>O associated active site (Ni-SIr), most likely after entering the active site pocket *via* a hydrophobic gas channel as HCN, producing a weakly bound Ni<sup>2+</sup>-CN species. Cyanide is a better nucleophile than OH<sup>-</sup> and much better than H<sub>2</sub>O,<sup>177,178</sup> and there are numerous examples of Ni(II)-CN complexes in the literature.<sup>179,180</sup> The second step involves an electron transfer to result in a Ni<sup>3+</sup> species. This species is transient and an internal proton transfer reaction regenerates HCN and places OH<sup>-</sup> in the bridging position resulting in Ni-B.

Oxidation of Ni<sup>2+</sup> to Ni<sup>3+</sup> requires the provision of another donor ligand, which is where CN<sup>-</sup>, a strong  $\sigma$ -donor, offers a large advantage over OH<sup>-</sup> and particularly H<sub>2</sub>O. In addition, HCN is a stronger acid than H<sub>2</sub>O. Consequently, a Ni<sup>3+</sup>-CN species can be formed much more readily than a Ni<sup>3+</sup>-OH species. However, studies of Ni<sup>3+</sup>-cyanido complexes have shown that the Ni<sup>3+</sup>-CN bond is labile,<sup>181,182</sup> and a further factor finally dictates the course of events; when two metal ions lie in close proximity, OH<sup>-</sup> is a very much better *bridging* ligand than CN<sup>-</sup>. There is no evidence of a cyanide ligand bridging two metal ions in a  $\mu_2$ -C,C conformation<sup>25,179,183</sup> (although there are some instances in which cyanide bridges in a  $\mu_3$ -C,C,N fashion).<sup>183</sup> On the other hand, examples of cyanide ligands forming linear bridges ( $\mu_2$ -C,N) are well known<sup>179,180</sup> however the small 2.6 Å space between the metal atoms within the active site<sup>174</sup> makes this an unlikely possibility. Conversely, hydroxyl is an excellent

$\mu_2$ -O,O bridging ligand across 2.6 Å and numerous examples of a hydroxide ligand acting in this way can be found.<sup>25</sup>

The mechanism outlined in Scheme 5.2 forms the basis of a rate law, a simple mathematical model that can be used to fit the experimental data. To simplify the derivation it is assumed that the rates are independent of pH (as observed in Figure 5.5) and electrode potential (which is true above a potential of 100 mV, see Figure 5.8). The free cyanide species in solution is assumed to be HCN (as also confirmed by ATR-IR under the same conditions). Representing the free inactive enzyme (Ni-SIr) as E, enzyme ‘bound’ to H<sub>2</sub> (and thus active in the catalytic cycle) as E:H<sub>2</sub> and enzyme bound to cyanide as E:HCN, the approximate concentrations of the active species are;



$$K_d^{H_2} = \frac{[E][H_2]}{[E : H_2]} \quad (5.3)$$

$$K_d^{HCN} = \frac{[E][HCN]}{[E : HCN]} \quad (5.4)$$

where  $K_d^{H_2}$  and  $K_d^{HCN}$  are the dissociation constants for H<sub>2</sub> and cyanide, respectively, leading back to the first inactive state (depicted in Scheme 5.2 as Ni-SIr).

In the absence of cyanide, the rate of Ni-B formation which occurs at high potential can be described by;



$$rate = k_1[E] \quad (5.6)$$

where  $k_1$  is the rate constant for the formation of Ni-B in the absence of cyanide.

If

$$E_{all} = [E] + [E : H_2] \quad (5.7)$$

then:

$$K_d^{H_2} = \frac{[E][H_2]}{[E_{all}] - [E]} \quad (5.8)$$

Rearranging and substituting the resulting expression for  $[E]$  into Equation 5.6 gives:

$$\text{rate} = k_1 \frac{K_d^{\text{H}_2} [E_{\text{all}}]}{(K_d^{\text{H}_2} + [\text{H}_2])} \quad (5.9)$$

The observed rate of Ni-B formation (monitored by the rate of decrease in current) in the absence of cyanide is then:

$$k_{\text{obs}}^{\text{Ni-B}} = k_1 \frac{K_d^{\text{H}_2}}{(K_d^{\text{H}_2} + [\text{H}_2])} \quad (5.10)$$

In the presence of cyanide, the rate of inactivation attributed to the pathway in Scheme 5.2 involving cyanide can be described by;



$$\text{rate} = k_2 [E : \text{HCN}] \quad (5.12)$$

where  $k_2$  describes both the oxidation of the Ni(II)-HCN species and the subsequent loss of HCN to form the bridging hydroxyl species Ni-B.

If

$$[E'_{\text{tot}}] = [E] + [E : \text{HCN}] \quad (5.13)$$

then rearrangement and substitution of  $[E]$  into Equation 5.4 gives:

$$K_d^{\text{HCN}} = \frac{([E'_{\text{tot}}] - [E : \text{HCN}])[\text{HCN}]}{[E : \text{HCN}]} \quad (5.14)$$

Further rearrangement yields;

$$[E : \text{HCN}] = \frac{[E'_{\text{tot}}][\text{HCN}]}{K_d^{\text{HCN}} + [\text{HCN}]} \quad (5.15)$$

which can be substituted into Equation 5.12 to give:

$$\text{rate} = k_2 \frac{[E'_{\text{tot}}][\text{HCN}]}{K_d^{\text{HCN}} + [\text{HCN}]} \quad (5.16)$$

If

$$[E_{\text{tot}}^0] = [E : H_2] + [E'_{\text{tot}}] \quad (5.17)$$

then by substituting Equation 5.17 into Equation 5.3 and rearranging one obtains:

$$[E'_{\text{tot}}] = \frac{K_d^{H_2}[E_{\text{tot}}^0]}{[H_2] + K_d^{H_2}} \quad (5.18)$$

This expression for  $[E'_{\text{tot}}]$  can be substituted into Equation 5.16 to give:

$$\text{rate} = \frac{k_2 K_d^{H_2} [HCN] [E_{\text{tot}}^0]}{(K_d^{HCN} + [HCN])(K_d^{H_2} + [H_2])} \quad (5.19)$$

The observed rate of Ni-B formation due to the effect of cyanide is then:

$$k_{\text{obs}}^{HCN} = \frac{k_2 K_d^{H_2} [HCN]}{(K_d^{HCN} + [HCN])(K_d^{H_2} + [H_2])} \quad (5.20)$$

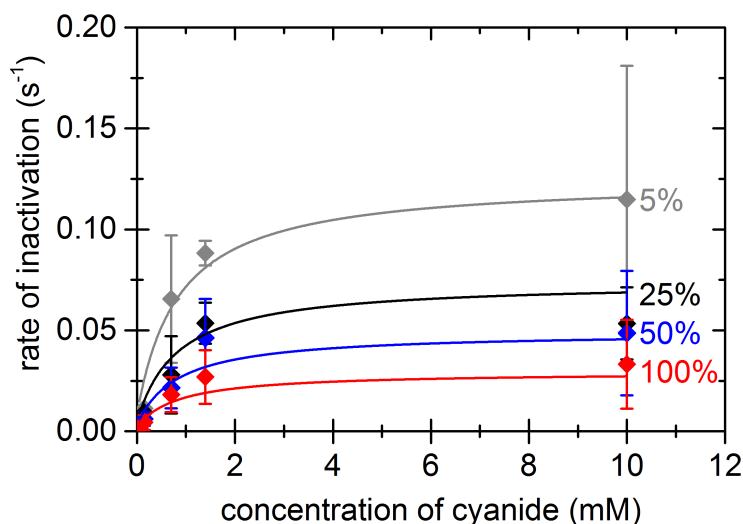
Therefore, accounting for the observed rate of Ni-B formation which usually occurs at high potential,  $k_{\text{obs}}^{\text{Ni-B}}$  (Equation 5.10), and the increase in observed rate due to cyanide,  $k_{\text{obs}}^{HCN}$  (Equation 5.20), the total observed rate of Ni-B formation in the presence of cyanide,  $k_{\text{obs}}$ , can be described by:

$$k_{\text{obs}} = k_{\text{obs}}^{\text{Ni-B}} + k_{\text{obs}}^{HCN} \quad (5.21)$$

$$k_{\text{obs}} = \frac{k_1 K_d^{H_2}}{(K_d^{H_2} + [H_2])} + \frac{k_2 K_d^{H_2} [HCN]}{(K_d^{HCN} + [HCN])(K_d^{H_2} + [H_2])} \quad (5.22)$$

Equation 5.22 was used to fit to data obtained in a similar way to that in Section 5.2.2.1 (Figure 5.3). However, unlike in Figures 5.3 and 5.4A where the rate of inactivation was found from the single exponential fit to the decrease in current observed upon cyanide introduction, here, the *initial* rate of inactivation, that is, the rate obtained within the first few seconds of the reaction before complications such as HCN flushing from the cell become significant, was used as the rate of Ni-B formation. To ensure thorough mixing of the cyanide solution during the short time that the data was collected, an electrode rotation rate of 3500 rpm was employed.

Experiments were carried out at 807 mV above  $E_{2\text{H}^+/\text{H}_2}$  under 5%, 25%, 50% and 100%  $\text{H}_2$ , the the total gas flow rate set to 500 sccm. In each case, the rate was obtained from the gradient of the slope fitted over the initial linear decrease in current, which ranged from the first 1.5 s to 5 s of data, depending upon the KCN concentration used (Figure 5.15).



**Figure 5.15:** The relationship between the initial rate of inactivation of *EcHyd2* and the concentration of cyanide injected into the cell under different  $\text{H}_2$  atmospheres as indicated in the figure. An aliquot of cyanide solution was injected into the mixed hydrogenase buffer cell solution during a chronoamperometry experiment at 807 mV above  $E_{2\text{H}^+/\text{H}_2}$  resulting in a cyanide concentration of 0.05, 0.15, 0.70, 1.40 and 10 mM in the electrochemical cell immediately after the injection. In each case, the rate was obtained from a linear fit to the initial part of the inactivation current. Lines show a global fit of Equation 5.22 to the data. The value of  $k_1$  was estimated to be  $0.01 \text{ s}^{-1}$ . Using this value, the values of the other parameters obtained from the fit are as follows:  $K_{\text{d}}^{\text{H}_2} = 0.24 \text{ bar}$ ,  $K_{\text{d}}^{\text{HCN}} = 0.84 \text{ mM}$  and  $k_2 = 0.14 \text{ s}^{-1}$ . Conditions: pH 7,  $25^\circ\text{C}$ ,  $\omega = 3500 \text{ rpm}$ , total gas flow = 500 rpm. Figure adapted from Reference 166.

Figure 5.15 shows that the rate of inactivation increases with increasing cyanide concentration from 0.05 mM to 1.4 mM then levels off, and decreases as the  $\text{H}_2$  concentration is increased. The solid lines show a global fit to the data, with values of  $K_{\text{d}}^{\text{H}_2}$ ,  $K_{\text{d}}^{\text{HCN}}$  and  $k_2$  held constant over the data obtained from all  $\text{H}_2$  concentrations studied. Equation 5.22, although derived using a simplified version of the mechanism proposed in Scheme 5.2, gives a good overall fit to the data and is instructive. According to the analysis, cyanide gives at least a 14-fold increase in the rate of

Ni-B formation (0.14/0.01). The value  $K_d^{H_2} = 0.24$  bar reflects the relatively low affinity of  $H_2$  for the Ni-SIr state compared to the much higher affinity of  $H_2$  for the active states as reflected in  $K_M$  values.<sup>122</sup> The value  $K_d^{HCN} = 0.84$  mM is also consistent with the weak (but fast) binding of cyanide.

## 5.6 Conclusions and Perspectives

In summary, the reaction of *EcHyd2* with cyanide outlines the importance of both kinetic and thermodynamic factors in determining a reaction pathway. The observed increase in the rate of the formation of the Ni-B state in the presence of cyanide can be attributed to the  $Ni^{3+}$ -CN species being the *kinetically* preferred intermediate relative to a  $Ni^{3+}$ -OH species. Cyanide is a much better nucleophile than  $OH^-$  and therefore facilitates the oxidation of  $Ni^{2+}$  to  $Ni^{3+}$  which depends upon an additional donor ligand being coordinated. However, the greater stability afforded when  $CN^-$  is subsequently replaced by  $OH^-$ , which can form a stable bridge between the Ni and the Fe, results in the rapid conversion to the Ni-B state which is the *thermodynamically* favoured product relative to a non-bridging  $Ni^{3+}$ -CN species. An analogous observation is made for *EcHyd1* indicating that accelerated formation of the Ni-B state in the presence of cyanide is not specific to *EcHyd2*.

This study provides interesting new possibilities for the formation of the Ni-B state, a state which is considered to be a ‘safe’ state for the enzyme: The favoured formation of this state in the presence of oxygen over the more slowly reactivating Unready states is regarded to be a key feature in the higher  $O_2$  tolerance of the so-called  $O_2$ -tolerant [NiFe]-hydrogenases.<sup>4,69</sup>

Finally, the PFE experiments outlined in this chapter show clearly that the inhibition caused by cyanide is simply reversed upon reduction, contrary to observations made previously.<sup>150</sup> These experiments serve as an example of how PFE, with its special capability for examining the catalytic ability of a miniscule sample of immobilised enzyme as a function of potential as well as time, can help resolve processes which remain hidden when probed by conventional techniques.





## Chapter 6

# Investigating the Nature of the Ni-L State of Hydrogenase-1 from *Escherichia coli*



## Abstract

This chapter details experiments that investigate the relationship between the Ni-C and Ni-L states of Hydrogenase-1 from *Escherichia coli* (*EcHyd1*). The conversion of the Ni-C state into a Ni-L state upon illumination is a phenomenon that has been observed in a number of [NiFe]-hydrogenases, however, the significance of the Ni-L state is still unclear and it is considered by many to be an light-induced artefact. Here Electron Paramagnetic Resonance spectroscopy and Attenuated Total Reflectance Infrared spectroelectrochemistry are used to probe the active states formed when samples of *EcHyd1* are prepared in conditions free of visible light.

In contrast to most other [NiFe]-hydrogenases, in *EcHyd1* a number of Ni-L states are detected even in samples which have not been exposed to visible light. These states are formed reversibly and the enzyme remains catalytically active after their formation, calling into question the assertion that the Ni-L state is artefactual. It is proposed that, unlike in other hydrogenases studied to date, in *EcHyd1* the Ni-L states are lower in energy than the Ni-C state and so are observed spectroscopically even in the dark.

## 6.1 Introduction

The [NiFe]-hydrogenase Ni-C state is widely observed to be photolabile at low temperature, the state being formed upon photolysis being termed Ni-L. Although several Ni-L states have been observed and characterised in a large number of [NiFe]-hydrogenases, the Ni-L state was, and often still is, considered a low temperature artefact which forms in the presence of light. More recent characterisations of O<sub>2</sub>-tolerant [NiFe]-hydrogenases show that for these enzymes, exposure to ambient light conditions is sufficient for the spectroscopically identified Ni-L state to be detected, calling into question the assertion that this state is artefactual.

This chapter discusses Electron Paramagnetic Resonance (EPR) and Attenuated Total Reflectance Infrared spectroscopy (ATR-IR) studies investigating the presence of the Ni-L state in the [NiFe]-hydrogenase, Hydrogenase-1 from *Escherichia coli* (*EcHyd1*). All ATR-IR experiments described in this chapter were undertaken by Dr. Philip Ash working within the research group of Prof. Kylie Vincent, University of Oxford.

### 6.1.1 Previous Studies Investigating the [NiFe]-hydrogenase Ni-L State

Theoretical and experimental studies have led to the proposition that the light sensitivity of the Ni<sup>3+</sup> Ni-C state at cryogenic temperatures is due to the photodissociation of the bridging hydride ligand as a proton to form a Ni<sup>+</sup> Ni-L species.<sup>39,60,184,185</sup> The conversion is reversible, and annealing the sample at a slightly higher temperature speeds up the rate at which the dissociated proton is able to move back to its original position, recovering the original Ni-C spectrum. Electron Paramagnetic Resonance (EPR) and Fourier-transform infrared (FTIR) studies of [NiFe]-hydrogenases have observed a number of states which appear upon illumination of the Ni-C state under cryogenic conditions.<sup>184,186,187</sup> Identified by slightly differing FTIR and EPR signatures, the various states may differ in terms of the site at which the dissociated

proton is bound, proposed binding sites including various amino acid residues close to the active site or a sulfur of a Ni coordinated cysteine ligand (see Figure 1.2).<sup>60,185</sup>

Although this light-dependence, first detected in the 1980s,<sup>72</sup> is a general feature of hydrogenases, recent studies have highlighted some important differences in the behaviour of the spectroscopically-defined Ni-L states between O<sub>2</sub>-sensitive and O<sub>2</sub>-tolerant [NiFe]-hydrogenases.

Studies of the O<sub>2</sub>-sensitive hydrogenase from *Desulfovibrio gigas* found that illumination of the Ni-C state at temperatures below 60 K caused the disappearance of the signals associated with the Ni-C state and the simultaneous appearance of two sets of signals which the authors referred to as Ni-C1\* and Ni-C2\*.<sup>186</sup> At temperatures above 60 K, only the Ni-C2\* signal was observed upon light irradiation. This photolytic process was irreversible below 100 K but completely reversible at temperatures above 120 K.<sup>186</sup> The authors concluded that the two light-induced signals indicated two forms of the active site, differing slightly in their coordination environments, and that one state was an intermediate in the formation of the other.

A more recent study of O<sub>2</sub>-sensitive hydrogenase from *Desulfovibrio vulgaris* Miyazaki F found that at 150 K illumination of the enzyme resulted in conversion of the Ni-C state to a Ni-L state, a process which was reversed in the dark.<sup>184</sup> The temperature dependence of the rate constants for the interconversion followed a linear Arrhenius dependence and extrapolation of the data gave a lifetime for the Ni-L state of 57  $\mu$ s at 297 K.<sup>184</sup>

The first study investigating the nature of a Ni-L state in an O<sub>2</sub>-tolerant hydrogenase was completed on the membrane-bound [NiFe]-hydrogenase from *Ralstonia eutropha* H16 (*ReMBH*).<sup>187</sup> In its native environment, this hydrogenase is tightly bound to its primary electron acceptor, a membrane-integral cytochrome *b*. Saggiu and co-workers published a detailed account of the spectroscopic states of *ReMBH*, both bound to and separated from the cytochrome *b* protein, as detected by FTIR and EPR spectroscopy. In contrast to all previous studies on O<sub>2</sub>-sensitive hydrogenases, EPR studies of the cytochrome-free *ReMBH* found that at 80 K nearly

equal amounts of Ni-C and Ni-L states could be detected, even without any prior illumination of the sample except for the normal light conditions in the laboratory. Conversion of the Ni-L state into the Ni-C state was not possible, even at elevated temperatures in the dark.<sup>187</sup> By using FTIR spectroscopy the Ni-C and Ni-L states can be monitored at much higher temperatures than is possible with EPR. Using FTIR the authors found that at 283 K a mixture of Ni-C and Ni-L states were observed.<sup>187</sup> Conversely, EPR spectra of the cytochrome-bound *ReMBH* showed only Ni-C signals prior to illumination. Complete conversion of the Ni-C state into three different Ni-L states could be achieved by illuminating the sample with white light for 20 minutes at 80 K and complete back-conversion was seen after 15 minutes in the dark at 200 K.<sup>187</sup> The authors concluded that the unusual behaviour of the Ni-C and Ni-L states in the cytochrome-free *ReMBH* resulted from the non-native structure and/or heterogeneity of the protein in this state and was a nonphysiological property.

Electron paramagnetic resonance studies of the O<sub>2</sub>-tolerant Hydrogenase-I from *Aquifex aeolicus* found that the Ni-C state of this enzyme converted to two light-induced forms, Ni-L1 and Ni-L2, after illumination at 170 K and 100 K respectively.<sup>73,188</sup> Similar to the cytochrome-free *ReMBH*, in Hydrogenase-I the Ni-L1 state could also be observed at temperatures below 100 K even in the absence of light.<sup>73</sup> A <sup>2</sup>H Mims ENDOR study of the Ni-C state found a smaller hyperfine interaction of a <sup>2</sup>H nuclei than what had previously been reported for O<sub>2</sub>-sensitive hydrogenases, suggesting a larger bond distance and weaker binding between the Ni and the hydride and a more asymmetric bridging situation between the Ni and the Fe, consistent with the increased light sensitivity of the Ni-C state.<sup>73</sup> A third light-induced state, not observed by EPR, could be resolved in FTIR studies of Hydrogenase-I.<sup>73</sup>

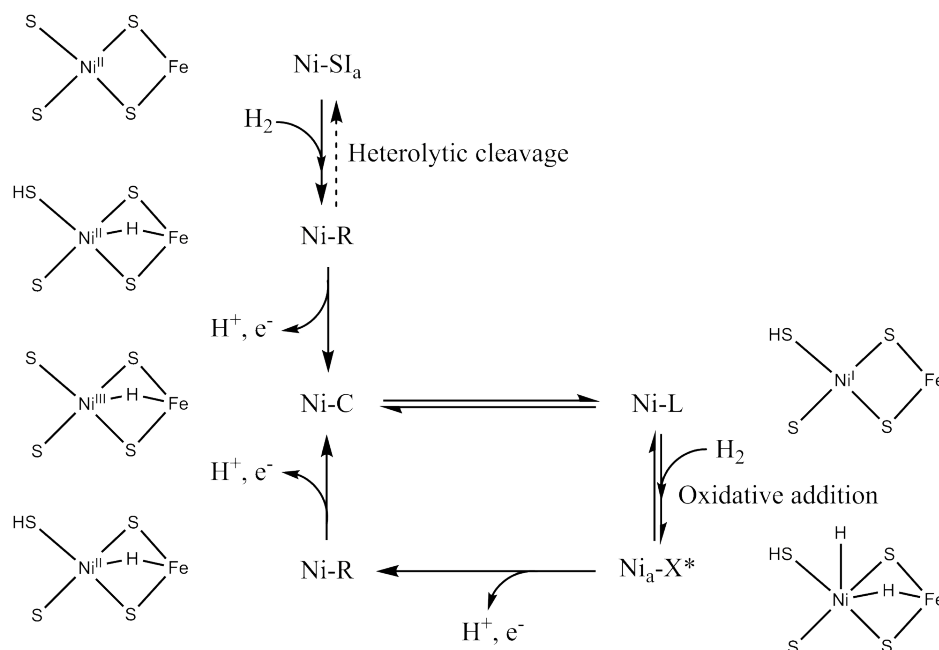
Finally, EPR studies of the O<sub>2</sub>-tolerant [NiFe]-hydrogenase Hydrogenase-1 from *Escherichia coli* (*EcHyd1*) have found that without any prior illumination of the sample, except that from the ambient laboratory light, a signal consistent with the

Ni-L1 state of Hydrogenase-I from *Aquifex aeolicus* can be observed at 80 K.<sup>34</sup> However, unlike for *Aquifex aeolicus*, there was no evidence of a Ni-C-like species even after the sample was left in the dark at 250 K for two hours.<sup>34</sup> Infrared spectroelectrochemical studies of *EcHyd1* carried out at ambient temperature have detected two Ni-L states (Ni-L2 and Ni-L3) together with a Ni-C state. All three states were observed upon H<sub>2</sub>-reduction of as-isolated enzyme implying that the Ni-L state is a key intermediate in the catalytic cycle of this enzyme.<sup>189</sup>

Various possible mechanisms of H<sub>2</sub> cycling by [NiFe]-hydrogenases based on theoretical studies have been published, and a number of these incorporate a Ni<sup>+</sup> state with no bridging ligand present along with a protonated cysteine ligand, a structure consistent with that proposed for the Ni-L state (see, for example, Scheme 1.2).<sup>74,190</sup> Lill and Siegbahn<sup>191</sup> have proposed the first mechanism which explicitly includes the Ni-L state as an intermediate in the catalytic cycle of [NiFe]-hydrogenases. The proposed mechanism, shown in Scheme 6.1, is initiated by heterolytic cleavage of H<sub>2</sub> by the Ni-SI<sub>a</sub> prior to the first catalytic cycle, yielding the Ni-R state. A proton coupled electron transfer then forms the Ni-C state which is in equilibrium with a Ni<sup>+</sup> Ni-L state, formed upon proton transfer. The Ni-L state can undergo oxidative addition of H<sub>2</sub> resulting in the formation of a currently uncharacterised intermediate which the authors referred to as Ni<sub>a</sub>-X\*. Two proton coupled electron transfer steps follow, first generating the Ni-R state and then the Ni-C state to complete the cycle. Subsequent catalytic cycles involve the Ni-C, Ni-L, Ni<sub>a</sub>-X\* and Ni-R states only.

## 6.2 Attenuated Total Reflectance Infrared Spectroelectrochemical Studies on *EcHyd1*

When held at a certain potential, a redox-active sample will adopt a redox-composition where the concentrations of reduced and oxidised species are dictated by the Nernst equation (Equation 2.5). Infrared (IR) spectroelectrochemical studies were performed to investigate the various redox states of *EcHyd1* which occur at



**Scheme 6.1:** The mechanism proposed by Lill and Siegbahn<sup>191</sup> which includes the Ni-L state as a catalytic intermediate. The nomenclature has been changed to that used in this thesis, with the exception of the  $\text{Ni}_a\text{-X}^*$  state which is unprecedented. The active site structure corresponding to each state is also included. This figure has been adapted from Reference 191.

different potentials.

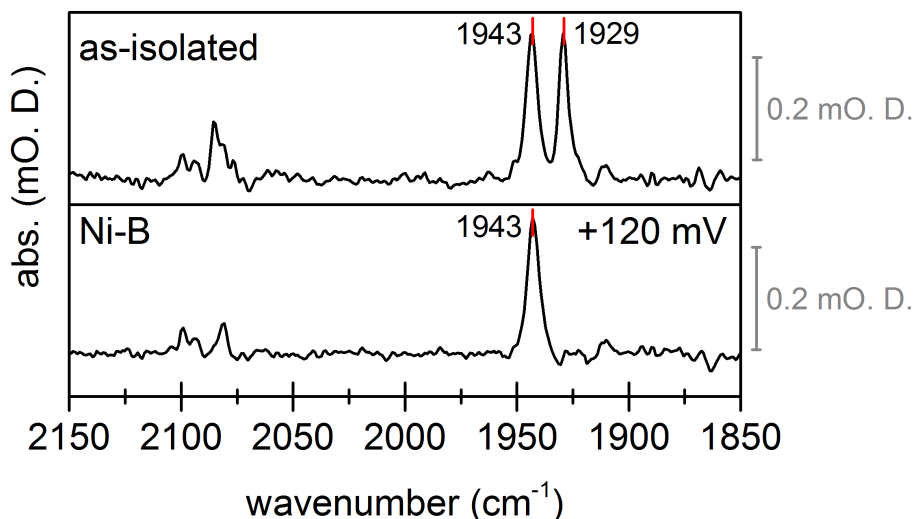
Using an Attenuated Total Reflectance Infrared (ATR-IR) setup developed by Healy and co-workers,<sup>171</sup> used previously in Chapter 5 Section 5.4 and shown in Figure 5.13, a sample of *Ec*Hyd1 was studied at potentials between  $-400$  mV and  $+120$  mV. The ATR-IR setup allows unmediated electrochemical control of the enzyme sample through the use of a three-dimensional carbon particle working electrode, and once the enzyme sample is placed inside the spectroelectrochemical cell, the sample is kept completely free from visible light. Additionally, the ATR-IR setup allows a complete redox titration to be obtained for a single enzyme sample and allows the change in the concentration of different redox species with potential to be monitored in ‘real-time’.

A sample of as-isolated *Ec*Hyd1 was mixed with Nafion dissolved in phosphate buffer (pH 6) containing carbon particles. This mixture was applied as a ‘film’ to the top of the Si ATR prism and covered with carbon paper. The remainder of the ATR-IR spectroelectrochemical cell was then assembled above the prism (see



Figure 5.13) and an ‘as-isolated’ infrared spectrum was recorded (Figure 6.1).

In order to obtain a ‘clean’ spectrum, the enzyme sample was activated within the ATR-IR cell by poisoning the sample at a low potential ( $-400$  mV) for 4 hours. The sample was then reoxidised at  $+120$  mV and a second was spectrum recorded (Figure 6.1).



**Figure 6.1:** ATR-IR spectrum of as-isolated *EcHyd1* and reoxidised *EcHyd1* (after initial reductive activation), as indicated in the figure. The  $\nu_{CO}$  values at the dominant peak growth positions are indicated and correspond to the following states: Ni-B,  $1943\text{ cm}^{-1}$ ; Ni-SI,  $1929\text{ cm}^{-1}$ . All spectra were recorded in  $N_2$ -saturated phosphate buffer, pH 6.

The as-isolated spectrum in Figure 6.1 shows a number of states present, with peaks corresponding to mainly the Ni-B ( $\nu_{CO}=1943\text{ cm}^{-1}$ ) and Ni-SI ( $\nu_{CO}=1929\text{ cm}^{-1}$ ) states. Currently only one Ni-SI state has been detected by IR for *EcHyd1* and it has not yet been possible to determine whether this is the Ni-SI<sub>a</sub> or Ni-SI<sub>r</sub> state<sup>171</sup> thus, in this chapter, this state will simply be referred to as Ni-SI. After activation and reoxidation only the Ni-B state is observed.

In order to monitor the potential-dependent transitions between the active site states, the potential of the solution was gradually decreased from  $+120$  mV to  $-400$  mV and spectra were recorded at various potential intervals. Once the sample had been fully reduced and a spectrum had been recorded at  $-400$  mV, the potential was then raised back to  $+120$  mV, with spectra being recorded at the same potentials

as before. The resulting spectra, taken at each potential step, are displayed as difference spectra with respect to the previously recorded spectrum in Figure 6.2.

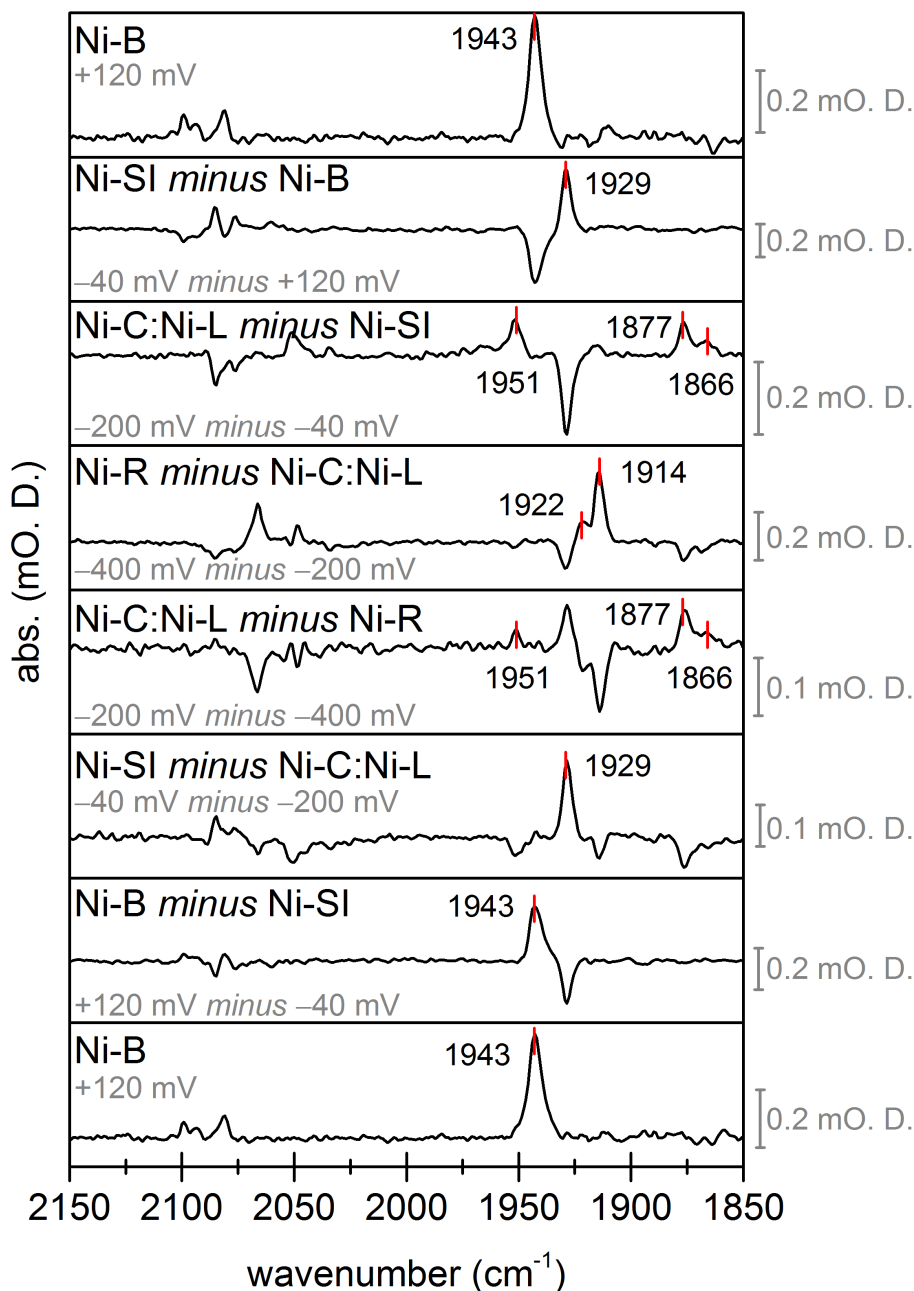
Figure 6.2 shows the reversible reduction of enzyme in the Ni-B state to the Ni-R state passing through the Ni-SI, Ni-C ( $\nu_{CO}=1951\text{ cm}^{-1}$ ) and Ni-L states. Two Ni-L states (Ni-L<sub>II</sub>,  $\nu_{CO}=1877\text{ cm}^{-1}$  and Ni-L<sub>III</sub>,  $\nu_{CO}=1866\text{ cm}^{-1}$ ) and two Ni-R states (Ni-R<sub>II</sub>,  $\nu_{CO}=1922\text{ cm}^{-1}$  and Ni-R<sub>III</sub>,  $\nu_{CO}=1914\text{ cm}^{-1}$ ) were detected, consistent with previous ATR-IR studies of *EcHyd1*.<sup>189</sup> During the spectroelectrochemical titrations, the Ni-C and Ni-L states, which occur within the same potential window, are formed reversibly.

Following the reoxidation of the sample to achieve only Ni-B, H<sub>2</sub>-saturated buffer was introduced into the ATR-IR cell (Figure 6.3).

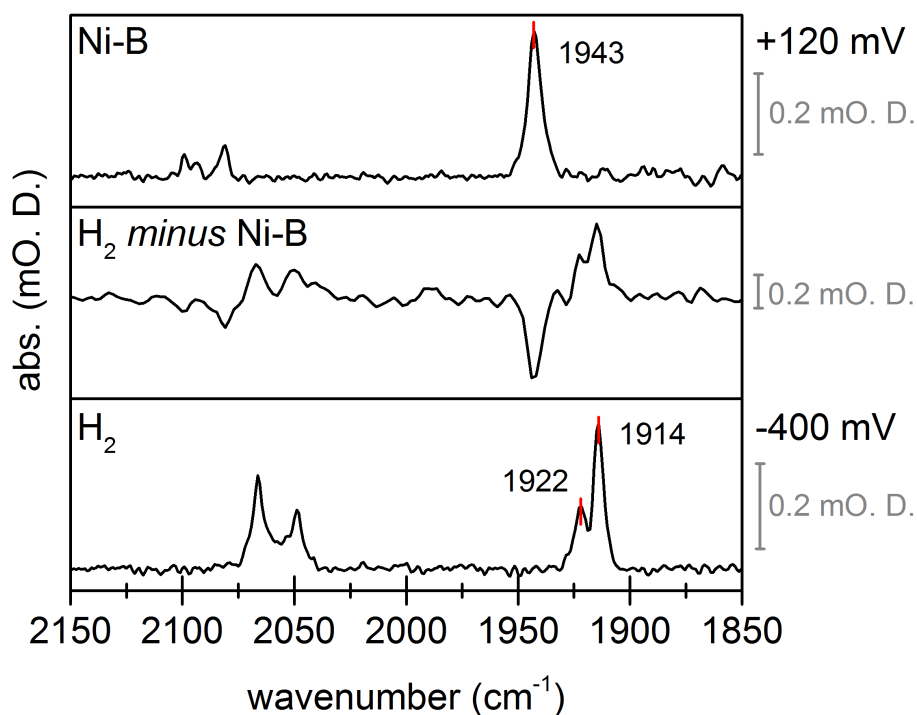
Before exposure, only the Ni-B state is observed, indicated by  $\nu_{CO}=1943\text{ cm}^{-1}$ . After exposure to H<sub>2</sub> all enzyme is reduced to form the Ni-R state, as shown by comparison of the difference spectra obtained after H<sub>2</sub> exposure (middle panel) and the absolute spectrum showing the complete electrochemical conversion of the enzyme into the Ni-R<sub>II</sub> and Ni-R<sub>III</sub> states ( $\nu_{CO}=1922\text{ cm}^{-1}$  and  $1914\text{ cm}^{-1}$  respectively). This complete conversion suggests that the enzyme remains active during and after the redox titration, *i.e.* both before and after passing through the Ni-L state. Subtraction of the electrochemically-reduced spectrum from the H<sub>2</sub>-reduced spectrum yields a flat line (not shown).

### 6.3 Electron Paramagnetic Resonance Studies of *EcHyd1*

To resolve the Ni signals associated with the [NiFe]-hydrogenase active site, Electron Paramagnetic Resonance (EPR) measurements were undertaken under cryogenic conditions. When preparing a sample for measurement by EPR, a sample would usually be frozen whilst exposed to the ambient light conditions of the laboratory. In this chapter, in order to evaluate the effect of light exposure upon the



**Figure 6.2:** Redox-triggered ATR-IR spectra of *EcHyd1*. A spectrum was measured at +120 mV before a series of potential steps and measurements were carried out as follows: -40 mV, -200 mV, -400 mV, -200 mV, -40 mV and +120 mV. The very top and bottom panels show the absolute spectra obtained at +120 mV before and after the potential sequence, all other panels show difference spectra as indicated in the figure. The  $\nu_{\text{CO}}$  values at the dominant peak growth positions are indicated for each panel and correspond to the following states: Ni-B, 1943  $\text{cm}^{-1}$ ; Ni-SI, 1929  $\text{cm}^{-1}$ ; Ni-C, 1951  $\text{cm}^{-1}$ ; Ni-L<sub>II</sub>, 1877  $\text{cm}^{-1}$ ; Ni-L<sub>III</sub>, 1866  $\text{cm}^{-1}$ ; Ni-R<sub>II</sub>, 1922  $\text{cm}^{-1}$ ; Ni-R<sub>III</sub>, 1914  $\text{cm}^{-1}$ . All spectra were recorded in  $\text{N}_2$ -saturated phosphate buffer, pH 6.



**Figure 6.3:** ATR-IR spectrum of as-isolated *EcHyd1* before (top panel) and after (middle and bottom panels) exposure to  $\text{H}_2$ . The top and bottom panels show absolute spectra whilst the middle panel shows a difference spectrum (bottom panel minus top panel). The  $\nu_{\text{CO}}$  values at the dominant peak growth positions are indicated for each panel and correspond to the following states: Ni-B,  $1943\text{ cm}^{-1}$ ; Ni-R<sub>II</sub>,  $1922\text{ cm}^{-1}$ ; Ni-R<sub>III</sub>,  $1914\text{ cm}^{-1}$ . All spectra were recorded in  $\text{N}_2$ -saturated or  $\text{H}_2$ -saturated phosphate buffer, pH 6.

active site states observed in a sample of *EcHyd1*, samples were prepared, frozen and transferred to the resonator under conditions free of visible light.

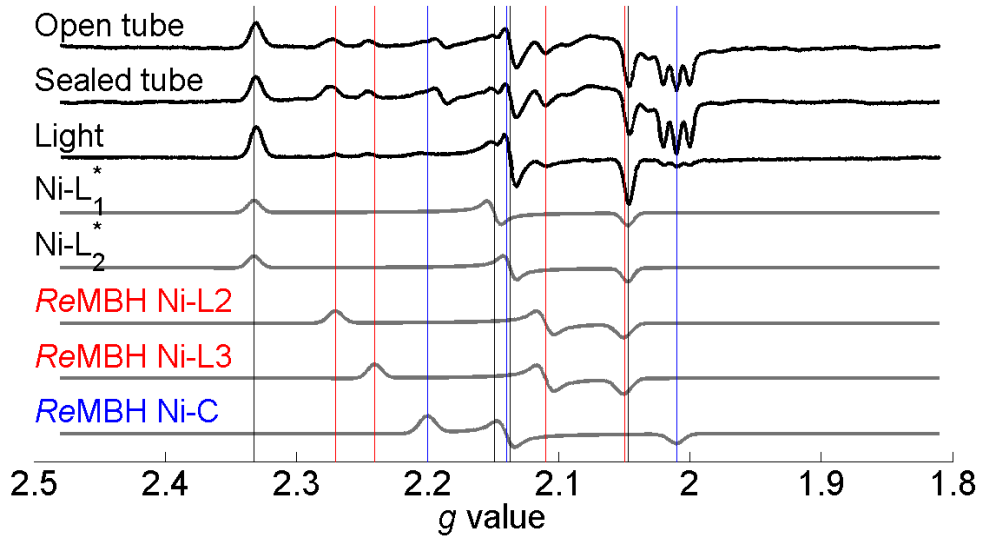
Sample preparation was completed within a glove box ( $< 2$  ppm  $O_2$ ). To make the samples, 1  $\mu$ L each of the redox mediators benzyl viologen and 2-hydroxyl-1,4-naphthoquinone (both 50  $\mu$ M) were added to 400  $\mu$ L of *EcHyd1* solution (4.42 mg/mL, in pH 6 100 mM MES/100 mM NaCl buffer) along with 1  $\mu$ L  $Na_2SO_4$  (400  $\mu$ M, made up in pH 8 100 mM borate buffer). The whole mixture was then placed within a titration cell (see Section 2.3.2.1) before the enzyme solution was activated under a flow of  $H_2$  for 4 hours. After activation, the  $H_2$  was flushed out of the solution with a flow of Ar for *ca.* 1 hour before 0.05–0.1  $\mu$ L aliquots of 200 mM  $Na_2SO_4$  were added to the solution until the potential, measured via chronopotentiometry with the current set at 0 nA, stabilised at  $-214$  mV.

The final sample was then split into two, each portion being placed within a separate EPR sample tube. One tube was flame-sealed outside of the glove box, carefully keeping the enzyme anaerobic throughout the procedure. Once sealed the environment inside this tube was permanently oxygen-free. The other tube was sealed using a removable gas tight seal. Both samples were then kept in the dark, within a glove box, overnight.

The use of a night vision camera allowed both samples to be removed from the glove box and placed within the EPR spectrometer without exposure to visible light. The procedure for measuring each sample was as follows: The tube with the temporary seal was frozen in liquid nitrogen and then placed into the resonator. The sample in the flame-sealed tube was placed into the resonator, where it was frozen under a flow of liquid He. Once in the resonator the samples were shielded from the ambient light of the laboratory.

The spectra measured for these samples are shown in Figure 6.4 along with, for comparison, a spectrum obtained from a sample made and frozen under the ambient light conditions of the laboratory as in ‘standard’ experiments.

Both samples frozen in the dark show signals previously associated with the



**Figure 6.4:** Spectra measured for *EcHyd1*, prepared under conditions free from visible light. Two ‘dark’ samples were made; one in an open tube, frozen outside the resonator in liquid  $N_2$ , and one in a sealed tube, frozen inside the resonator; labelled ‘Open tube’ and ‘Sealed tube’, respectively, in the figure. For comparison a sample of *EcHyd1* prepared under the ambient light conditions of the laboratory, labelled ‘Light’, is also shown. All other spectra are simulations and are as follows;  $Ni-L_1^*$  and  $Ni-L_2^*$  show the  $g$ -values of the Ni-L-like states previously detected for *EcHyd1*;  $Ni-L_2$  and  $Ni-L_3$  show the  $g$ -values previously obtained for two of the light-dependent Ni-L states observed in *ReMBH*; finally, Ni-C denotes the  $g$ -values obtained for the Ni-C state of *ReMBH*.

Ni-L<sub>1</sub>\* and Ni-L<sub>2</sub>\* states observed in *EcHyd1* ( $g_{xyz} = 2.33, 2.15, 2.05$  and  $g_{xyz} = 2.33, 2.14, 2.05$  respectively). Two other  $g_x$  signals are prominent at  $g = 2.27$  and  $g = 2.25$ . These correspond closely to the  $g_x$  values reported for the *ReMBH* Ni-L2 and Ni-L3 states ( $g_x = 2.27$  and  $g_x = 2.24$  respectively). Values of  $g_y = 2.11$  and  $g_z = 2.05$  were reported for the *ReMBH* Ni-L2 and Ni-L3 states and corresponding signals are observed in the open tube and sealed tube experimental data. Signals at  $g$ -values previously reported for the Ni-L<sub>1</sub>\* and Ni-L<sub>2</sub>\* states in *EcHyd1* are also observed in the sample of *EcHyd1* frozen under ambient light conditions (labelled as ‘Light’ in Figure 6.4). Very small signals at  $g = 2.27$  and  $g = 2.25$  can also be seen. Finally, a derivative-like signal can be observed at  $g = 2.19$  in the spectra resulting from the samples that were frozen under dark conditions. This signal does not correspond to any signal observed previously in hydrogenase EPR spectra. No signals consistent with a Ni-C state can be observed in any of the experimental spectra. For comparison purposes, a simulation of the Ni-C signal observed for *ReMBH* ( $g_{xyz} = 2.20, 2.14, 2.01$ ) is shown in Figure 6.4. All Ni-C states reported fall within  $\pm 0.01$  of those reported for *ReMBH*.

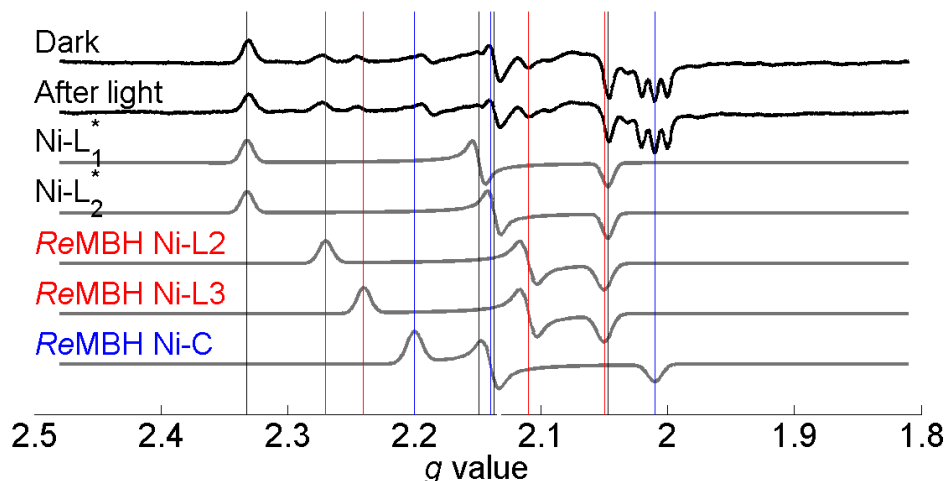
The samples corresponding to the ‘Open tube’ and ‘Sealed tube’ spectra shown in Figure 6.4 were defrosted and then subjected to the ambient light conditions of the laboratory (whilst ensuring that both samples were kept under anaerobic conditions throughout). The samples were then refrozen and EPR spectra were recorded. The resulting spectrum for the open tube sample is shown in Figure 6.5.

After placing the samples under ambient light for 17 hours the resulting EPR spectra look identical to that recorded previously (data not shown for the ‘Sealed tube’ sample).

## 6.4 Discussion

The results presented in this chapter confirm that Ni-L-like states of *EcHyd1* can be formed in the absence of visible light.

Spectra obtained using ATR-IR spectroscopy show evidence of the formation of



**Figure 6.5:** The effect of ambient light upon a sample of *EcHyd1* previously shown to form a number of Ni-L-like states under dark conditions. The sample labelled ‘Dark’ is the same spectrum as the ‘Open tube’ spectrum shown in Figure 6.4. The sample labelled ‘After light’ shows the resulting spectrum taken after the same sample was placed in ambient light for 17 hours. All other spectra shown are simulations and are as for Figure 6.4.

both the Ni-C and two Ni-L states (referred to as Ni-L<sub>II</sub> and Ni-L<sub>III</sub>). Formation of the Ni-C, Ni-L<sub>II</sub> and Ni-L<sub>III</sub> states occurs within the same potential window. After the formation of the Ni-L<sub>II</sub> and Ni-L<sub>III</sub> states, the enzyme is still catalytically active and able to react with H<sub>2</sub>.

Measurements made using EPR show a number of species with *g*-values consistent with those previously reported for Ni-L species, namely the Ni-L<sub>1</sub>\* and Ni-L<sub>2</sub>\* states previously observed in *EcHyd1* and the Ni-L<sub>2</sub> and Ni-L<sub>3</sub> states observed in *ReMBH*.<sup>187</sup> The relative proportion of these states varies depending upon whether the sample is frozen immediately after its preparation under ambient light or left in the dark overnight and then frozen under conditions free from visible light. This may indicate that the various Ni-L states detected, thought to be different protonation states of the enzyme, are in an equilibrium, the position of which is dependent upon light exposure. The energy difference between the states involved in this equilibrium, which would also involve the Ni-C state, may vary between hydrogenases. The observation of Ni-L states in the absence of visible light is consistent with the Ni-L states being more energetically favoured than the Ni-C state in *EcHyd1* rela-



tive to other hydrogenase enzymes. The very small signals at  $g = 2.27$  and  $g = 2.25$  in the spectrum associated with the sample frozen in the light, *i.e.* under ‘usual’ conditions, may be the reason that these states have not previously been reported for *EcHyd1*.

The observation of a Ni-C-like state *via* ATR-IR but not *via* EPR is consistent with previous reports.<sup>34,73,189</sup> Electron paramagnetic resonance samples are prepared in a bulk solution in which the potential is controlled by redox mediators. This results in slow electron-transfer between the redox mediator and the enzyme, relative to the rate of catalysis, enabling the enzyme to stabilise in a particular redox state. However, when measuring a sample *via* ATR-IR, the enzyme is adsorbed directly onto the carbon particle electrode network. This results in fast electron-transfer between the enzyme and the electrode which, together with the presence of protons in the surrounding aqueous solution, enables the hydrogenase to continuously turnover, passing through a number of transient catalytic intermediates which may, in turn, be detected using this method.

## 6.5 Conclusions and Perspectives

The results presented in this chapter are consistent with the Ni-L state being a catalytic intermediate detectable by both ATR-IR and EPR, and not merely an artefact that forms when a [NiFe]-hydrogenase active site is exposed to light.

The varying behaviour of the Ni-L states relative to the Ni-C state in different [NiFe]-hydrogenases is consistent with the energy difference between these states differing within the class. In *EcHyd1*, the Ni-L states are lower in energy and this hydrogenase will adopt the Ni-L state even in the dark. In other hydrogenases, the Ni-C state is the favoured state and population of the Ni-L states is only possible upon excitation by visible light. The results presented in this chapter, along with observations made on the O<sub>2</sub>-tolerant [NiFe]-hydrogenases *ReMBH*<sup>187</sup> and Hydrogenase-I from *Aquifex aeolicus*,<sup>73</sup> in which Ni-L states were detected in enzyme samples that had only been exposed to the ambient light conditions of the

laboratory, may indicate that in general, the energy of the Ni-L states are closer to that of the Ni-C state in the O<sub>2</sub>-tolerant [NiFe]-hydrogenases relative to the O<sub>2</sub>-sensitive enzymes. *EcHyd1* is an extreme case in which the Ni-L states are lower in energy than the Ni-C state.

A more thorough investigation is needed to determine why the energies of the Ni-C and Ni-L states may differ between different hydrogenases. Considering that the active site structures of all [NiFe]-hydrogenases are the same, density functional theory could be used to investigate how the second sphere ligands surrounding the active site may affect the energy of the different states. A temperature dependent ATR-IR study may also help to determine the relationship between the Ni-L and Ni-C states in *EcHyd1*.

## Chapter 7

# Electrochemical Investigations of the Spontaneous Activation of [FeFe]-Hydrogenases by an Inorganic [2Fe] Active Site Mimic



## Abstract

Apo-hydrogenase, that is, hydrogenase protein that incorporates a preassembled [4Fe4S]-cluster but does not include a [2Fe] binuclear sub-cluster to complete the formation of the H-cluster, is unable to interconvert  $\text{H}_2$  and  $\text{H}^+$ . This chapter details Protein Film Electrochemistry (PFE) experiments that investigate the spontaneous *in vitro* incorporation of a synthetic [2Fe] complex, homologous to the [2Fe] binuclear sub-cluster of the H-cluster, into apo-hydrogenase. This incorporation results in fully functional enzyme with a voltammetric waveshape indistinguishable from that of the native enzyme.

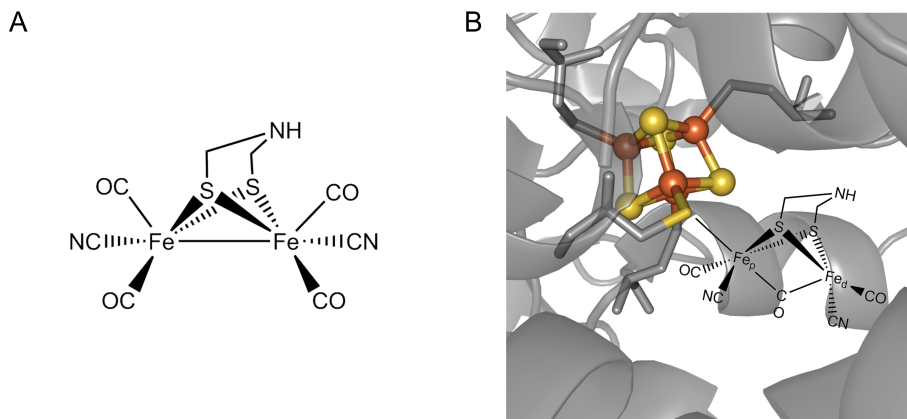
As studied by PFE, the formation of active hydrogenase from apo-hydrogenase corresponds directly to an increase in observed catalytic current over time. In this chapter chronoamperometry measurements provide a quantitative description of the effect of potential, temperature and [2Fe]-complex concentration upon the rate of synthetic complex incorporation into apo-hydrogenase from both *Chlamydomonas reinhardtii* and *Clostridium pasteurianum*. Significant differences are observed between the two enzymes suggesting the importance that protein structure has upon the mechanism.

This work provides the first electrochemical account of the incorporation of an active site structural analogue into apo-hydrogenase and as such it highlights the possibilities that PFE provides for investigating the spontaneous incorporation of other [2Fe]-subcluster mimics.

## 7.1 Introduction

Biomimetic chemistry can provide a valuable tool for understanding the chemically and/or thermodynamically challenging reactions catalysed by many metalloenzymes.<sup>192</sup> The importance of the reactions carried out by these enzymes has inspired many researchers to create functional analogues designed to mimic the catalytic processes. Whereas the structure of functional analogues can differ substantially from the natural co-factor, structural analogues are exact chemical reproductions.<sup>193</sup> Structural analogues can be used to reconstitute the activity of an isolated apo-enzyme (an enzyme without its co-factors) and can provide substantial insights into its structure-function relationship. The reconstitution of apo-enzymes can generate novel semisynthetic enzymes, with enhanced or even entirely new functionality.<sup>192</sup> Reconstitution has been successfully demonstrated for [FeFe]-hydrogenases.<sup>45,46</sup> The native H-cluster has not been extracted to date and these studies instead used a chemically synthesised mimic homologous to the [2Fe] binuclear subcluster of the H-cluster<sup>194</sup> shown in Figure 7.1A. The first study successfully loaded the [2Fe] synthetic mimic (henceforth referred to as  $[\text{Fe}]^{\text{MIM}}$ ) onto the maturase enzyme HydF from *Thermotoga maritima*,<sup>45</sup> the scaffold protein upon which the [2Fe]-moiety is usually assembled *in vivo*.<sup>45</sup> The holo-HydF entity formed is able to transfer the [2Fe]-subcluster to a hydrogenase precursor from *Chlamydomonas reinhardtii* (apo-HydA1), consisting of the hydrogenase protein and a preassembled [4Fe4S]-cluster. Reconstitution of  $[\text{Fe}]^{\text{MIM}}$  and apo-HydA1 then resulted in holo-HydA1 of full hydrogenase activity<sup>45</sup> (Figure 7.1B). A further study demonstrated that *in vitro*  $[\text{Fe}]^{\text{MIM}}$  could activate apo-HydA1 even in the absence of HydF or any other helper proteins.<sup>46</sup> Furthermore,  $[\text{Fe}]^{\text{MIM}}$  could also be used to activate the apo-hydrogenase from *Megasphaera elsdenii* (HYDAME) and *Clostridium pasteurianum* (CpI) *in vitro* forming fully functional holo-HYDAME and holo-CpI respectively. Spontaneous chaperone-independent assembly of functional hydrogenases offers great possibility: Not only does it mean that attempts to incorporate other structural and functional analogues into the natural protein environment may

prove successful, but it may also lead the way to engineering and testing artificial protein environments. In the future entirely artificial hydrogenases may be possible, engineered with specific technological applications in mind.<sup>193</sup>



**Figure 7.1:** (A) Representation of the synthetic [2Fe]-subcluster mimic ( $[\text{Fe}]^{\text{MIM}}$ ) found to spontaneously activate apo-hydrogenase, forming fully functional holo-enzyme.  $[\text{Fe}]^{\text{MIM}}$  differs from the native [2Fe]-subcluster by an additional CO ligand. (B) Representation of the [2Fe]- and [4Fe4S]-subclusters, together forming the H-cluster, bound within the protein structure (PDB code 3C8Y, some amino acids have been deleted to provide an unobstructed view of the H-cluster). The [2Fe]-subcluster is shown schematically, whilst the [4Fe4S]-cluster is shown in ball and stick representation. The protein is shown in grey using a ribbon representation. Colours represent the following atoms: Fe orange, S yellow, coordinating cysteines are shown in grey.

This chapter details the first electrochemical study investigating the spontaneous incorporation of a [2Fe]-subcluster mimic into apo-hydrogenase entities from both *Chlamydomonas reinhardtii* (CrHydA1) and *Clostridium pasteurianum* (CpI) in an attempt to elucidate details of this mechanism and to further the work described above.

### 7.1.1 The synthetic [2Fe]-subcluster complex ( $[\text{Fe}]^{\text{MIM}}$ )

The work presented in this chapter was completed in collaboration with Julian Esselborn working within the laboratory of Prof. Thomas Happe at the Ruhr-Universität in Bochum, Germany.  $[\text{Fe}]^{\text{MIM}}$  was made by Julian Esselborn using a previously published protocol<sup>194</sup> and was supplied as a 15.5 mM stock solution in phosphate buffer (see Section 2.3.4 for specific details regarding the buffer compo-

sition). The stock solution was stored at  $-80\text{ }^{\circ}\text{C}$  and was diluted with phosphate buffer prior to use, with care taken to chill the buffer beforehand in order to keep the sample cold. In this chapter, a 5000 x dilution of the stock solution, resulting in a  $3.1\text{ }\mu\text{M}$  solution of  $[\text{Fe}]^{\text{MIM}}$ , was most commonly used. During an experiment, an injection of 0.16 mL of this solution into 2 mL of cell solution would give a final concentration of  $0.2296\text{ }\mu\text{M}$   $[\text{Fe}]^{\text{MIM}}$ .

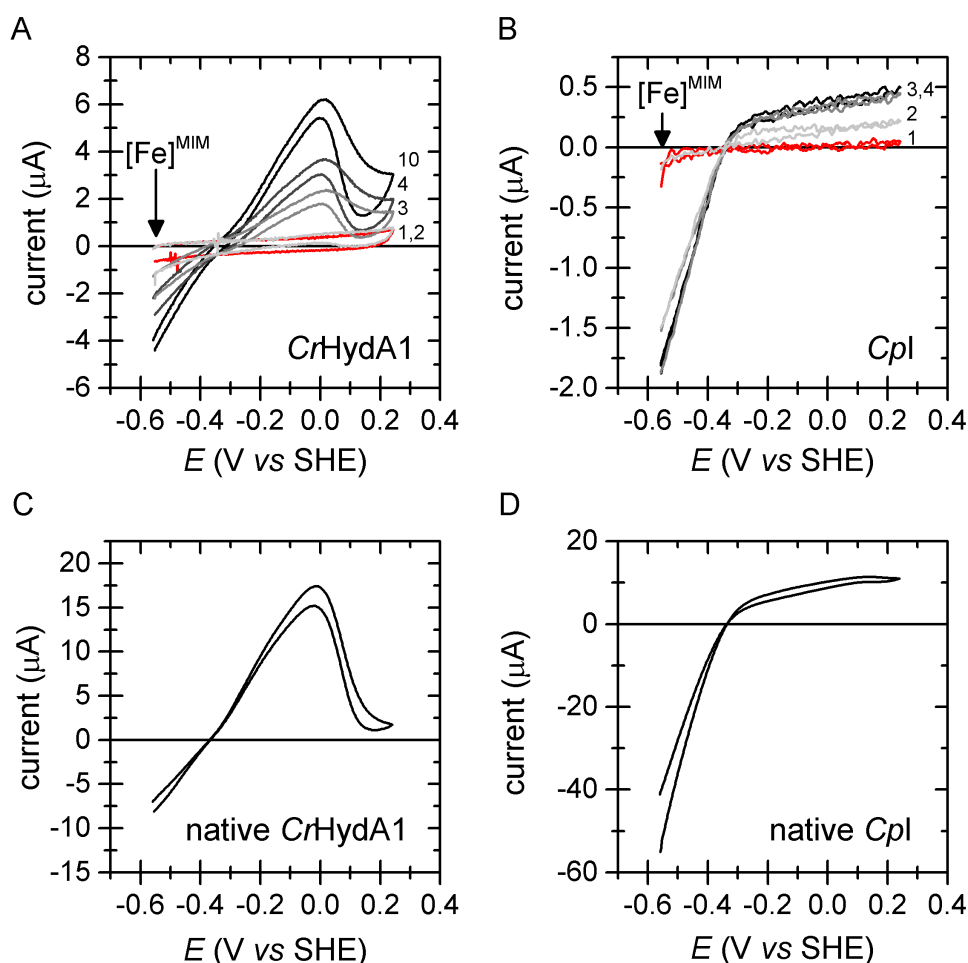
## 7.2 Results

### 7.2.1 Cyclic Voltammetry Studies on the Spontaneous Activation of Apo-Hydrogenase by a [2Fe]-Subcluster Mimic

In order to monitor the spontaneous incorporation of a [2Fe]-subcluster mimic into the apo-protein of either *Chlamydomonas reinhardtii* (CrHydA1) or *Clostridium pasteurianum* (CpI), a film of the apo-hydrogenase protein was applied to a PGE electrode (see Sections 2.3.1.1 and 2.3.1.3). The potential was then swept between  $-560\text{ mV}$  and  $+240\text{ mV}$  completing a full scan before an aliquot of buffer containing the mimic  $[\text{Fe}]^{\text{MIM}}$  (Figure 7.1A) was injected into the cell to give a final concentration of  $0.2296\text{ }\mu\text{M}$  of  $[\text{Fe}]^{\text{MIM}}$ . The incorporation of  $[\text{Fe}]^{\text{MIM}}$  into the apo-protein was then monitored as a change in current over subsequent cyclic voltammetry scans (Figure 7.2).

Before the introduction of  $[\text{Fe}]^{\text{MIM}}$  into the cell no catalytic current is observed (Figures 7.2A and 7.2B, red traces) and the current *vs* potential traces resemble a ‘blank’ scan with the observed current being attributed to non-Faradaic current only (obtained when no electroactive species is present, see Section 2.1.3.1). The considerably smaller non-Faradaic current observed in Figure 7.2B compared to Figure 7.2A is due to the much slower scan rate used in the experiment shown in Figure 7.2B.<sup>111</sup> Upon the introduction of  $[\text{Fe}]^{\text{MIM}}$  a gradual increase in current can be observed. In both Figures 7.2A and 7.2B the waveshapes observed in the third





**Figure 7.2:** The effect of  $[\text{Fe}]^{\text{MIM}}$  upon apo-hydrogenase from (A) *CrHydA1* and (B) *Cpl* when introduced at  $-560$  mV during a set of cyclic voltammetry scans. The scan prior to the introduction of  $[\text{Fe}]^{\text{MIM}}$  is shown in red and those after are shown in shades of grey. In both cases  $[\text{Fe}]^{\text{MIM}}$  was introduced at the start of the second scan as indicated by the arrows (the potential was swept from low to high), to give a final concentration of  $0.2296 \mu\text{M}$  of  $[\text{Fe}]^{\text{MIM}}$ . The scan number is indicated alongside each plot. Conditions for (A): pH 6.1,  $5^\circ\text{C}$ ,  $\nu = 30 \text{ mV s}^{-1}$ ,  $\omega = 1000 \text{ rpm}$ , 100%  $\text{H}_2$ . Conditions for (B) as for (A) except:  $15^\circ\text{C}$ ,  $\nu = 2 \text{ mV s}^{-1}$ . Data in (B) have been smoothed using a Savitzky-Golay function with 10 points of window. Panels (C) and (D) show cyclic voltammetry scans obtained from native holo-enzyme samples of *CrHydA1* and *Cpl* respectively. Conditions for (C): pH 6,  $18^\circ\text{C}$ ,  $\nu = 5 \text{ mV s}^{-1}$ ,  $\omega = 2500 \text{ rpm}$ , 100%  $\text{H}_2$ . Conditions for (D): pH 6,  $15^\circ\text{C}$ ,  $\nu = 2 \text{ mV s}^{-1}$ ,  $\omega = 1000 \text{ rpm}$ , 100%  $\text{H}_2$ .

scans have the same characteristic features as the waveshapes obtained from the native holo-enzymes of *CrHydA1* and *CpI* respectively (Figures 7.2C and 7.2D).

The current continues to increase over a number of cycles until eventually subsequent scans overlay or, due to the effects of film loss, start to decrease in magnitude.

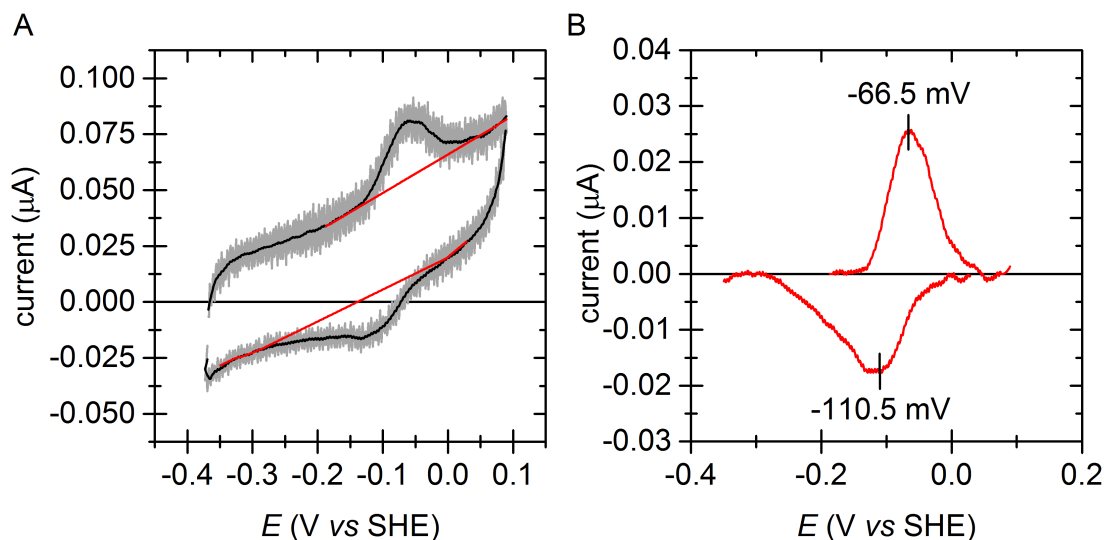
Once a stable current had been reached, the electrode was removed from the cell and rinsed with ultrapure water. The cell solution was then exchanged for fresh buffer and the electrode placed back into the cell, before a second cyclic voltammetry experiment was carried out. The voltammograms obtained were identical to those obtained prior to the buffer exchange, with any film loss occurring at a similar rate to before (not shown). This is consistent with the incorporation of  $[\text{Fe}]^{\text{MIM}}$  into the apo-protein being irreversible, resulting in stable holo-enzyme.

### 7.2.2 The Electrochemistry of $[\text{Fe}]^{\text{MIM}}$

In order to monitor the redox activity of  $[\text{Fe}]^{\text{MIM}}$  alone, a 2.2963  $\mu\text{M}$  solution of  $[\text{Fe}]^{\text{MIM}}$  was placed inside the electrochemical cell. Using a ‘blank’ PGE electrode (*i.e.* one which had not been modified with protein) cyclic voltammetry sweeps were undertaken over the potential region studied in this chapter.

In the cyclic voltammogram shown in Figure 7.3A the potential was swept between  $-370$  mV and  $+90$  mV. The resulting voltammogram shows a reversible redox peak at  $-66.5$  mV on the oxidative sweep and  $-110.5$  mV on the reductive sweep. The 44 mV difference between these peaks is consistent with them being attributed to a one electron redox process.<sup>111</sup> No other features are seen in the voltammetry. A  $[\text{Fe}]^{\text{MIM}}$  concentration ten times higher than that used in the majority of experiments with apo-protein in this chapter was necessary for any redox activity to be observed.

Further experiments where a wider potential region was monitored ( $-560$  mV to  $+240$  mV) did not show any other signals (not shown).

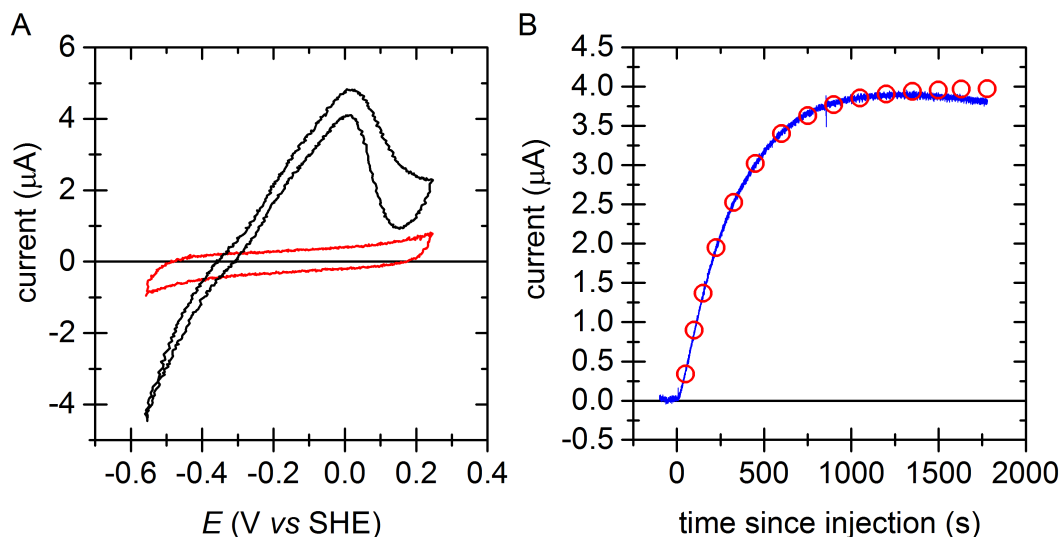


**Figure 7.3:** (A) Cyclic voltammogram of  $[\text{Fe}]^{\text{MIM}}$  in solution (grey). To find accurate peak positions the raw data was smoothed using a Savitzky-Golay method with 20 points of window (black line). A baseline was then fitted to the data (red line) and subtracted from the data. (B) The baseline subtracted data using the data and baseline shown in panel A. Peak positions are as highlighted in the panel. Conditions: pH 6.1, 15 °C,  $\nu = 7 \text{ mV s}^{-1}$ , concentration of  $[\text{Fe}]^{\text{MIM}} = 2.2963 \text{ } \mu\text{M}$ , no electrode rotation.

### 7.2.3 Chronoamperometry Studies on the Spontaneous Incorporation of $[\text{Fe}]^{\text{MIM}}$ into Apo-Hydrogenase

Chronoamperometry allows the rate of  $[\text{Fe}]^{\text{MIM}}$  incorporation into apo-hydrogenase adsorbed onto an electrode to be determined. As  $[\text{Fe}]^{\text{MIM}}$  is incorporated into the apo-protein, and fully functional holo-protein is formed, a current will be produced proportional to the turnover rate of the active enzyme. By measuring the rate of the increase in current over time, the rate of incorporation can be found; in this way, the effect of the external conditions upon the incorporation of  $[\text{Fe}]^{\text{MIM}}$  into apo-hydrogenase adsorbed onto an electrode can be determined. Figure 7.4B shows the increase in current observed upon addition of a 0.16 mL aliquot of  $[\text{Fe}]^{\text{MIM}}$  into the electrochemical cell solution (original volume 2 mL), resulting in a final concentration of  $0.2296 \text{ } \mu\text{M}$  of  $[\text{Fe}]^{\text{MIM}}$ . In this example apo-hydrogenase from *CrHydA1* was adsorbed onto the electrode and the potential was held at  $-40 \text{ mV}$  throughout.

As seen in Figure 7.4B, the introduction of  $[\text{Fe}]^{\text{MIM}}$  into the cell solution resulted in an observable increase in current over time, consistent with  $[\text{Fe}]^{\text{MIM}}$  being incor-



**Figure 7.4:** (A) The current observed during a cyclic voltammetry experiment before (red line) and 1750 s after (black line) an injection of  $[\text{Fe}]^{\text{MIM}}$  into the cell solution, as shown in panel (B). (B) The current response (blue line) observed upon injection of a 0.16 mL aliquot of  $[\text{Fe}]^{\text{MIM}}$  at time  $t = 0$  s giving a final concentration of  $0.2296 \mu\text{M}$  of  $[\text{Fe}]^{\text{MIM}}$  inside the cell.  $E = -40$  mV throughout. The first 1400 s following the introduction of  $[\text{Fe}]^{\text{MIM}}$  has been fitted to a single exponential (red circles) giving a rate of  $0.003 \text{ s}^{-1}$ . Conditions: pH 6.1,  $15^\circ\text{C}$ ,  $\omega = 1000$  rpm, 100%  $\text{H}_2$ . Cyclic voltammetry undertaken with  $\nu = 30 \text{ mV s}^{-1}$ .

porated into the apo-protein resulting in functional holo-enzyme. The increasing current started to plateau after *ca.* 1000 s following the injection and after *ca.* 1400 s, the current is seen to decrease. This is consistent with the rate of film loss now becoming dominant over the increase in current that results from  $[\text{Fe}]^{\text{MIM}}$  uptake by the apo-protein. A single exponential can be fitted to the increasing current, prior to the point where film loss becomes visible, as shown by the red circles in Figure 7.4B (giving a rate of  $0.003 \text{ s}^{-1}$  in this case).

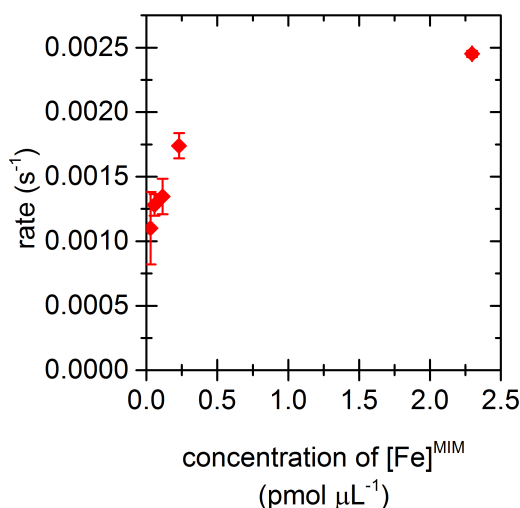
Figure 7.4A demonstrates that the increase in current seen in Figure 7.4B can be attributed to catalytic activity by functional *CrHydA1*. Prior to the introduction of  $[\text{Fe}]^{\text{MIM}}$  only non-Faradaic current is observed (red trace), whilst a cyclic voltammogram taken immediately after the chronoamperometry experiment (black trace) shows the characteristic waveshape expected from the holo-protein of *CrHydA1*, as observed in Figure 7.2C.

By adsorbing apo-hydrogenase from *CpI* onto an electrode, analogous experiments can be undertaken to investigate the incorporation of  $[\text{Fe}]^{\text{MIM}}$  into apo-

hydrogenase from this enzyme. Sections 7.2.3.1, 7.2.3.2 and 7.2.3.3 compare  $[\text{Fe}]^{\text{MIM}}$  incorporation into the apo-protein from *CrHydA1* and *CpI* using chronoamperometry experiments similar to those described in this section.

### 7.2.3.1 The Concentration Dependence of $[\text{Fe}]^{\text{MIM}}$ Incorporation into Apo-Hydrogenase from *CpI*

Aliquots of  $[\text{Fe}]^{\text{MIM}}$  of varying concentrations were introduced into the cell solution during experiments analogous to those described in Section 7.2.3. A summary of these results are shown in Figure 7.5.



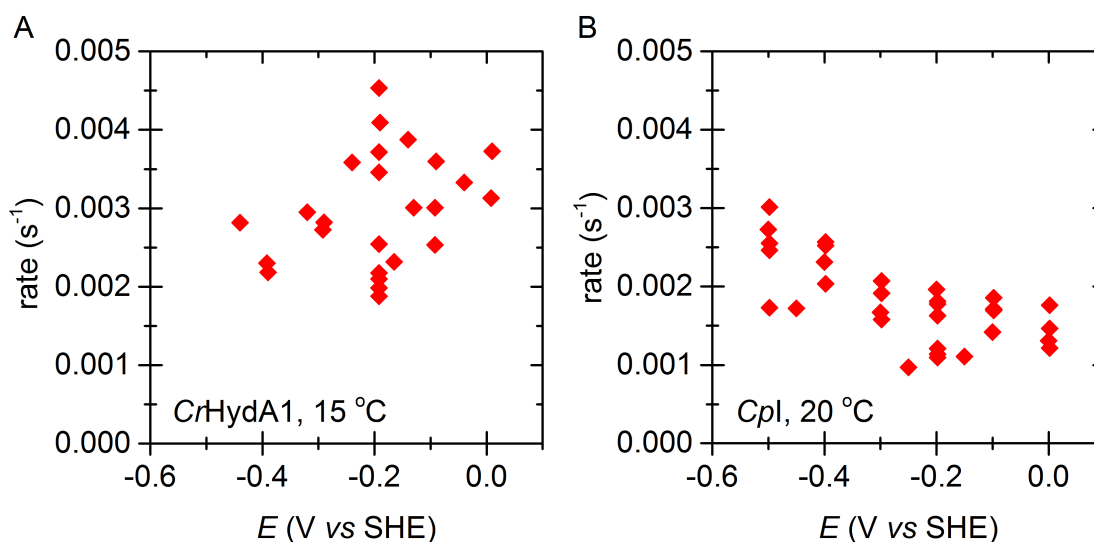
**Figure 7.5:** A summary of the results obtained from chronoamperometry experiments investigating the effect of the concentration of  $[\text{Fe}]^{\text{MIM}}$  on the rate of  $[\text{Fe}]^{\text{MIM}}$  incorporation into apo-hydrogenase from *CpI*. Diamonds reflect the values obtained by fitting the raw data to a single exponential as shown in Figure 7.4B. Conditions: pH 6.1, 20 °C,  $\omega = 1000$  rpm, 100%  $\text{H}_2$ ,  $E = -198$  mV. In each experiment a 0.16 mL aliquot of  $[\text{Fe}]^{\text{MIM}}$  was injected into the cell resulting in final concentrations of 2.2963, 0.2296, 0.1148, 0.0574 and 0.0287  $\mu\text{M}$  of  $[\text{Fe}]^{\text{MIM}}$ , representing an excess of 100x, 10x, 5x, 2.5x and 1.2x of  $[\text{Fe}]^{\text{MIM}}$  relative to apo-hydrogenase respectively. The error bars reflect the standard deviation of three repeats.

The data shown in Figure 7.5 are consistent with an increase in the rate of incorporation of  $[\text{Fe}]^{\text{MIM}}$  into *CpI* with increasing concentration of  $[\text{Fe}]^{\text{MIM}}$ . As the concentration of  $[\text{Fe}]^{\text{MIM}}$  in the cell solution increases above *ca.* 0.2296  $\mu\text{M}$  the rate of increasing incorporation with  $[\text{Fe}]^{\text{MIM}}$  concentration starts to decrease. In light of this result, a final concentration of 0.2296  $\mu\text{M}$   $[\text{Fe}]^{\text{MIM}}$  was chosen as the standard

concentration and was used in the remaining studies outlined in this chapter.

### 7.2.3.2 The Potential Dependence of $[\text{Fe}]^{\text{MIM}}$ Incorporation into Apo-Hydrogenase

Chronoamperometry experiments were completed to determine the effect of potential on the rate of incorporation of  $[\text{Fe}]^{\text{MIM}}$  into apo-hydrogenase from *CrHydA1* and *CpI*. Experiments were completed analogous to those described in Section 7.2.3 with potentials between  $-500$  mV and  $0$  mV applied (Figure 7.6). Conversion of the newly formed holo-enzyme into the catalytically inactive  $\text{H}_{\text{ox}}^{\text{inact}}$  state at high potentials (Section 1.8), especially significant in the case of *CrHydA1*, dictated that these experiments could not be performed above  $0$  mV.



**Figure 7.6:** A summary of the results obtained from chronoamperometry experiments investigating the effect of potential on the rate of  $[\text{Fe}]^{\text{MIM}}$  incorporation into apo-hydrogenase of (A) *CrHydA1* and (B) *CpI*. Diamonds reflect the rates obtained by fitting the raw data to a single exponential as shown in Figure 7.4B. Conditions: pH 6.1,  $\omega = 1000$  rpm, 100%  $\text{H}_2$ . In each experiment  $0.16$  mL of  $3.1 \mu\text{M}$   $[\text{Fe}]^{\text{MIM}}$  was injected into the cell resulting in a final concentration of  $0.2296 \mu\text{M}$  of  $[\text{Fe}]^{\text{MIM}}$ . Film loss occurs rapidly on films of *CrHydA1* and in order to minimism this the experiments summarised in panel A were carried out at  $15^\circ\text{C}$ . Film loss occurs more slowly on films of *CpI* and the experiments in panel B were conducted at  $20^\circ\text{C}$  accordingly.

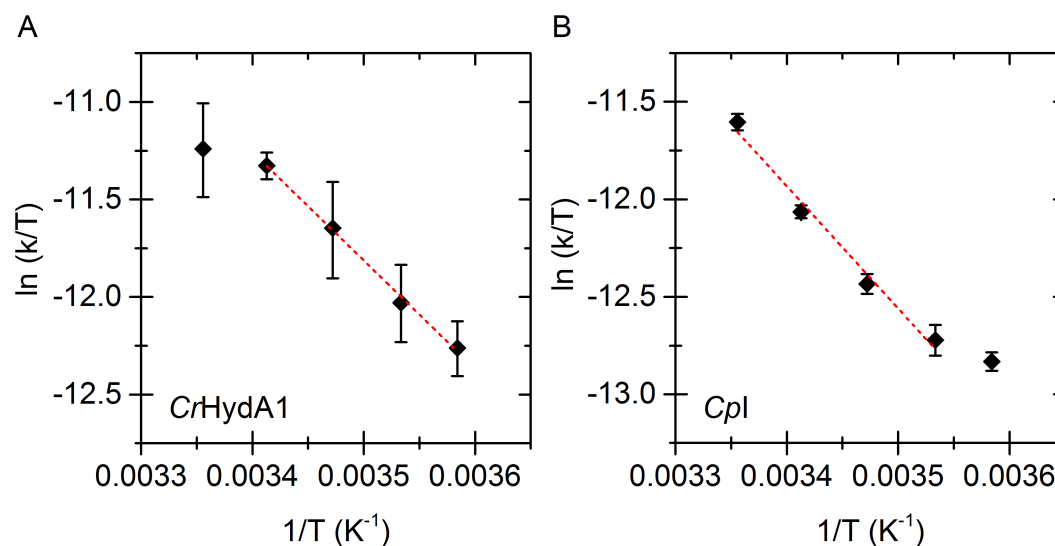
Experiments on *CrHydA1* (Figure 7.6A) show little potential dependence, although the large amount of scatter in the data means that it is difficult to draw a firm conclusion. The larger amount of scatter observed in the data for *CrHydA1* com-

pared to that for *CpI* can be attributed to the higher rate of film loss which occurs for films of *CrHydA1* adsorbed onto a PGE electrode. Experiments on *CpI* (Figure 7.6B) show *ca.* 50% decrease in rate as the potential is increased from  $-500$  mV to  $0$  mV.

### 7.2.3.3 The Activation Energy Requirement for $[\text{Fe}]^{\text{MIM}}$ Incorporation into Apo-Hydrogenase

The effect of temperature on the rate of  $[\text{Fe}]^{\text{MIM}}$  incorporation into apo-hydrogenase from *CrHydA1* and *CpI* can be determined by carrying out experiments analogous to those described above. By finding the rate of incorporation, which corresponds to the rate of increasing current, at a number of different temperatures an Eyring analysis (see Appendix B) can be completed (Figure 7.7). From the gradients of the Eyring plots the activation energy required for the incorporation of  $[\text{Fe}]^{\text{MIM}}$  into apo-hydrogenase adsorbed onto an electrode can be determined. Accounting for the Nernstian dependence of  $E_{2\text{H}^+/\text{H}_2}$  with temperature, all experiments were carried out at  $150$  mV above  $E_{2\text{H}^+/\text{H}_2}$  ( $E_{2\text{H}^+/\text{H}_2} = -348$  mV at pH 6.1,  $15^\circ\text{C}$ , under 100%  $\text{H}_2$ ). This potential provides sufficient driving force to maintain considerable  $\text{H}_2$  oxidation activity whilst being below the potential at which oxidative inactivation to form the  $\text{H}_{\text{ox}}^{\text{inact}}$  state occurs.

In both Figures 7.7A and 7.7B, the Eyring plots do not show a linear dependence across the entire temperature range studied. However, the data obtained for *CrHydA1* does show a strong linear dependence between  $6^\circ\text{C}$  ( $1/T (\text{K}^{-1}) = 0.00358$ ) and  $20^\circ\text{C}$  ( $1/T (\text{K}^{-1}) = 0.00341$ ) and the gradient found from a line of best fit to these points gives a value of  $\Delta H^\ddagger = 46.2 \text{ kJ mol}^{-1}$  ( $r^2 = 0.99$ ). The larger error in the data obtained for *CrHydA1* (as seen by the considerably larger error bars in Figure 7.7A compared to Figure 7.7B) can be attributed to the higher and more variable rate of film loss which occurs between experiments for this enzyme. The rate of film loss increases with increasing temperature and this may account for the decrease in gradient observed above  $20^\circ\text{C}$ .



**Figure 7.7:** Eyring plots obtained from chronoamperometry experiments investigating the effect of temperature on the rate of  $[\text{Fe}]^{\text{MIM}}$  incorporation into the apo-protein of (A) *CrHydA1* and (B) *Cpl*. Diamonds reflect the mean and error bars reflect the standard deviation of either three or four repeats. A line of best fit, indicated by a red dashed line, has been applied to the linear portion of each dataset (see text) giving a gradient which equates to a value of  $\Delta H^\ddagger = 46.2 \text{ kJ mol}^{-1}$  and  $\Delta H^\ddagger = 52.1 \text{ kJ mol}^{-1}$  for *CrHydA1* and *Cpl* respectively. Conditions: pH 6.1,  $\omega = 1000 \text{ rpm}$ , 100%  $\text{H}_2$ . In each experiment 0.16 mL of  $3.1 \mu\text{M}$   $[\text{Fe}]^{\text{MIM}}$  was injected into the cell resulting in a final concentration of  $0.2296 \mu\text{M}$  of  $[\text{Fe}]^{\text{MIM}}$ . All experiments were carried out at 150 mV above  $E_{2\text{H}^+/\text{H}_2}$  accounting for the Nernstian dependence of  $E_{2\text{H}^+/\text{H}_2}$  with temperature.



The data obtained for *CpI* deviates from linearity most pronouncedly at lower temperatures. The gradient found from a linear fit to the data obtained between 10 °C ( $1/T$  ( $\text{K}^{-1}$ ) = 0.00353) and 25 °C ( $1/T$  ( $\text{K}^{-1}$ ) = 0.00336) gives  $\Delta H^\ddagger = 52.1 \text{ kJ mol}^{-1}$  ( $r^2 = 0.97$ ).

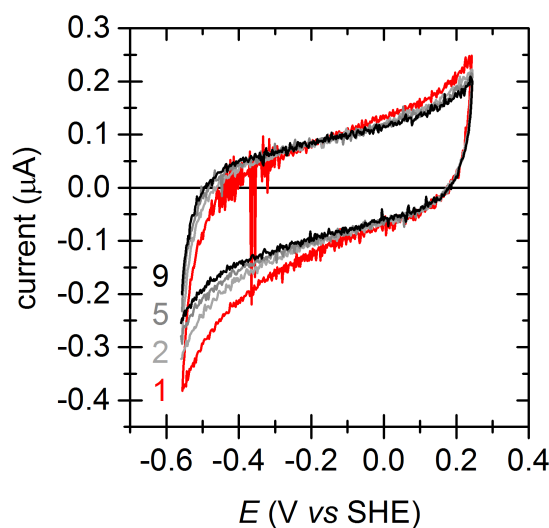
The intercept obtained from an Eyring plot can be used to determine  $\Delta S^\ddagger$ . The linear fits shown in Figure 7.7 give  $\Delta S^\ddagger = -134 \text{ J K}^{-1} \text{ mol}^{-1}$  and  $\Delta S^\ddagger = -120 \text{ J K}^{-1} \text{ mol}^{-1}$  for *CrHydA1* and *CpI* respectively.

### 7.2.4 Searching for Non-Turnover Signals

Infrared studies have shown that other synthetic [2Fe]-subcluster analogues, similar to the one shown in Figure 7.1A but with carbon or oxygen containing dithiolato ligands of the type  $-\text{SCH}_2\text{XCH}_2\text{S}-$  where  $\text{X} = \text{CH}_2$  ('pdt-[Fe]<sup>MIM</sup>') or O ('odt-[Fe]<sup>MIM</sup>') instead of the azadithiolate bridge of [Fe]<sup>MIM</sup>, could also be incorporated into apo-hydrogenase.<sup>45</sup> The resulting spectra show 'H-cluster like' FTIR signatures, with the pdt-[Fe]<sup>MIM</sup> incorporated complex showing bands with strong similarities to those observed from native holo-enzyme in the  $\text{H}_{\text{ox}}$  state. However, when either pdt-[Fe]<sup>MIM</sup> and odt-[Fe]<sup>MIM</sup> were used the resultant protein showed no catalytic activity as determined by a solution assay.<sup>45</sup>

In the absence of substrate turnover, the oxidation and reduction of the [FeS]-clusters that comprise the electron relay chain within an enzyme can result in the observation of non-turnover signals when viewed by cyclic voltammetry (Section 2.1.3.5). These signals can provide information on the redox activity of these clusters. In the case of hydrogenases, this information can shed light upon the reasons behind the different behaviour characteristics seen from one enzyme to another.<sup>4,128</sup> The ability to perform  $\text{H}_2$  production under minimal driving force using the protons found in aqueous buffer as the substrate mean that these signals are notoriously difficult to obtain for hydrogenases. The ability to form a structurally complete enzyme, but one which is unable to undergo catalysis, may aid experiments designed to resolve these signals.

*CrHydA1* contains no auxiliary [FeS]-clusters (Section 1.5.3) apart from the [4Fe4S]-cluster which, together with the [2Fe]-subcluster, forms the catalytically active H-cluster. This [4Fe4S]-cluster forms the entry point at which electrons, either from the [FeS]-cluster electron relay or, in the case of *CrHydA1*, from outside the protein, enter the bimetallic active site. In order to monitor the redox properties of the crucial [4Fe4S]-cluster, a film of apo-hydrogenase protein from *CrHydA1* was applied to a PGE electrode. A cyclic voltammetry experiment, similar to that described in Section 7.2.3, was then carried out. In this case,  $[\text{Fe}]^{\text{MIM}}$  was substituted with odt- $[\text{Fe}]^{\text{MIM}}$  and a 0.16 mL aliquot of odt- $[\text{Fe}]^{\text{MIM}}$  was introduced into the cell during a chronoamperometry step at  $-198$  mV (not shown). This resulted in a final concentration of  $0.2296 \mu\text{M}$  of odt- $[\text{Fe}]^{\text{MIM}}$ . The redox activity of the protein film was then monitored by a change in current over subsequent cyclic voltammetry scans.



**Figure 7.8:** The current observed during a cyclic voltammetry experiment before (red line) and after (grey lines) an injection of a 0.16 mL aliquot of odt- $[\text{Fe}]^{\text{MIM}}$  into the cell solution resulting in a final concentration of  $0.2296 \mu\text{M}$  of odt- $[\text{Fe}]^{\text{MIM}}$ . Scan numbers are indicated by colour alongside each plot. Conditions: pH 6.1,  $15^\circ\text{C}$ ,  $\omega = 1000$  rpm,  $\nu = 30 \text{ mV s}^{-1}$ , 100%  $\text{H}_2$ .

Consistent with the results of photospectroscopic assays previously published which concluded that the incorporation of odt- $[\text{Fe}]^{\text{MIM}}$  into apo-hydrogenase resulted in protein unable to evolve  $\text{H}_2$ ,<sup>45</sup> the introduction of odt- $[\text{Fe}]^{\text{MIM}}$  into the cell solution did not result in catalytic activity being observed. No other redox activity was

observed, notably, non-turnover signals cannot be identified.

Subsequent scans show a decrease in the magnitude of the non-Faradaic current observed. This may be indicative of film loss (Section 2.3.1.5), the desorption of the protein film from the electrode over time leading to a decrease in the surface area of the electrode. In an attempt to minimise the rate of film loss, and so maintain a high concentration of protein molecules on the electrode surface, the experiment was repeated at 5 °C (experiment not shown). Non-turnover signals were still not observed.

Although monomeric *CrHydA1* is significantly smaller than all other hydrogenases studied in this thesis, it is still relatively large compared to a molecular catalyst. This limits the number of catalytic sites that can be present on an electrode surface and the low concentration of sites may be accountable for the inability to resolve non-turnover signals.

## 7.3 Discussion

Protein film electrochemistry enables the spontaneous incorporation of  $[\text{Fe}]^{\text{MIM}}$  into apo-hydrogenase from both *CrHydA1* and *CpI* to be monitored in ‘real time’, the observed increase in catalytic current corresponding directly to the increasing ratio of holo-enzyme to apo-enzyme present on the electrode surface.

The cyclic voltammetry studies described herein have shown that incorporation of  $[\text{Fe}]^{\text{MIM}}$  into apo-hydrogenase from both *CrHydA1* and *CpI* results in a waveshape that is indistinguishable from native holo-enzyme (Figure 7.2). This is consistent with FTIR and EPR work previously published which finds that the reconstituted H-cluster is virtually identical to the native enzyme spectroscopically,<sup>45,46</sup> as well as in terms of activity.<sup>46</sup>

Solution electrochemistry of  $[\text{Fe}]^{\text{MIM}}$  (Figure 7.3) found that this model complex was redox active over the potential region  $-370$  mV to  $+90$  mV, with one reversible redox peak visible that was centred around  $-88.5$  mV. Previous work has suggested that, like other di-iron  $[\text{FeFe}]$ -hydrogenase  $[2\text{Fe}]$ -cluster mimics,<sup>195</sup>  $[\text{Fe}]^{\text{MIM}}$  consists

of Fe ions in a Fe(I)Fe(I) redox state.<sup>45</sup> The peak seen in the solution electrochemistry may reflect the reversible oxidation of one of the Fe ions from Fe(I) to Fe(II). [Fe]<sup>MIM</sup> by itself did not show any signs of catalytic hydrogenase activity within this potential range, consistent with previous work,<sup>46,196</sup> therefore the increase in current observed in experiments involving apo-hydrogenase is solely due to the activation of the apo-enzyme by [Fe]<sup>MIM</sup>.

Chronoamperometry allowed for a quantitative analysis of the spontaneous incorporation of [Fe]<sup>MIM</sup> into apo-hydrogenase to be undertaken. The increase in current seen upon the introduction of [Fe]<sup>MIM</sup> into the electrochemical cell showed a single exponential dependence, up until the point where the rate of film loss out competed the rate of formation of catalytically active holo-enzyme (Figure 7.4B). This single exponential represents the rate determining step, however, it is not until the very last step of the activation process is complete, resulting in catalytically active enzyme, that a catalytic current is observed *via* PFE (*i.e.* there may be some delay between the completion of the rate determining step and the onset of catalysis).

Figure 7.5 shows how the concentration of [Fe]<sup>MIM</sup> in the cell solution affects the rate of incorporation. The observed rate continues to increase with [Fe]<sup>MIM</sup> concentration even as it exceeds 10 times that of the apo-protein (corresponding to a [Fe]<sup>MIM</sup> concentration of 0.2296  $\mu$ M), although the increase in rate seen with increasing [Fe]<sup>MIM</sup> concentration does start to become less great at concentrations above this. This is consistent with the uptake of [Fe]<sup>MIM</sup> into the apo-protein being a rate determining step for *CpI*.

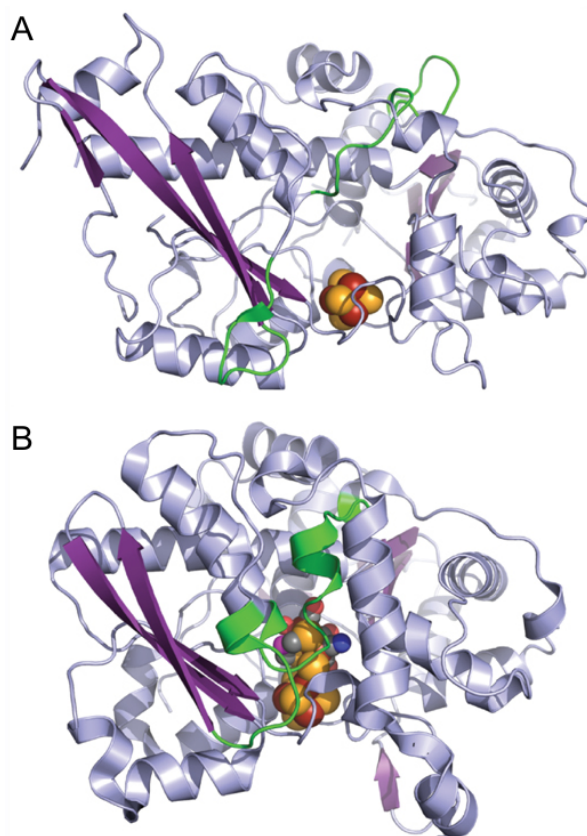
Studies investigating the effect of potential upon the rate of the spontaneous incorporation of [Fe]<sup>MIM</sup> into the apo-protein from *CrHydA1* and *CpI* showed little dependence over the 500 mV range studied in each case (Figure 7.6), although the large amount of scatter in the data recorded for *CrHydA1* means that a small potential dependence may not have been resolved for this protein. Data from *CpI* show a small increase in the rate of incorporation with decreasing potential below *ca.* -200 mV (Figure 7.6B). This corresponds with the onset of redox activity of [Fe]<sup>MIM</sup> as

observed in Figure 7.3. Previous work has suggested that when CO attacks [FeFe]-hydrogenases, it is the  $H_{\text{red}}$  species, with the [2Fe]-subcluster in a Fe(I)Fe(I) state, that reacts irreversibly with CO<sup>197</sup> (see Section 1.8 for details on the redox states of the H-cluster involved in catalysis). Theoretical calculations involving the reaction between CO and a model of the H-cluster in the  $H_{\text{red}}$  state have indicated that this reaction is accompanied by an elongation of the bond between the  $Fe_p$  atom and the sulfur of the cysteine ligand ( $S_{\text{cys}}$ ) of more than 0.5 Å, indicating a cleavage of the Fe- $S_{\text{cys}}$  bond.<sup>197</sup> This cleavage, which would form apo-hydrogenase and  $[Fe]^{\text{MIM}}$ , is the reverse of the incorporation reaction studied in this chapter. The principle of microscopic reversibility would then suggest that  $[Fe]^{\text{MIM}}$  would bind most efficiently to the apo-protein when in a Fe(I)Fe(I) state, which would be followed by the loss of CO. This may explain the small increase in the rate of incorporation observed in *CpI* at potentials below -200 mV. According to Figure 7.3B, at potentials above 0 mV the majority of  $[Fe]^{\text{MIM}}$  is oxidised suggesting that the rate of formation of catalytically active holo-enzyme may significantly decrease at potentials above this. However, this potential corresponds to the onset of the formation of the  $H_{\text{ox}}^{\text{inact}}$  state of the enzyme, a catalytically inactive state and so unsuitable for study by PFE. More suitable techniques for the study of the possible formation of holo-enzyme in this potential region may be spectroelectrochemical FTIR and potentiometric EPR spectroscopy, techniques which are able to provide details regarding the protein structure.

Temperature dependence studies found that  $\Delta H^\ddagger$  for  $[Fe]^{\text{MIM}}$  incorporation into the apo-protein is approximately 6 kJ mol<sup>-1</sup> higher for *CpI* than for *CrHydA1*. The Eyring plots (Figure 7.7) obtained for the incorporation of  $[Fe]^{\text{MIM}}$  into *CrHydA1* and *CpI* show a non-linear dependence that can be indicative of a change in the rate limiting step or a change in the conformation of the apo-protein across the temperature region studied.<sup>198</sup> The difference in  $\Delta H^\ddagger$  found for the incorporation of  $[Fe]^{\text{MIM}}$  into *CrHydA1* and *CpI* is consistent with the variations in the crystal structures obtained for apo-hydrogenase and holo-hydrogenase from *CrHydA1* and *CpI*: Whereas the

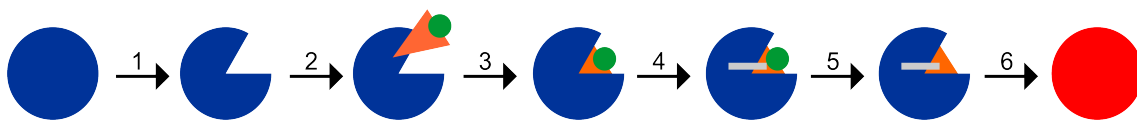
crystal structures obtained from apo-hydrogenase and holo-hydrogenase for *CpI* are very similar,<sup>199</sup> the crystal structure of apo-hydrogenase from *CrHydA1* shows an open structure around the site at which the [2Fe]-subcluster would bind,<sup>200</sup> as shown in Figure 7.9. Although the geometries found may only reflect the conformation of the protein which is allowed in the crystal lattice, it does suggest that differences may also be found in solution between the structure of apo-hydrogenase from *CrHydA1* and *CpI*. The larger value of  $\Delta H^\ddagger$  found for *CpI* may reflect the need of this apo-hydrogenase to first ‘open’ to allow incorporation of  $[\text{Fe}]^{\text{MIM}}$  into the protein, whereas the protein structure is already open ready for  $[\text{Fe}]^{\text{MIM}}$  incorporation in the case of *CrHydA1*. The plots shown in Figure 7.7 give  $\Delta S^\ddagger = -134 \text{ J K}^{-1} \text{ mol}^{-1}$  and  $\Delta S^\ddagger = -120 \text{ J K}^{-1} \text{ mol}^{-1}$  for the incorporation of  $[\text{Fe}]^{\text{MIM}}$  into *CrHydA1* and *CpI* respectively. The negative values of  $\Delta S^\ddagger$  reflect the intermolecular reaction which forms one molecule from two.<sup>201</sup> Protein folding into a more tightly bound ‘closed’ structure usually leads to an increase in entropy, as hydrophobic effects are minimised as non-polar residues are secluded from the surrounding water molecules diminishing any ordered arrangement of water molecules on the protein surface.<sup>201,202</sup> This effect will, to some extent, offset the negative values of  $\Delta S^\ddagger$  found.<sup>202</sup> The less negative value of  $\Delta S^\ddagger$  obtained for *CpI* is consistent with a greater increase in entropy being associated with protein closing for this enzyme, which may be as a result of the larger size of this protein.

There are many plausible mechanisms which may describe the spontaneous incorporation of  $[\text{Fe}]^{\text{MIM}}$  into apo-hydrogenase resulting in catalytically active holo-enzyme. As outlined in Figure 7.10, the mechanism must include apo-protein opening and closing, association of  $[\text{Fe}]^{\text{MIM}}$  with the protein, migration of  $[\text{Fe}]^{\text{MIM}}$  to the binding site, bond forming as  $[\text{Fe}]^{\text{MIM}}$  forms a bond to the sulfur of the cysteine ligand which connects the di-iron subcluster to the [4Fe4S]-cluster, and bond breaking as the CO ligand is lost from  $[\text{Fe}]^{\text{MIM}}$ . The results outlined in this chapter go some way to shedding more light on which of these steps may limit the rate of incorporation of  $[\text{Fe}]^{\text{MIM}}$  into apo-hydrogenase and the differences between the pathways



**Figure 7.9:** X-ray crystal structure of apo-hydrogenase from *CrHydA1* determined to a resolution of 1.97 Å (PDB code: 3LX4), compared to holo-hydrogenase from *CpI* (PDB code: 3C8Y). (A) Ribbon representation of the overall apo-hydrogenase structure from *CrHydA1*. The two loop regions thought to undergo major conformational rearrangement, ‘closing’ upon incorporation of  $[\text{Fe}]^{\text{MIM}}$ , are coloured green. The  $[\text{4Fe4S}]$ -subcluster is shown in space filling representation. (B) Ribbon representation of the holo-hydrogenase *CpI* structure in the same orientation as the protein in panel A. The regions of *CpI* corresponding to the loop regions highlighted in panel A are shown in green. In both panels the colouring scheme is as follows: Fe dark red, S orange, C grey, N blue, O light red,  $\alpha$ -helices and loops shown in light blue and  $\beta$ -sheets in violet. Figure taken from Reference 200.

established in *CrHydA1* and *CpI*.



**Figure 7.10:** The steps required for  $[\text{Fe}]^{\text{MIM}}$  incorporation into apo-hydrogenase to form holo-hydrogenase. The steps are as follows: (1) opening of the apo-hydrogenase protein (blue); (2)  $[\text{Fe}]^{\text{MIM}}$  (orange and green) association with the protein; (3) migration of  $[\text{Fe}]^{\text{MIM}}$  to the binding site; (4) bond formation (grey); (5) dissociation of a CO ligand (green) and; (6) protein closing to form holo-hydrogenase (red). Steps *in vivo* may not necessarily occur in the order outlined in the figure, for example, protein closing may occur before the dissociation of CO.

The small dependence of formation of active holo-enzyme upon electrode potential suggests that the formation of a bond between the apo-hydrogenase structure and  $[\text{Fe}]^{\text{MIM}}$  is not the rate determining step. The higher rate generally observed for *CrHydA1* (see Figure 7.6 noting that experiments were carried out at 15 °C and 20 °C for *CrHydA1* and *CpI* respectively) is consistent with the rate determining step being protein dependent, indicating that opening, closing,  $[\text{Fe}]^{\text{MIM}}$  migration or CO dissociation may be the rate determining step. The 6 kJ mol<sup>-1</sup> higher activation energy required for the incorporation of  $[\text{Fe}]^{\text{MIM}}$  into *CpI* compared to *CrHydA1* is consistent with the need for *CpI* to unfold and then refold to incorporate  $[\text{Fe}]^{\text{MIM}}$  at the correct position within the protein. Protein conformational changes are often considered to be relatively slow, occurring within a milli- to microsecond timescale.<sup>203</sup> They are also temperature dependent, consistent with the non-linear temperature dependence observed in the Eyring plots obtained from the temperature studies of *CrHydA1* and *CpI* (Figure 7.7).

The possibility to minimise the influence of mass transport and diffusion, and the ability to work in the absence of any redox-mediators, means that PFE is an invaluable tool for studying the rates of the reactions detailed in this chapter. However, it is important to note that the rates obtained for  $[\text{Fe}]^{\text{MIM}}$  incorporation may be different when measured by PFE, where apo-hydrogenase is adsorbed onto the surface of an electrode, to those measured when apo-hydrogenase is free in solution. Nevertheless, the site at which  $[\text{Fe}]^{\text{MIM}}$  binds within the protein is protected from the



influence of the electrode surface by the surrounding protein shell and the influences of external conditions, such as potential and temperature, will most likely have the same relative effect when monitored either way.

## 7.4 Conclusions and Perspectives

The experiments presented in this chapter have investigated the spontaneous incorporation of  $[\text{Fe}]^{\text{MIM}}$  into the apo-protein from *CrHydA1* and *CpI*, forming fully functional holo-enzymes, indistinguishable from the native holo-enzyme itself. Although a detailed mechanism cannot yet be proposed, a number of dependences have been identified allowing conclusions to be drawn and key areas for future work to be outlined.

Significant differences observed between *CrHydA1* and *CpI* suggests that the thermodynamics of  $[\text{Fe}]^{\text{MIM}}$  incorporation into apo-hydrogenase is determined largely by the protein structure itself, suggesting that protein opening and/or closing, migration of  $[\text{Fe}]^{\text{MIM}}$  into place within the protein or CO dissociation out of the protein may be the rate determining step.

Experiments investigating the effects of salt concentration or pH, which would affect protein-protein interactions, may shed light on whether protein conformational changes are the key barrier to model-complex incorporation. Investigating the effects that very low levels of CO have in solution upon the rate of  $[\text{Fe}]^{\text{MIM}}$  incorporation may help clarify whether CO migration out of the protein is a rate determining step. The effect of light, which has previously been shown to increase the rate of CO dissociation from the H-cluster, reactivating the enzyme after CO inhibition of the native holo-enzyme,<sup>50,190</sup> may also have a role to play here.

Finally, work in this chapter has highlighted the possibilities that PFE provides for investigating the spontaneous incorporation of other  $[\text{2Fe}]$ -subcluster mimics into apo-hydrogenase. Advantages to using PFE over conventional activity assays include: the near instantaneous nature of the result, the very small amount of apo-protein and synthetic complex required, the ability to investigate the affect of applied

potential upon the complex formed without the presence of redox mediators, along with the effects of external conditions such as temperature and pH.

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# Appendices

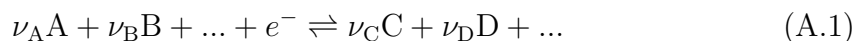




# Appendix A

## The Nernst Equation

Consider the reaction:<sup>204</sup>



where  $\nu_J$  is the stoichiometric coefficient of J. At equilibrium, the total electrochemical potential of the products will equal that of the reactants, so;

$$\nu_A \bar{\mu}_A + \nu_B \bar{\mu}_B + \dots + \bar{\mu}_{e^-} = \nu_C \bar{\mu}_C + \nu_D \bar{\mu}_D + \dots \quad (\text{A.2})$$

where  $\bar{\mu}_J$  is the electrochemical potential of species J. The electrochemical potential of a species is related to the chemical potential of that species by;

$$\bar{\mu}_J = \mu_J + z_J F \Phi \quad (\text{A.3})$$

where  $\mu_J$  is the chemical potential of species J,  $z_J$  is the charge on species J,  $F$  is Faraday's constant and  $\Phi$  corresponds to the potential of the electrode or solution in which species J resides. Together,  $z_J F \Phi$  describes the electrical energy of J.

Substituting this relationship into Equation A.2 gives:

$$\begin{aligned} \nu_A(\mu_A + z_A F \Phi_S) + \nu_B(\mu_B + z_B F \Phi_S) + \dots + (\mu_{e^-} - F \Phi_M) = \\ \nu_C(\mu_C + z_C F \Phi_S) + \nu_D(\mu_D + z_D F \Phi_S) + \dots \end{aligned} \quad (\text{A.4})$$

Conservation of electrical charge requires that:

$$\nu_A z_A + \nu_B z_B + \dots - 1 = \nu_C z_C + \nu_D z_D + \dots \quad (\text{A.5})$$

Hence, by rearranging Equation A.4 and then substituting in Equation A.5 we get:

$$F(\Phi_M - \Phi_S) = \nu_A \mu_A + \nu_B \mu_B + \dots - \nu_C \mu_C - \nu_D \mu_D + \mu_{e^-} \quad (\text{A.6})$$

If,  $\mu_J = \mu_J^\theta + RT \ln a_J$ , then:

$$\begin{aligned} F(\Phi_M - \Phi_S) = \nu_A(\mu_A^\theta + RT \ln a_A) + \nu_B(\mu_B^\theta + RT \ln a_B) + \dots \\ - \nu_C(\mu_C^\theta + RT \ln a_C) - \nu_D(\mu_D^\theta + RT \ln a_D) + \mu_{e^-} \end{aligned} \quad (\text{A.7})$$

By substituting in the term;

$$F \Delta \Phi^\theta = (\nu_A \mu_A^\theta + \nu_B \mu_B^\theta + \dots) - (\nu_C \mu_C^\theta + \nu_D \mu_D^\theta + \dots) + \mu_{e^-} \quad (\text{A.8})$$

where  $F \Delta \Phi^\theta$  is constant at a fixed temperature and pressure, then Equation A.7 becomes:

$$F(\Phi_M - \Phi_S) = F \Delta \Phi^\theta + RT(\nu_A \ln a_A + \nu_B \ln a_B + \dots - \nu_C \ln a_C - \nu_D \ln a_D - \dots) \quad (\text{A.9})$$

This expression can be rearranged to give the Nernst equation:

$$\Phi_M - \Phi_S = \Delta \Phi^\theta + \frac{RT}{F} \ln \left\{ \frac{a_A^{\nu_A} a_B^{\nu_B} \dots}{a_C^{\nu_C} a_D^{\nu_D} \dots} \right\} \quad (\text{A.10})$$

In general use, the activities of chemical species can be replaced by their concentrations. If  $E = \Phi_{\text{M}} - \Phi_{\text{S}}$  and  $E^{\ominus} = \Delta\Phi^{\theta}$ , Equation A.10 can be written as;

$$E = E^{\ominus} + \frac{RT}{nF} \ln \frac{[\text{Ox}]}{[\text{Red}]} \quad (\text{A.11})$$

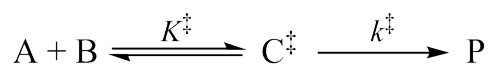
which, when at equilibrium potential ( $E = E_{\text{eqm}}$ ) corresponds to Equation 2.5 in Section 2.1.3.2.



# Appendix B

## The Eyring Equation

The Eyring equation is an expression for the rate constant of a reaction which allows the values for the standard entropy of activation ( $\Delta S^\ddagger$ ) and enthalpy of activation ( $\Delta H^\ddagger$ ) for the transition state to be determined. It is based on Transition State Theory, also known as Activated Complex Theory, in which the assumption is made that a reaction between A and B proceeds *via* the formation of an activated complex,  $C^\ddagger$  which then subsequently falls apart by unimolecular decay into products such that,<sup>205</sup>



where the equilibrium constant  $K^\ddagger$  can be expressed by;

$$K^\ddagger = \frac{[C^\ddagger]}{[A][B]} \quad (\text{B.1})$$

and the rate at which the activated complex decays into products,  $v$ , can be described by the rate constant  $k^\ddagger$ :

$$v = k^\ddagger [C^\ddagger] \quad (\text{B.2})$$

Through rearrangement of Equation B.1, Equation B.2 can be written as;

$$v = k^\ddagger K^\ddagger [A][B] \quad (\text{B.3})$$

and if we let  $k_r = k^\ddagger K^\ddagger$ ;

$$v = k_r[A][B] \quad (\text{B.4})$$

The activated complex,  $C^\ddagger$ , can form products,  $P$ , if it passes through a transition state. The rate at which a complex does this,  $k^\ddagger$ , is proportional to the vibrational frequency along the reaction coordinate;

$$k^\ddagger = \kappa v \quad (\text{B.5})$$

where  $\kappa$  is the transmission coefficient and can usually be assumed to be *ca.* 1 so that  $k^\ddagger = v$ . The frequency of the vibration, and in turn  $k^\ddagger$  can be given by;

$$v = \frac{k_B T}{h} \quad (\text{B.6})$$

where  $k_B$  is the Boltzmann's constant,  $T$  is the temperature in Kelvin and  $h$  is Planck's constant. Substituting this expression into the expression for  $k_r$  we get:

$$k_r = \frac{k_B T}{h} K^\ddagger \quad (\text{B.7})$$

Now considering the thermodynamic relationships;<sup>198</sup>

$$\Delta G^\ddagger = -RT \ln K^\ddagger \quad (\text{B.8})$$

$$\Delta G^\ddagger = \Delta H^\ddagger - T \Delta S^\ddagger \quad (\text{B.9})$$

combining Equations B.8 and B.9 to solve for  $\ln K^\ddagger$  we get:

$$\ln K^\ddagger = -\frac{\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R} \quad (\text{B.10})$$

Finally, substituting Equation B.10 into Equation B.7 gives:

$$k_r = \frac{k_B T}{h} \exp \left( \frac{-\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R} \right) \quad (\text{B.11})$$

This is the Eyring equation, in which  $k_r$  is the rate constant for the reaction,  $k_B$  is the Boltzmann constant,  $T$  is the temperature in Kelvin,  $h$  is the Planck constant and  $R$  is the gas constant and  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$  are the standard enthalpy and entropy of activation, respectively.

Applying a logarithmic transform gives;

$$\ln \left( \frac{k_r}{T} \right) = \frac{-\Delta H^\ddagger}{RT} + \ln \left( \frac{k_B}{h} \right) + \frac{\Delta S^\ddagger}{R} \quad (\text{B.12})$$

Therefore a plot of  $\ln(k_r/T)$  *vs.*  $1/T$  will give a straight line with a gradient of  $-\Delta H^\ddagger/R$  and a  $y$ -intercept of  $[\ln(k_B/h) + (\Delta S^\ddagger/R)]$ .





# Appendix C

## Derivation of Equation 3.12

In Chapter 3, the net current,  $j$ , is given by Equation 3.6, shown below:

$$j = nF\Gamma_m[k_{1a}[\text{Red}] - k_{1c}[\text{Ox}]] \quad (\text{C.1})$$

where  $n$  is the number of electrons required for the stoichiometric catalytic reaction,  $F$  is the Faraday constant,  $\Gamma_m$  is the total electroactive coverage of the enzyme on the electrode surface,  $[\text{Ox}]$  and  $[\text{Red}]$  are the proportion of enzyme in which the electrochemical control centre is, respectively, oxidised and reduced, and  $k_{1a}$  and  $k_{1c}$  can be described by,

$$k_{1a} = k^0 \exp \left[ \alpha_a \frac{nF}{RT} (E - E_{\text{Ox/R}}^0) \right] = k^0 e_1^{1-\alpha_c} \quad (\text{C.2})$$

$$k_{1c} = k^0 \exp \left[ -\alpha_c \frac{nF}{RT} (E - E_{\text{Ox/R}}^0) \right] = k^0 e_1^{-\alpha_c} \quad (\text{C.3})$$

where  $k^0$  is the standard rate constant for the electron transfer process,  $\alpha_a$  and  $\alpha_c$  are the respective transfer coefficients (assumed to be equal to 0.5),  $E_{\text{Ox/R}}^0$  is the reduction potential of the electrochemical control centre,  $R$  is the ideal gas constant,  $T$  is the temperature,  $E$  is the potential of the electrode, and:

$$e_1 = \exp \left[ \frac{nF}{RT} (E - E_{\text{Ox/R}}^0) \right] = \frac{k_{1a}}{k_{1c}} \quad (\text{C.4})$$

If  $P$  represents the fraction of enzyme in the reduced state, and  $(1 - P)$  represents the fraction of enzyme in the oxidised state, Equation C.1 will become:

$$j = nF\Gamma_m[k_{1a}P - k_{1c}(1 - P)] \quad (\text{C.5})$$

The coverage of enzyme in the reduced ( $P$ ) state will change with time according to:

$$\frac{dP}{dt} = k_{1c}(1 - P) - k_{1a}P - k_{2c}P + k_{2a}(1 - P) \quad (\text{C.6})$$

Applying the steady-state approximation,  $P$  can be written as:

$$P = \frac{k_{1c} + k_{2a}}{k_{1a} + k_{1c} + k_{2a} + k_{2c}} \quad (\text{C.7})$$

By substituting this expression for  $P$  into Equation C.6 and again assuming a steady-state,  $(1 - P)$  becomes:

$$(1 - P) = \frac{k_{1a} + k_{2c}}{k_{1a} + k_{1c} + k_{2a} + k_{2c}} \quad (\text{C.8})$$

Substituting Equations C.7 and C.8 into Equation C.5 gives:

$$j = nF\Gamma_m \frac{k_{1a}k_{2a} - k_{1c}k_{2c}}{k_{1a} + k_{1c} + k_{2a} + k_{2c}} \quad (\text{C.9})$$

If we then divide the numerator and denominator by  $k_{1c}$ , then, remembering that  $e_1 = k_{1a}/k_{1c}$  (Equation C.4):

$$j = nF\Gamma_m \frac{k_{2a}e_1 - k_{2c}}{1 + e_1 + \frac{k_{2a} + k_{2c}}{k_{1c}}} \quad (\text{C.10})$$

If we let;

$$p = \frac{k_{2a} + k_{2c}}{k^0} = \frac{k_{2a}(1 + e_2)}{k^0} \quad (\text{C.11})$$

then substituting Equation C.3 into Equation C.10 gives:

$$j = nF\Gamma_m \frac{k_{2a}e_1 - k_{2c}}{1 + e_1 + pe_1^{\alpha_c}} \quad (\text{C.12})$$

Once again remembering that  $e_1 = k_{1a}/k_{1c}$  (Equation C.4) and that;

$$e_2 = \frac{k_{2c}}{k_{2a}} \quad (\text{C.13})$$

then by substituting in;

$$j_{\text{lim}} = nF\Gamma_m k_{2a} \quad (\text{C.14})$$

where  $j_{\text{lim}}$  is in this case defined as the limiting current for an oxidative process, then Equation C.12 becomes;

$$j = j_{\text{lim}} \frac{e_1 - e_2}{1 + e_1 + pe_1^{\alpha_c}} \quad (\text{C.15})$$

which is the same as Equation 3.7 in Chapter 3.

To account for the dispersion in interfacial electron-transfer rate constants caused by the various orientations that a molecule may adopt upon an electrode surface, an effective tunnelling factor  $\beta d_0$  is introduced, where  $\beta$  is a medium-dependent decay constant and  $d_0$  is the distance above closest approach of the donor and acceptor. This leads to a distribution in interfacial electron-rate constants between the limiting values,  $k_{\text{min}} = k^0 \exp(-\beta d_0)$  and  $k_{\text{max}} = k^0$ , where  $k^0$  is the standard rate constant for interfacial electron transfer.<sup>115,137</sup>

If we let;

$$a = e_1 - e_2 \quad (\text{C.16})$$

$$b = 1 + e_1 \quad (\text{C.17})$$

$$c = k_{2a}(1 + e_2)e_1^{\alpha_c} \quad (\text{C.18})$$

then:

$$j = j_{\text{lim}} \frac{a}{b + \frac{c}{k^0}} \quad (\text{C.19})$$

To obtain the overall current, we must integrate over all possible rate constants;

$$j = j_{\text{lim}} \int_{k_{\text{min}}}^{k_{\text{max}}} \frac{a}{b + \frac{c}{k}} p(k) dk \quad (\text{C.20})$$

where  $p(k)dk$  is the probability of a particular enzyme molecule having a rate constant between  $k$  and  $(k + dk)$ . For a uniform distribution of distances,  $p(k)dk = 1/\beta d_0 k$  if  $k_{\text{min}} < k < k_{\text{max}}$  otherwise  $p(k)dk = 0$ . Therefore:

$$j = j_{\text{lim}} \int_{k_{\text{min}}}^{k_{\text{max}}} \frac{a}{\beta d_0 b k + c \beta d_0} dk \quad (\text{C.21})$$

If we let;

$$u = \beta d_0 b k + c \beta d_0 \quad (\text{C.22})$$

then;

$$du = \beta d_0 b dk \quad (\text{C.23})$$

and:

$$dk = \frac{du}{\beta d_0 b} \quad (\text{C.24})$$

Substituting  $u$  (Equation C.22) and  $dk$  (Equation C.24) into Equation C.21 gives;

$$j = \frac{j_{\text{lim}}}{\beta d_0 b} \int_{k_{\text{min}}}^{k_{\text{max}}} \frac{a}{u} dk \quad (\text{C.25})$$

which once integrated gives:

$$j = \frac{j_{\text{lim}}}{\beta d_0 b} [a \ln u]_{k_{\text{min}}}^{k_{\text{max}}} \quad (\text{C.26})$$

If we then substitute  $u = \beta d_0 b k + c \beta d_0$  back into the Equation C.26 and rearrange

we get;

$$j = \frac{j_{\text{lim}}}{\beta d_0 b} a [\ln(\beta d_0 b k_{\text{max}} + c \beta d_0) - \ln(\beta d_0 b k_{\text{min}} + c \beta d_0)] \quad (\text{C.27})$$

which can be rearranged to:

$$j = \frac{j_{\text{lim}}}{\beta d_0 b} a \ln \left( \frac{b k_{\text{max}} + c}{b k_{\text{min}} + c} \right) \quad (\text{C.28})$$

Substituting in values for  $a$ ,  $b$ ,  $c$ ,  $k_{\text{max}}$  and  $k_{\text{min}}$  gives;

$$j = \frac{j_{\text{lim}}}{\beta d_0} \frac{e_1 - e_2}{1 + e_1} \ln \frac{k_{2a}(1 + e_2)e_1^{\alpha_c} + (1 + e_1)k^0}{k_{2a}(1 + e_2)e_1^{\alpha_c} + (1 + e_1)k^0 \exp(-\beta d_0)} \quad (\text{C.29})$$

which can finally be simplified to:

$$j = \frac{j_{\text{lim}}}{\beta d_0} \frac{e_1 - e_2}{1 + e_1} \ln \frac{p e_1^{\alpha_c} + (1 + e_1)}{p e_1^{\alpha_c} + (1 + e_1) \exp(-\beta d_0)} \quad (\text{C.30})$$

which is the same as Equation 3.12 as shown in Chapter 3. This equation can be used to describe a current *vs.* potential waveform for a system which reaches a limiting current in the oxidative direction.



# Appendix D

## The Calculation of $\Delta H_{\text{Ox}}^\ddagger$ and $\Delta H_{\text{ET}}^\ddagger$ , as shown in Figure 3.7

|                | T (K) | $j_{\text{lim}}$ | $k_{2a}$             | $E_{2\text{H}^+/\text{H}_2}$ | $E_{\text{Ox/R}}^0$ | $e_2$ | $p$   | $k_0$                |
|----------------|-------|------------------|----------------------|------------------------------|---------------------|-------|-------|----------------------|
| <i>CrHydA1</i> | 291.3 | 0.975            | 1.68x10 <sup>8</sup> | −0.346                       | −0.342              | 0.699 | 0.790 | 1.40x10 <sup>8</sup> |
| Repeat 1       | 288.7 | 0.898            | 1.55x10 <sup>8</sup> | −0.343                       | −0.339              | 0.703 | 2.039 | 1.30x10 <sup>8</sup> |
|                | 286.1 | 0.792            | 1.37x10 <sup>8</sup> | −0.340                       | −0.336              | 0.704 | 2.126 | 1.10x10 <sup>8</sup> |
|                | 283.4 | 0.636            | 1.10x10 <sup>8</sup> | −0.337                       | −0.333              | 0.701 | 2.264 | 8.25x10 <sup>7</sup> |
|                | 280.8 | 0.564            | 9.74x10 <sup>7</sup> | −0.334                       | −0.330              | 0.739 | 2.574 | 6.58x10 <sup>7</sup> |
| <i>CrHydA1</i> | 288.5 | 1.158            | 2.00x10 <sup>8</sup> | −0.343                       | −0.338              | 0.665 | 1.151 | 1.10x10 <sup>8</sup> |
| Repeat 2       | 291.2 | 1.215            | 2.10x10 <sup>8</sup> | −0.346                       | −0.341              | 0.658 | 3.636 | 9.57x10 <sup>7</sup> |
|                | 285.9 | 1.040            | 1.80x10 <sup>8</sup> | −0.340                       | −0.334              | 0.616 | 4.380 | 6.63x10 <sup>7</sup> |
|                | 283.2 | 0.953            | 1.65x10 <sup>8</sup> | −0.337                       | −0.331              | 0.623 | 4.614 | 5.79x10 <sup>7</sup> |
|                | 280.5 | 0.810            | 1.40x10 <sup>8</sup> | −0.334                       | −0.329              | 0.641 | 4.395 | 5.22x10 <sup>7</sup> |
| <i>CrHydA1</i> | 288.2 | 1.206            | 2.08x10 <sup>8</sup> | −0.343                       | −0.340              | 0.805 | 0.805 | 1.28x10 <sup>8</sup> |
| Repeat 3       | 290.9 | 1.279            | 2.21x10 <sup>8</sup> | −0.346                       | −0.344              | 0.848 | 3.419 | 1.19x10 <sup>8</sup> |
|                | 285.5 | 1.034            | 1.79x10 <sup>8</sup> | −0.339                       | −0.337              | 0.872 | 4.038 | 8.28x10 <sup>7</sup> |
|                | 282.8 | 0.905            | 1.56x10 <sup>8</sup> | −0.336                       | −0.335              | 0.903 | 3.872 | 7.68x10 <sup>7</sup> |
|                | 280.0 | 0.802            | 1.39x10 <sup>8</sup> | −0.333                       | −0.332              | 0.941 | 4.112 | 6.54x10 <sup>7</sup> |

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|          | T (K) | $j_{\text{lim}}$ | $k_{2a}$           | $E_{2\text{H}^+/\text{H}_2}$ | $E_{\text{OX/R}}^0$ | $e_2$ | $p$   | $k_0$              |
|----------|-------|------------------|--------------------|------------------------------|---------------------|-------|-------|--------------------|
| CaHydA   | 290.1 | 0.480            | $8.29 \times 10^7$ | -0.345                       | -0.362              | 3.822 | 0.790 | $5.06 \times 10^8$ |
| Repeat 1 | 285.4 | 0.375            | $6.48 \times 10^7$ | -0.339                       | -0.356              | 4.027 | 0.833 | $3.91 \times 10^8$ |
|          | 280.8 | 0.304            | $5.25 \times 10^7$ | -0.334                       | -0.352              | 4.297 | 0.864 | $3.22 \times 10^8$ |
|          | 299.6 | 0.726            | $1.25 \times 10^8$ | -0.356                       | -0.374              | 4.051 | 0.703 | $9.02 \times 10^8$ |
|          | 294.8 | 0.575            | $9.93 \times 10^7$ | -0.351                       | -0.369              | 4.042 | 0.753 | $6.65 \times 10^8$ |
|          |       |                  |                    |                              |                     |       |       |                    |
| CaHydA   | 290.1 | 0.577            | $9.97 \times 10^7$ | -0.345                       | -0.357              | 2.577 | 1.151 | $3.10 \times 10^8$ |
| Repeat 2 | 285.2 | 0.479            | $8.27 \times 10^7$ | -0.339                       | -0.350              | 2.490 | 1.318 | $2.19 \times 10^8$ |
|          | 294.8 | 0.695            | $1.20 \times 10^8$ | -0.351                       | -0.364              | 2.800 | 1.028 | $4.44 \times 10^8$ |
|          | 280.9 | 0.408            | $7.05 \times 10^7$ | -0.334                       | -0.345              | 2.525 | 1.518 | $1.64 \times 10^8$ |
|          | 299.6 | 0.799            | $1.38 \times 10^8$ | -0.356                       | -0.371              | 3.219 | 0.969 | $6.01 \times 10^8$ |
|          |       |                  |                    |                              |                     |       |       |                    |
| CaHydA   | 280.7 | 0.337            | $5.82 \times 10^7$ | -0.343                       | -0.349              | 3.333 | 0.844 | $2.99 \times 10^8$ |
| Repeat 3 | 299.5 | 0.743            | $1.28 \times 10^8$ | -0.356                       | -0.373              | 3.656 | 0.650 | $9.19 \times 10^8$ |
|          | 294.7 | 0.634            | $1.10 \times 10^8$ | -0.350                       | -0.366              | 3.446 | 0.740 | $6.58 \times 10^8$ |
|          | 290.0 | 0.538            | $9.29 \times 10^7$ | -0.345                       | -0.360              | 3.269 | 0.796 | $4.98 \times 10^8$ |
|          | 285.4 | 0.431            | $7.44 \times 10^7$ | -0.339                       | -0.354              | 3.278 | 0.854 | $3.73 \times 10^8$ |
|          |       |                  |                    |                              |                     |       |       |                    |
| EcHyd2   | 309.5 | 0.935            | $1.61 \times 10^8$ | -0.368                       | -0.348              | 0.219 | 0.556 | $3.54 \times 10^8$ |
| Repeat 1 | 314.4 | 1.127            | $1.95 \times 10^8$ | -0.374                       | -0.356              | 0.244 | 0.640 | $3.37 \times 10^8$ |
|          | 304.8 | 0.715            | $1.24 \times 10^8$ | -0.362                       | -0.339              | 0.171 | 0.459 | $3.15 \times 10^8$ |
|          | 300.0 | 0.553            | $9.56 \times 10^7$ | -0.357                       | -0.332              | 0.150 | 0.394 | $2.79 \times 10^8$ |
|          | 295.3 | 0.415            | $7.17 \times 10^7$ | -0.351                       | -0.326              | 0.140 | 0.315 | $2.59 \times 10^8$ |
|          | 290.5 | 0.296            | $5.12 \times 10^7$ | -0.345                       | -0.320              | 0.137 | 0.262 | $2.22 \times 10^8$ |
|          | 285.9 | 0.211            | $3.64 \times 10^7$ | -0.340                       | -0.316              | 0.148 | 0.246 | $1.70 \times 10^8$ |
|          |       |                  |                    |                              |                     |       |       |                    |
|          |       |                  |                    |                              |                     |       |       |                    |
| EcHyd2   | 309.5 | 0.900            | $1.56 \times 10^8$ | -0.368                       | -0.347              | 0.202 | 0.547 | $3.42 \times 10^8$ |
| Repeat 2 | 314.4 | 1.102            | $1.90 \times 10^8$ | -0.374                       | -0.355              | 0.242 | 0.651 | $3.63 \times 10^8$ |
|          | 304.8 | 0.698            | $1.20 \times 10^8$ | -0.362                       | -0.339              | 0.170 | 0.440 | $3.20 \times 10^8$ |
|          | 300.0 | 0.532            | $9.19 \times 10^7$ | -0.357                       | -0.333              | 0.156 | 0.374 | $2.84 \times 10^8$ |
|          | 295.3 | 0.398            | $6.87 \times 10^7$ | -0.351                       | -0.327              | 0.150 | 0.303 | $2.60 \times 10^8$ |
|          |       |                  |                    |                              |                     |       |       |                    |

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|               | T (K) | $j_{\text{lim}}$ | $k_{2a}$           | $E_{2\text{H}^+/\text{H}_2}$ | $E_{\text{OX}/\text{R}}^0$ | $e_2$              | $p$    | $k_0$              |
|---------------|-------|------------------|--------------------|------------------------------|----------------------------|--------------------|--------|--------------------|
| <i>EcHyd2</i> | 309.3 | 0.913            | $1.58 \times 10^8$ | -0.368                       | -0.349                     | 0.236              | 0.665  | $2.93 \times 10^8$ |
| Repeat 3      | 314.0 | 1.127            | $1.95 \times 10^8$ | -0.373                       | -0.355                     | 0.257              | 0.675  | $3.63 \times 10^8$ |
|               | 304.5 | 0.715            | $1.24 \times 10^8$ | -0.362                       | -0.339                     | 0.179              | 0.504  | $2.89 \times 10^8$ |
|               | 299.7 | 0.544            | $9.40 \times 10^7$ | -0.356                       | -0.333                     | 0.163              | 0.411  | $2.66 \times 10^8$ |
|               | 295.0 | 0.406            | $7.01 \times 10^7$ | -0.351                       | -0.327                     | 0.153              | 0.350  | $2.31 \times 10^8$ |
| <i>EcHyd1</i> | 309.1 | 1.059            | $1.83 \times 10^8$ | -0.368                       | -0.220                     | $1.45 \times 10^5$ | 0.053  | $3.46 \times 10^9$ |
| Repeat 1      | 313.8 | 1.470            | $2.54 \times 10^8$ | -0.373                       | -0.222                     | $1.42 \times 10^5$ | 0.065  | $3.90 \times 10^9$ |
|               | 304.3 | 0.762            | $1.32 \times 10^8$ | -0.362                       | -0.219                     | $1.82 \times 10^5$ | 0.051  | $2.58 \times 10^9$ |
|               | 299.6 | 0.534            | $9.24 \times 10^7$ | -0.356                       | -0.217                     | $2.11 \times 10^5$ | 0.047  | $1.95 \times 10^9$ |
|               | 294.9 | 0.374            | $6.46 \times 10^7$ | -0.351                       | -0.215                     | $2.22 \times 10^5$ | 0.0455 | $1.44 \times 10^9$ |
| <i>EcHyd1</i> | 309.0 | 1.057            | $1.83 \times 10^8$ | -0.367                       | -0.220                     | $1.66 \times 10^5$ | 0.051  | $3.60 \times 10^9$ |
| Repeat 2      | 313.8 | 1.487            | $2.57 \times 10^8$ | -0.373                       | -0.223                     | $1.49 \times 10^5$ | 0.061  | $4.24 \times 10^9$ |
|               | 304.2 | 0.773            | $1.33 \times 10^8$ | -0.362                       | -0.219                     | $1.83 \times 10^5$ | 0.046  | $2.93 \times 10^9$ |
|               | 299.4 | 0.538            | $9.30 \times 10^7$ | -0.356                       | -0.217                     | $2.08 \times 10^5$ | 0.041  | $2.24 \times 10^9$ |
|               | 294.7 | 0.375            | $6.48 \times 10^7$ | -0.350                       | -0.215                     | $2.35 \times 10^5$ | 0.039  | $1.67 \times 10^9$ |

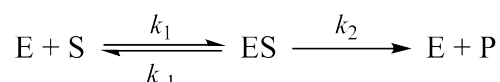
**Table D.1:** The values of  $j_{\text{lim}}$ ,  $k_{2a}$ ,  $E_{2\text{H}^+/\text{H}_2}$ ,  $E_{\text{OX}/\text{R}}^0$ ,  $e_2$ ,  $p$  and  $k_0$  found from the fits to voltammograms of *CrHydA1*, *CaHydA*, *EcHyd2* and *EcHyd1*, and used in the calculation of the values for  $\Delta H_{\text{OX}}^{\ddagger}$  and  $\Delta H_{\text{ET}}^{\ddagger}$  as shown in Figure 3.7



# Appendix E

## The Michaelis-Menten Equation

If we consider the enzyme-substrate reaction in which an enzyme molecule (E) binds with a substrate molecule (S) to form an enzyme-substrate complex (ES). This complex then reacts further to release free enzyme and product (P).



where  $k_1$ ,  $k_{-1}$  and  $k_2$  represent the rate constants for the individual reactions. If we consider that the variation in the concentration of ES is almost zero over the timecourse of the reaction, then the steady-state approximation can be applied to ES so that:

$$\frac{d[\text{ES}]}{dt} = k_1[\text{E}][\text{S}] - k_{-1}[\text{ES}] - k_2[\text{ES}] = 0 \quad (\text{E.1})$$

By rearrangement;

$$[\text{ES}] = \frac{k_1[\text{E}][\text{S}]}{(k_{-1} + k_2)} \quad (\text{E.2})$$

then, if  $K_M = (k_{-1} + k_2)/k_1$ ;

$$[\text{ES}] = \frac{[\text{E}][\text{S}]}{K_M} \quad (\text{E.3})$$

If we now consider the total amount of enzyme present;

$$[\text{E}]_0 = [\text{E}] + [\text{ES}] \quad (\text{E.4})$$

where  $[E]_0$  is the amount of free enzyme present at the start of the reaction. By rearranging Equation E.4 and substituting the value of  $[E]$  into Equation E.3 we obtain:

$$[ES] = \frac{[E]_0[S]}{K_M + [S]} \quad (\text{E.5})$$

The rate of the reaction,  $v$ , is given by;

$$v = k_2[ES] \quad (\text{E.6})$$

$$v = \frac{k_2[E]_0[S]}{K_M + [S]} \quad (\text{E.7})$$

and the maximum rate of the reaction,  $v_{\max}$ , is obtained when all the enzyme molecules are saturated with substrate so that  $[ES] = [E]_0$  and;

$$v_{\max} = k_2[E]_0 \quad (\text{E.8})$$

which can be substituted into Equation E.7 to give:

$$v = \frac{v_{\max}[S]}{K_M + [S]} \quad (\text{E.9})$$

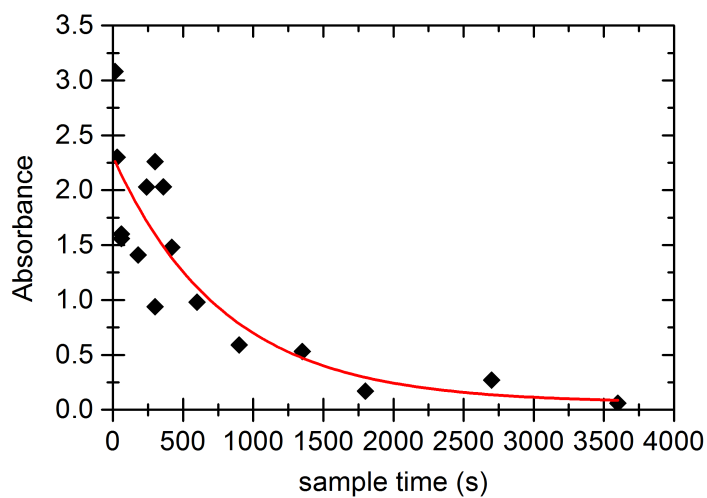
This is the Michaelis-Menten equation and from this equation it is simple to see that  $K_M$ , known as the Michaelis constant, gives the substrate concentration at which the reaction rate is half of  $v_{\max}$ .

There are a number of graphical methods for determining  $K_M$  and  $v_{\max}$  for an enzyme. The most widely used of these is the Lineweaver-Burk method in which  $1/v$  is plotted against  $1/[S]$ .<sup>173</sup> The result is a straight line, the gradient of which gives  $K_M/v_{\max}$ , the intercept on the  $y$ -axis gives  $1/v_{\max}$  and the line will reach the  $x$ -axis at a value corresponding to  $-1/K_M$ .

## Appendix F

# Monitoring the Rate of Decrease in $\text{CN}^-$ Concentration Following Injection into the Electrochemical Cell

Spectrophotometric determination of cyanide concentration was completed using the pyridine-pyrazolone method first proposed by Epstein,<sup>206</sup> but using the modifications later proposed by Nagashima.<sup>207</sup> This method makes use of the König reaction.



**Figure F.1:** A summary of UV-vis spectroscopic data measuring the decrease in cyanide concentration over time as HCN is flushed out of the electrochemical cell. Other conditions: pH 7, 25 °C, initial concentration of KCN in the cell is 1.4 mM (dissolved in mixed hydrogenase buffer, total volume = 2.15 mL), rate of  $\text{H}_2$  flushing through the cell = 500 sccm,  $\omega = 3500$  rpm. Fitting the data to a single exponential decay (shown in red) shows that, under these conditions,  $\text{CN}^-$  is removed from the cell (as HCN) with a half life of approximately 795 s.

